THE EFFECT OF CARBONATED SOFT DRINK COMPONENTS ON ISOLATED DETRUSOR MUSCLE CONTRACTION

Thesis submitted for the degree of
Doctor of Medicine
At the University of Leicester

By

Jaydip Dasgupta

Department of Cancer Studies and Molecular Medicine
Reproductive Sciences Section
University of Leicester

June 2007

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

THE EFFECT OF CARBONATED SOFT DRINK COMPONENTS ON ISOLATED DETRUSOR MUSCLE CONTRACTION

Jaydip Dasgupta

Introduction: Overactive bladder is a prevalent and disabling condition affecting millions of people worldwide with a negative impact on quality of life (Tubaro and Palleschi, 2005). A recent epidemiological study reported an association between consumption of carbonated soft drinks and development of overactive bladder symptoms (Dallosso et al., 2003a).

Objective of the study: The objective of our study was to investigate the hypothesis that carbonated soft drink components modulate detrusor muscle contraction. The effects of some of these individual ingredients on the contractile response of isolated rat detrusor muscle strips to nerve stimulation, receptor activation and Ca^{2+} channel activation were investigated to determine their mechanism of action on detrusor smooth muscle contraction.

Methods: Strips of rat detrusor muscle were placed in an organ bath and stimulated with electrical field stimulation (EFS) in the absence and presence of atropine, and with α, β methylene ATP, potassium, calcium and carbachol. The responses were repeated in the presence of carbonated soft drink components and the whole carbonated soft drink to compare with the control responses.
Whole protein extract from rat bladder and tongue were subject to SDS-PAGE, Western blotting and probing with antibodies specific for the T1R2 taste receptor protein.

Results: The artificial sweeteners, ascorbic acid and citric acid (10^{-7} M to 10^{-2} M) enhanced the contractile response to 10 Hz EFS compared to control ($p<0.01$). The sweeteners (acesulfame K 10^{-6} M, aspartame 10^{-7} M, sodium saccharin 10^{-7} M) increased the atropine resistant response to EFS marginally. Acesulfame K 10^{-6} M and sodium saccharin 10^{-7} M enhanced the maximum contractile response to α, β methylene ATP, to KCl and calcium significantly compared to control. Ascorbic acid significantly increased the atropine resistant response to EFS and inhibited contraction in response to carbachol. Both ascorbic acid and citric acid enhanced the contractile responses to α, β methylene ATP, KCl and calcium significantly compared to control. Whole carbonated soft drink (1:200) also enhanced the contractile responses to α,β methylene ATP, KCl and calcium. Although the atropine resistance response was significantly enhanced, the contraction due to carbachol was significantly inhibited in presence of carbonated soft drink.
Probing of the Western blot with specific T_1R_2 antibodies revealed a single, co-migrating band in rat tongue and bladder tissue with each antibody. The bands corresponded in size to the predicted size of the intact T_1R_2 protein.

Discussion: These results suggested that low concentrations of artificial sweeteners, ascorbic acid and citric acid, enhanced detrusor muscle contraction by augmenting Ca^{2+} influx by a mechanism yet to be defined. A similar result was obtained with the whole beverage. Western blot analysis suggested the presence of protein molecules in the rat bladder of similar size to the sweet taste receptor present in tongue. These data suggest that carbonated soft drink components may be acting via specific receptors to modulate the effects observed.

Declaration

The experimental work presented in this thesis was performed by me between February 2003 and June 2006, whilst working as a Clinical Research Fellow in Urogynaecology in the department of Obstetrics & Gynaecology, University Hospitals of Leicester and Honorary Fellow in the department of Cancer Studies and Molecular Medicine, Reproductive Science Section, University of Leicester. Analysis of data and writing of this thesis was also carried out by me.

Acknowledgements

My work on this thesis has been a journey of discovery in more ways than one -firstly, in acquiring extensive knowledge of my chosen subject and secondly, the awe-inspiring discovery of the immense support and guidance I have been privileged to receive from my supervisors, colleagues, family and friends, without whose help the journey would have been harder, far longer and lot less memorable.

I would first like to thank my supervisors Dr Doug Tincello and Dr Ruth Elliott, each of whom in their own inimitable way, have constantly guided me and tried to ensure that my work reaches highest standard possible. Ruth, my dear friend and mentor, has been the motivating force behind my work, without whose help none of this would have been possible. Not only has she patiently guided me through the rigours of laboratory work and the intricacies of statistics, she has also taught me almost everything I needed to know in computing technology for this project. And when the going got tough, she kept me going, helping me see the lighter side of life with our long discussions about photography and films.

My thesis came into existence as the brainchild of Doug Tincello, who conceptualised, helped obtain the research grant for, and inspired me to embark on this project. Like a guiding light he has constantly shown me the way ahead, reviewed and corrected mistakes and supported every stage from conception to delivery.

Thanks to Tony Taylor for guiding me in the technique of Western blotting. I would like to thank specially Mr Chris Mayne, who unfailingly championed my cause in national and international meetings and conferences. His supportive presence was always there to advice me, to cheer me up, boost my flagging spirits when necessary and help me manage my clinical obligations alongside the demands of the thesis. Thanks to the British Society of Urogynaecology (BSUG) for providing my research grant and to all my colleagues at work who have always been encouraging and supportive. Special thanks to my dearest scientist friend Vimal for his constant encouragement, support and constructive criticism.

I received a lot of help and cooperation from the staff at the department of Biomedical Sciences in University of Leicester, who assisted me by providing a steady supply of rat bladders for my experiments.

And last but not the least, thanks to my family- my wife Aditi for helping collating the chapters and proof reading, and my toddler son Rhik for his tolerance of his father's apparent preference of the laptop over him.

Finally I would like to dedicate this piece of work to my father, Late Dr Dilip Kumar Dasgupta and my mother Mrs Mira Dasgupta.

TABLE OF CONTENTS

CHAPTER/SECTION HEADING **PAGE**

Abstract 2

Declaration & Acknowledgements 3

Table of contents 5-10

List of Figures and Tables 11-14

CHAPTER 1: INTRODUCTION: RATIONALE FOR THESIS 15-18

CHAPTER 2: REVIEW OF LITERATURE (ANATOMY & PHYSIOLOGY) 19-85

2.1 Introduction 20
2.2 Anatomy
2.2.1 Bladder 20
2.2.2 The trigone 22
2.2.3 Bladder neck 23
2.2.4 Urethra 24
2.2.5 Pelvic floor muscles 26
2.3 Functional considerations 28
2.4 Innervation of lower urinary tract (LUT): urinary bladder and urethra 28
2.4.1 Parasympathetic pathway 29
2.4.2 Sympathetic pathway 32
2.4.3 Sensory pathway 35
2.4.4 Somatic pathway 36
2.4.5 Brain 37
2.5 Micturition cycle 39
2.5.1 The storage phase 39
2.5.2 The emptying phase 41
2.6 Innervation of mammalian urinary bladder 43
2.7 Ultrastructure of smooth muscle cell 46
2.7.1 Contractile proteins and filaments 47
2.7.2 Cytoskeleton and other structures 50
2.7.3 Interplay of actin-myosin 52
2.8 Mechanical properties of bladder smooth muscle 53
2.9 Excitation-contraction coupling 56
2.10 Electrical properties of bladder smooth muscle 58
2.10.1 Resting membrane potential and action potential 58
2.10.2 Role of ion channels in increasing free intracellular calcium 61
2.10.2.1 Ca^{2+} channels 61
a. Voltage-sensitive L-type Ca^{2+} channels 61
b. Receptor-operated & store-operated Ca^{2+} channels 63

2.10.2.2 K^+ channels 64
a. ATP-sensitive K^+ channels 64
b. Ca^{2+}-activated K^+ channels 65
2.10.3 Stretch activated channels 67
2.10.4 Ligand activated channels 67
2.11 Neural and Hormonal Control 68
2.11.1 Cholinergic mechanism 68
2.11.2 Adrenergic mechanism 75
a. α-adrenoceptors 75
b. β-adrenoceptors 75
2.11.3 Non-adrenergic non-cholinergic (NANC) mechanism 77
a. ATP 79
b. Nitric oxide 81
c. Neuropeptides 82
d. VIP 82
f. Endothelins 83
g. Prostanoids 84
2.12 Summary 84

CHAPTER 3. HISTORICAL BACKGROUND OF URINARY INCONTINENCE AND OVERACTIVE BLADDER 86

3.1 Introduction 87
3.2 Evolution of definitions 87
3.3 Stress urinary incontinence 88
3.4 Urge incontinence 89
3.5 Mixed incontinence 89
3.6 Lower urinary tract symptoms 90
3.7 Overactive bladder (OAB) 90
3.8 Epidemiology of urinary incontinence and overactive bladder 92
3.8.1 Prevalence 92
3.8.2 Prevalence of urinary incontinence 92
3.8.3 Prevalence of overactive bladder 94
3.9 Natural history and relationship between overactive bladder and detrusor overactivity (DO) 98
3.10 Risk factors of urinary incontinence and overactive bladder 100
3.10.1 Age 100
3.10.2 Sex 101
3.10.3 Pregnancy and childbirth 103
3.10.4 Body mass index 104
3.10.5 Race, ethnicity and country 105
3.10.6 Miscellaneous factors 106
3.10.7 Diet and lifestyle 107
3.10.7.1 Physical exercise 107
3.10.7.2 Psychological symptoms 108
3.10.7.3 Caffeine, alcohol and smoking 109

3.10.7.4 Diet 109
3.10.7.5 Leicestershire MRC Incontinence Study 110

CHAPTER 4. REVIEW OF LITERATURE: CARBONATED SOFT DRINKS 116

4.1 Introduction 117
4.2 Impact on human body 118
4.2.1 Effect on bone 118
4.2.2 Effect on dental health 121
4.2.3 Effect on gastrointestinal system 121
4.2.4 Effect on urinary system 122
4.3 Individual components of carbonated soft drinks 123
4.3.1 Caffeine 123
4.3.2 Artificial sweeteners 125
4.3.3 Acidulants, preservatives and antioxidants 131
4.3.3.1 General overview 131
4.3.3.2 Ascorbic acid 133
4.3.3.3 Citric acid 139

CHAPTER 5. MATERIALS AND METHODS 144

5.1 Introduction 145
5.2 Physiological background of methodology 145
5.2.1. Neurotransmitters and receptors 145
5.2.2. Agonists and antagonists 147
5.2.3. L-type voltage-gated Ca^{2+} channels 148
5.3 Preparation of tissues 149
5.4 Preparation of different concentrations of soft drink additives 152
5.5 Preparation of different concentrations of carbonated soft drink 153
5.6 10Hz Electrical Field Stimulation (EFS): a screening test 154
5.7 Spontaneous contractions 155
5.8 Time course experiments 155
5.9 Response curves 156
5.9.1 Frequency response curves 156
5.9.1.1 EFS at different frequencies 156
5.9.1.2 EFS in the absence and presence of atropine 10^{-6} M 157
5.9.2 Concentration response curves 157
5.9.2.1 Concentration response curves to carbachol 159
5.9.2.2 Concentration response curves to α, β- methylene ATP 159
5.9.2.3 Concentration response curves to potassium chloride 160
5.9.2.4 Concentration response curves to calcium chloride 160
5.10 Statistical analysis 162

CHAPTER 6. 10 Hz ELECTRICAL FIELD STIMULATION, SCREENING TESTS: RESULTS 163

6.1 Introduction 164
6.2 Artificial sweeteners 164
6.3 Ascorbic acid and citric acid 166
6.4 Other ingredients of carbonated soft drink 167

CHAPTER 7. ARTIFICIAL SWEETENERS: RESULTS & DISCUSSION 169

7.1 Introduction 170
7.2 Results 170
7.2.1 Spontaneous contractions 170
7.2.2 Frequency response curves 172
7.2.3 EFS in the absence and presence of atropine plus sweetener 174
7.2.4 Concentration response curves to carbachol 176
7.2.5 Concentration response curves to alpha-beta methylene ATP 177
7.2.6 Concentration response curves to potassium chloride 179
7.2.7 Concentration response curves to calcium chloride 181
7.3 Discussion 183

CHAPTER 8. ASCORBIC ACID & CITRIC ACID: RESULTS & DISCUSSION 188

8.1 Introduction 189
8.2 Results 189
8.2.1 Spontaneous contractions 189
8.2.2 Frequency response curves 191
8.2.3 EFS in the absence and presence of atropine plus ascorbic acid / citric acid 192
8.2.4 Concentration response curves to carbachol 194
8.2.5 Concentration response curves to alpha-beta methylene ATP 195
8.2.6 Concentration response curves to potassium chloride 196
8.2.7 Concentration response curves to calcium chloride 197
8.3 Discussion 198

CHAPTER 9. CARBONATED SOFT DRINK: RESULTS & DISCUSSION 204

9.1 Introduction 205
9.2 Results 205
9.2.1 Spontaneous contractions 205
9.2.2 Frequency response curves 206
9.2.3 EFS in the absence and presence of atropine plus carbonated soft drink 208
9.2.4 Concentration response curves to carbachol 209
9.2.5 Concentration response curves to alpha-beta methylene ATP 210
9.2.6 Concentration response curves to potassium chloride 211
9.2.7 Concentration response curves to calcium chloride 212
9.3 Discussion 213

CHAPTER 10. SWEET TASTE RECEPTORS 218

10.1 Introduction 219
10.2 Review of Literature 219
10.2.1. Taste perception 219
10.2.2. Taste receptors 220
10.2.3. Genetics of sweet taste receptors 226
10.3 Methodology 227
10.3.1. Tissue preparation 227
10.3.2. Preparation of reagents 228
10.3.3. Gel electrophoresis 230
10.3.3.1. Preparation of Western blot 230
10.3.3.2. Western transfer 230
10.3.3.3. Blocking 230
10.3.3.4. Detection 231
10.3.3.5. ECL chemiluminescent detection 231
10.4 Result and analysis 232
10.4.1 Western blot with V-20 antibody 232
10.4.2 Western blot with T-20 antibody 233
10.4.3 Interpretation of findings 234
10.5 Discussion 235

CHAPTER 11. DISCUSSION 240

CHAPTER 12. BIBLIOGRAPHY 255

APPENDIX: Published Abstracts and Papers

1. International Urogynaecological Association Meeting (IUGA), Buenos Aires, Argentina, October 2003
2. Joint meeting of the International Continence Society (ICS) and the International Urogynaecological Association, 34th Annual Meeting, Paris, France, August 2004
3. Blair Bell Research Society Meeting, Southampton, September 2004
4. ICS UK 12th Annual Scientific Meeting, Glasgow, April, 2005
5. International Urogynaecological Association Meeting, Montreal, Canada, Aug 2005
6. Research Urogynaecology Society (RUGS) meeting at Royal College of Obstetricians & Gynaecologists, November, 2005

7. *Toxicology and Applied Pharmacology* 2006. **217**: 216-224

LIST OF FIGURES

Figure 1 *Urinary bladder (showing trigone and bladder wall) and urethra*

Figure 2 *Sagittal section of midurethra*

Figure 3 *Lateral view of the pelvic floor structures related to urethral support*

Figure 4 *Innervation of lower urinary tract*

Figure 5a *Peripheral action of neurotransmitters on micturition cycle: storage phase*

Figure 5b *Peripheral action of neurotransmitters on micturition cycle: voiding phase*

Figure 6 *Neurotransmitter signal pathways via activation muscarinic receptors*

Figure 7 *Purinergic calcium transients mediated by $P2X_2$ receptor activation*

Figure 8 *Schematic diagram showing relationship between different types of urinary incontinence*

Figure 9 *Prevalence of patients having different types of urinary incontinence*

Figure 10 *Prevalence of three most common types of urinary incontinence by age*

Figure 11 *Chemical structure of acesulfame K and sodium saccharin*

Figure 12 *Chemical structure of aspartame*

Figure 13 *Chemical structure of ascorbic acid*

Figure 14 *Chemical structure of citric acid*

Figure 15 *Schematic diagram showing activation of different pathways by application of different stimuli to bladder muscle strips*

Figure 16 *Diagram showing incisions to cut bladder strips*

Figure 17 *Diagram of organ bath*

Figure 18 *A standard concentration-response curve*

Figure 19 *EC_{50} of a standard concentration-response curve*

Figure 20 *The effect of acesulfame K 10^{-8} M-10^{-2} M (A) sodium saccharin 10^{-8} M-10^{-3} M (B) and aspartame 10^{-8} M-10^{-2} M (C) on rat detrusor muscle contractile response to 10 Hz EFS*

Figure 21 *The effect of ascorbic acid 10^{-8} M-10^{-2} M (A) and citric acid 10^{-8} M-10^{-3} M (B) on rat detrusor muscle contractile response to 10 Hz EFS*

Figure 22 *The effect of phenylalanine 10^{-8} M-10^{-2} M (A), potassium sorbate 10^{-8} M-10^{-2} M (B), sodium citrate 10^{-8} M-10^{-2} M (C) and sodium benzoate 10^{-8} M-10^{-2} M (D) on rat detrusor muscle contractile response to 10 Hz EFS*

Figure 23 *Spontaneous contractions of bladder muscle before and after addition of (A) acesulfame k, (B) sodium saccharin and (C) aspartame*

Figure 24 *Frequency response curves: Time course experiment*

Figure 25 *The effect of acesulfame K 10^{-6} M (A), sodium saccharin 10^{-7} M (B) and aspartame 10^{-7} M (C) on rat detrusor muscle contractile response to EFS*

Figure 26 *The effect of acesulfame K 10^{-6} M (A), sodium saccharin 10^{-7} M (B) and aspartame 10^{-7} M (C) on rat detrusor muscle contractile response to EFS in the presence and absence of atropine 10^{-6} M*

Figure 27 *The effect of acesulfame K 10^{-6} M (A), sodium saccharin 10^{-7} M (B) and aspartame 10^{-7} M (C) on rat detrusor muscle contractile response to carbachol*

Figure 28 *The effect of acesulfame K 10^{-6} M (A), sodium saccharin 10^{-7} M (B) and aspartame 10^{-7} M (C) on rat detrusor muscle contractile response to α, β methylene ATP*

Figure 29 *The effect of acesulfame K 10^{-6} M (A), sodium saccharin 10^{-7} M (B) and aspartame 10^{-7} M (C) on rat detrusor muscle contractile response to KCl*

Figure 30 *The effect of acesulfame K 10^{-6} M (A), sodium saccharin 10^{-7} M (B) and aspartame 10^{-7} M (C) on rat detrusor muscle contractile response to calcium*

Figure 31 *Spontaneous contractions of bladder muscle before and after addition of ascorbic acid 10^{-4} M (A) and citric acid 10^{-5} M (B)*

Figure 32 *The effect of ascorbic acid 10^{-4} M (A) and citric acid 10^{-5} M (B) on rat detrusor muscle contractile response to EFS*

Figure 33 *The effect of ascorbic acid 10^{-4} M (A) and citric acid 10^{-5} M (B) on rat detrusor muscle contractile response to EFS in the presence and absence of atropine 10^{-6} M*

Figure 34 *The effect of ascorbic acid 10^{-4} M (A) and citric acid 10^{-5} M (B) on rat detrusor muscle contractile response to carbachol*

Figure 35 *The effect of ascorbic acid 10^{-4} M (A) and citric acid 10^{-5} M (B) on rat detrusor muscle contractile response to α, β methylene ATP*

Figure 36 *The effect of ascorbic acid 10^{-4} M (A) and citric acid 10^{-5} M (B) on rat detrusor muscle contractile response to KCl*

Figure 37 *The effect of ascorbic acid 10^{-4} M (A) and citric acid 10^{-5} M (B) on rat detrusor muscle contractile response to calcium*

Figure 38 *Spontaneous contractions of bladder muscle before and after addition of the carbonated soft drink 1:200*

Figure 39 *The effect of the carbonated soft drink 1:300 (A, 1:200 (B) and 1:100 (C) on rat detrusor muscle contractile response to EFS*

Figure 40 *The effect of the carbonated soft drink 1:200 on rat detrusor muscle contractile response to EFS in the presence and absence of atropine 10^{-6} M*

Figure 41 *The effect of the carbonated soft drink 1:200 on rat detrusor muscle contractile response to carbachol*

Figure 42 *The effect of the carbonated soft drink 1:200 on rat detrusor muscle contractile response to α, β methylene ATP*

Figure 43 *The effect of the carbonated soft drink 1:200 on rat detrusor muscle contractile response to KCl*

Figure 44 *The effect of the carbonated soft drink 1:200 on rat detrusor muscle contractile response to calcium*

Figure 45 *Sweet taste receptors: T1R3 monomer and T1R2/T1R3 heterodimer*

Figure 46 *Signal transduction of sweet taste*

Figure 47 *Western blot using V-20*

Figure 48 *Western blot using T-20*

LIST OF TABLES

Table 1	*Prevalence of overactive bladder in different studies*
Table 2	*Molecular weights of different carbonated soft drink components and other chemicals involved in the experiments*
Table 3	*Concentration of individual soft drink components for detail investigations*
Table 4	*Summary of the effects of acesulfame K 10^{-6}M, sodium saccharin 10^{-7} M and aspartame 10^{-6} M on the muscle contraction in response to different stimuli*
Table 5	*Summary of the effects of ascorbic acid 10^{-4}M and citric acid 10^{-5} M on the muscle contraction in response to different stimuli*
Table 6	*Summary of the effects of carbonated soft drink (1:200) on the muscle contraction in response to different stimuli*

CHAPTER 1

INTRODUCTION: RATIONALE FOR THESIS

Urinary incontinence is a common condition within adult populations in the community and is therefore a public health and primary care concern. People suffering from urinary incontinence have poorer health status and consequently need greater health and social care (Roe et al., 2000). Detrusor muscle overactivity is a common cause of urinary incontinence with a socioeconomic impact comparable to diabetes mellitus (Hampel et al., 2003). The disease is characterised by spontaneous muscle contraction within the bladder wall, causing symptoms of urinary urgency, frequency and incontinence. The specific mechanisms underlying the pathophysiology of the overactive bladder have been investigated over the past several years, and more detailed understanding has begun to emerge. Several studies have assessed the association of incontinence and overactive bladder symptoms with lifestyle factors. The reported modifiable risk factors include cigarette smoking (Bump & McClish, 1992; Hannestad et al., 2003b), caffeine consumption (Bryant et al., 2002; Tomlinson et al., 1999), obesity (Larrieu et al., 2004; Sampselle et al., 2002), and high-impact sports or exercise (Thyssen et al., 2002).

The Leicestershire MRC Incontinence Study investigated dietary and lifestyle factors associated with urinary incontinence and overactive bladder, and demonstrated that daily consumption of carbonated soft drinks was one factor independently associated with the onset of OAB symptoms and urinary incontinence within the next twelve months (Dallosso et al., 2003a). Many of these carbonated soft drinks are rich sources of sugar and others have high levels of artificial sweeteners such as sodium saccharin, aspartame and acesulfame K. Chemicals used in the manufacture of carbonated soft drinks also include colorants, stabilizers, antioxidants (such as ascorbic acid) and preservatives (commonly citric acid or phosphoric acid). The association of overactive bladder

symptoms with carbonated drinks is of great concern, as their consumption has increased dramatically in Britain over the past 20 years and is particularly high in younger people (Euromonitor International, 2002). Whether cutting down on carbonated soft drink consumption has any impact on overactive bladder symptoms in later life is a question that has not been explored so far.

The exact pathophysiology of the overactive bladder is complex and not fully understood yet. Goldberg and Sand (2002) have described two main theories related to the development of OAB: neurogenic and myogenic. Neurogenic causes mainly include altered bladder wall innervation (Drake et al., 2003a), pathologically enhanced cell coupling and enhanced sensitivity with activation of secondary excitatory pathways (C-fibers). Myogenic causes are related to increased density of elastin and collagen, increased gap junctions, enhanced activity of interstitial cells and finally ischaemia and hypoxia at cellular level (Goldberg and Sand, 2002). Although it has been unanimously accepted that purinergic (atropine resistant) component is absent in normally functioning bladders, its presence in pathological bladder tissue was suggested by several investigators. A significant residual nerve-mediated bladder contraction was found to be present in strips of bladder from patients diagnosed with urodynamically proven detrusor instability, which persisted in the presence of atropine and was abolished by pre-treatment with α, β-methylene-ATP, an agent which desensitizes the purinergic receptor (Palea et al., 1993; Fry et al., 1999).

Recent studies have shown that stretching of bladder muscle can stimulate release of acetylcholine (ACh) from the urothelial cells (non-neuronal) which may play a role in urinary bladder function (Yoshida et al., 2006; Hanna-Mitchell et al., 2007; Lips et al.,

2007). This non-neuronal ACh was found to be different from that released from the nerve endings with respect to molecular components of the ACh synthesis and release machinery (Lips et al., 2007).

In spite of this the bladder muscle maintains its ability to cause a substantial phasic rise in pressure. This may occur through various pathways, such as nerve stimulation of those still intact, post synaptic receptor activation, increase of Ca^{2+} influx through L-type voltage-gated calcium channels or some other, as yet unknown mechanism possibly derived from the urothelium. An important factor common to these pathways is that an increase in intracellular calcium concentration plays a key role in causing the detrusor muscle to contract (Rüegg, 1992). Looking at the association between carbonated soft drink consumption and development of overactive bladder, it would be logical to speculate that soft drink components, either individually or in combination, modulate detrusor muscle contraction by influencing any of the above mechanisms. This forms the basis of our **hypothesis**, i.e., ***carbonated soft drink components modulate detrusor muscle contraction.***

This study therefore ***aimed*** to investigate the effects of individual components of a popular carbonated soft drink on the contractile response of rat bladder smooth muscle to various stimuli using an organ bath and also determines the probable mechanism of action of the soft drink components and the carbonated soft drink as a whole on bladder muscle function.

CHAPTER 2

REVIEW
OF
LITERATURE
ANATOMY & PHYSIOLOGY

2.1 Introduction

The study of biology repeatedly illustrates the link between structure and function of various organs. This is true in the case of the lower urinary tract (LUT), which consists of the bladder and the urethra. This structural unit serves two important functions: storage and voiding of urine. The detrusor smooth muscle of the bladder wall allows the bladder to expand without any rise in pressure during the filling phase, and is also responsible for bladder contraction during voiding (Abrams, 2003). As urinary bladder relaxes during the filling phase, urine is stored in bladder at low pressure (Damaser, 1999). The lower urinary tract is regulated by a complex interplay between the central and peripheral nervous systems and hormonal control systems (Andersson and Arner, 2004). Different anatomical, physiological and pharmacological aspects are discussed in this chapter.

2.2 Anatomy

2.2.1 Bladder

The urinary bladder is a hollow, muscular organ which acts as a compliant reservoir for urine. Two ureteral orifices form an important landmark inside the bladder; the part located above the ureteral orifices is the 'dome' of the bladder, while the triangular area, the apices of which are formed by two ureteral orifices and the internal urinary meatus, is called the 'trigone' (Fig. 1). The bladder wall consists of the detrusor muscle, covered on its outer aspect by adventitia, and partly by peritoneal serosa and partly by fascia over its dome. The detrusor muscle layer is lined internally by a submucosa and transitional cell epithelium. Abrams (2002a) described the detrusor muscle as an interlacing network of smooth muscle bundles. These bundles of smooth muscle cells of different size are

surrounded by connective tissue containing collagen and are arranged in diverse directions (Drake et al, 2003a). Within the main bundles, the smooth muscle cells exist in groups of small functional units, or fascicles (Drake et al, 2001). However, in general, this muscle layer can be divided into an outer and inner longitudinal muscle layer with an intervening circular-oblique layer (DeLancey, 2006). Thus from the functional viewpoint, the detrusor muscle comprises an integrated unit of interconnected muscle bundles which, on contraction, will cause a reduction in all dimensions of the bladder lumen (Gosling, 1979). Posteriorly, some of the outer longitudinal muscle fibers extend over the bladder base and merge with the capsule of the prostate in men and with the anterior vaginal wall in women. Some of the muscle fibers extend onto the anterior aspect of the rectum to form the rectovesical muscle. Anteriorly, some of the outer longitudinal muscle fibers continue into the pubovesical ligaments and contribute to the muscular component of these structures (Gil Vernet, 1968).

Regarding the embryologic development of female urothelium, Berrocal et al. (2002) described it to be derived from the ventral chamber created by the division of the cloaca by the urorectal septum. The connective tissue and smooth muscle in bladder are subsequently developed from the adjacent mesenchyme. Baskin et al. (2001) demonstrated the mechanism of transformation of the mesenchyme into smooth muscle, which was initiated by the growth factors secreted by the urothelium. Further development of detrusor smooth muscle was found to be related to complex changes in muscle-specific protein expression and cell turnover (Smeulders et al., 2002).

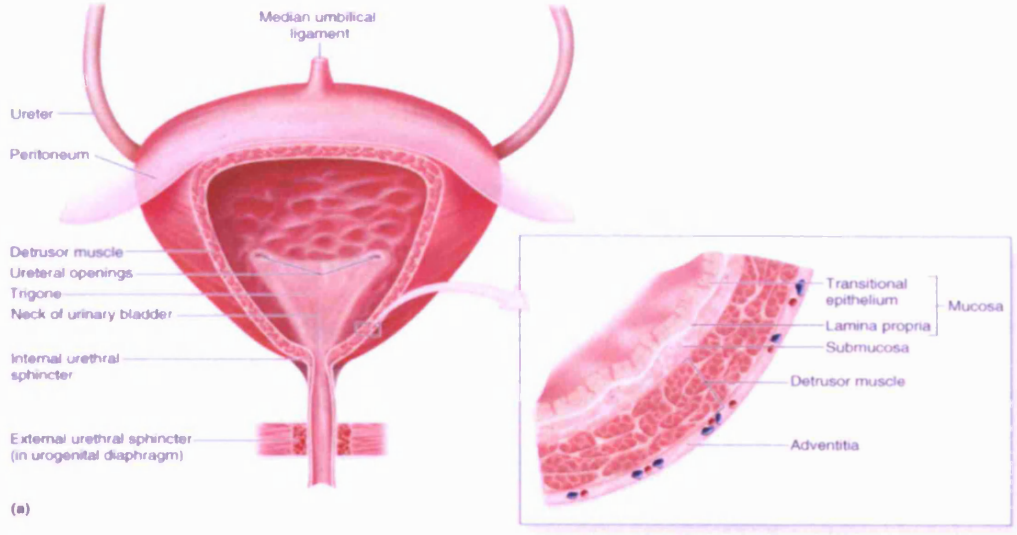


Fig. 1 *Urinary bladder (showing trigone and bladder wall) and urethra (Reproduced from Human Anatomy by Herbrandson C, URL A)*

2.2.2 The Trigone

The trigone is a special muscular triangular area, lying at the posterior wall of the bladder, next to the bladder neck (Fig. 1). The three apices of this triangular area are formed by two ureteral orifices and the internal urinary meatus. The interureteric ridge, which forms the base of the triangle, is a useful landmark in cystoscopic identification of the ureteric orifices (DeLancey, 2006). The triangular elevated area is due to the presence of a separate specialized group of muscle fibres within the detrusor, which has a different developmental history compared to detrusor smooth muscle (DeLancey, 2006). The embryological background of the trigone was always a point of interest among the anatomists. The trigone was traditionally thought to be of mesodermal origin (Lassmann and Zderic, 2004). However, recent studies using transgenic mice suggested it to be of

endodermal origin (Thomas et al., 2005). The smooth muscle in this area was found to have two distinct layers, often termed as superficial and deep trigonal muscles (Dixon and Gosling, 1990). The deep muscle cells are indistinguishable from the detrusor and termed as trigonal detrusor muscle. The specialized muscle fibres were found to be continuous with the ureteral smooth muscle above with urethra below (Lewis, 2000). These muscle fibres also form a ring within the detrusor at the level of the internal urinary meatus (Huisman, 1983). The specialized smooth muscle fibres of the trigone are clearly distinguishable from those of the detrusor by smaller size of their fascicles and greater density of surrounding connective tissue (Oswald et al., 2006). John et al. (2001) suggested that contraction of trigonal muscle has a supportive role in normal micturition, by moving the terminal ureters to the bladder base helping efflux of urine into urethra while protecting reflux of urine into the ureters.

2.2.3 Bladder neck

The smooth muscle of this region is histologically, histochemically and pharmacologically distinct from that of detrusor smooth muscle (Gosling et al., 1983). It denotes the area at the base of bladder, where the urethral lumen traverses through the thickened musculature of bladder base (DeLancey, 2006). The arrangement of smooth muscles in this region is quite different in males and females. Muscle bundles of large diameter, typical of detrusor, are replaced by those of small diameter. Unlike the circularly oriented smooth muscle in male, the majority of smooth muscle bundles in female bladder neck extend obliquely or longitudinally into the urethral wall (Gosling et al., 1983). The functional importance will be discussed later in the chapter.

2.2.4 The Urethra

The urethra helps retention of urine in the bladder and thus plays an important role in maintenance of continence. The female urethra is a fibro-muscular tube approximately 3.5 cm long. The mucosal layer is continuous with the transitional epithelium of the bladder and below with the non-keratinizing squamous epithelium of the vestibule. Except during the passage of urine, the anterior and posterior walls of the urethra are in apposition, and the epithelium forms extensive longitudinal folds in the mucosal layer which provide the urethral lumen with a stellate appearance (Gosling et al., 1983). A well-developed vascular plexus forms the submucosal layer (Berkow, 1953). The surface tension of this mucosal and the submucosal layer contribute to urethral closing pressure (Rud et al., 1980). The wall of the female urethra comprises an outer sleeve of striated muscle (the rhabdosphincter) and an inner coat of smooth muscle fibres (Gosling, 1979). The female rhabdosphincter is anatomically separate from the adjacent periurethral striated muscle of the anterior pelvic floor (pubococcygeus). In its upper two-thirds, the sphincter fibers are arranged circularly, while distally, the fibers are extended either to encircle the vaginal wall as the urethrovaginal sphincter or along the inferior pubic ramus above the urogenital diaphragm as the compressor urethrae (DeLancey, 2006). The muscle cells are histochemically all of slow twitch variety (Gosling et al., 1981), which is ideal for maintaining a basal constant tone of the muscle. Activation of this muscle voluntarily should augment urethral constriction when an increased closure pressure is required. Studies of skeletal muscle blockade suggest that this muscle is responsible for approximately one-third of resting urethral closure pressure (Rud et al., 1980).

Two urethral sphincter mechanisms are involved in controlling urine flow in women: the

bladder neck smooth muscle sphincter and the striated muscle sphincter, also called the rhabdosphincter (Fig. 2). Fluoroscopic examination showed that the proximal part or urethra and bladder neck are fairly mobile structures, whereas the distal part of the urethra remains fixed in position (Westby et al., 1982). Most of the studies showed that urethra opens during the first phase of normal micturition, but it is still unclear whether it is an active or passive phenomenon. The smooth muscle sphincter in the wall of bladder neck and proximal urethra can not be anatomically recognized, and it is more than likely that active contraction of this component is not an important factor for the continence of urine in women (Tanagho and Smith, 1966). However, the bladder neck and proximal urethra possess in their walls innumerable elastic fibres which are particularly important for producing passive occlusion of the urethral lumen (Gosling, 1979). The presence and pharmacologic response of α_1-adrenergic receptors in the smooth muscle of the trigone and proximal urethra of both men and women would suggest a role for these receptors in the initiation of voiding. Strong evidence suggested nitric oxide may mediate active relaxation of proximal urethral smooth muscle (Bennet et al., 1993).

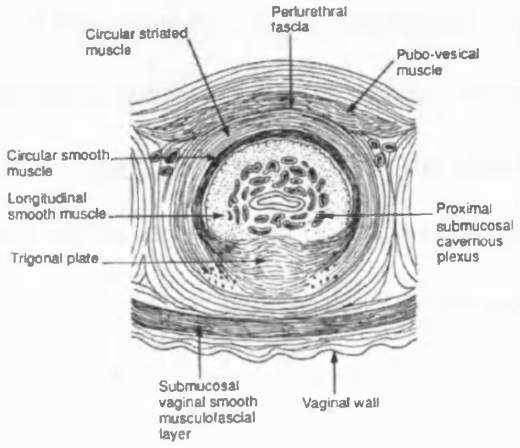


Fig. 2 *Sagittal section of the mid-urethra modified from Huisman, 1983 [from Strohbehn and DeLancey]*

2.2.5 The Pelvic Floor Muscles

The position and mobility of bladder and urethra are important factors in maintenance of urinary continence (Hodgkinson, 1953). The pelvic floor muscles, levator ani, support and maintain the position of the bladder neck and urethra, and allow compression of the urethra against the vaginal wall. DeLancey has described the 'pelvic floor' to be comprised of several components lying between the pelvic peritoneum and the vulvar skin, which contains the peritoneum, endopelvic fascia, levator ani muscles, perineal membrane and external genital muscles (DeLancey, 2006) (Fig. 3). The medial parts of the levator ani muscles (sphincter vaginae) are related to (but structurally separate from) the urethral wall. These fibres consist of a mixture of large-diameter fast and slow twitch fibres (Gosling et al., 1981), and hence unlike the rhabdosphincter described above, these periurethral muscle fibres possesses morphological features that are similar to other 'typical' voluntary muscles (Gosling, 1979). The levator ani muscles and the fascia covering both anterior and posterior surfaces are together termed as 'pelvic diaphragm' (Kearney et al., 2004). The levator ani consists of two parts: pubovisceral and iliococcygeal (Lawson, 1974). Laterally, this iliococcygeal muscle spans out like a horizontal sheet and provides a premise on which pelvic viscerae can rest. The medial part, pubovisceral muscle is aligned in U-shaped form, the ends of which arise from the pubic bones on either side of the midline and inserts into or forms a sling around, the urethra, vagina and rectum. This part includes both pubococcygeus and puborectalis portion of levator ani (Lawson, 1974).

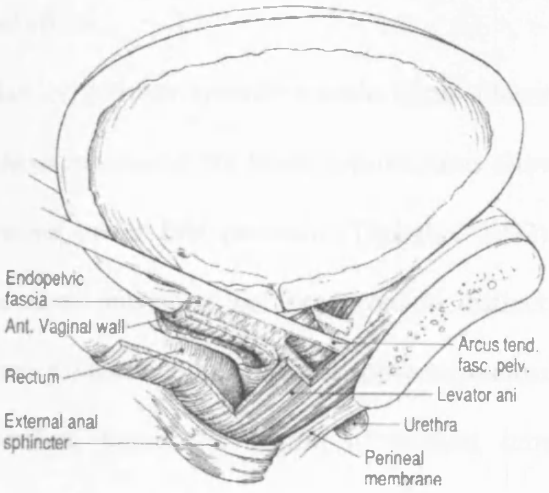


Fig. 3 *Lateral view of the pelvic floor structures related to urethral support (as seen from the side in the standing position, cut just lateral to the midline). The windows have been cut in the levator ani muscles, vagina, and endopelvic fascia so that the urethra and anterior vaginal walls can be seen. (DeLancey 2006)*

The levator ani muscle fibres are arranged in a fashion to form an opening, called urogenital hiatus through which the urethra and vagina pass (DeLancey, 2006). The rectum also passes through this opening, but as the levator ani fibers are attached to rectum directly, it is not considered part of the 'hiatus'. The hiatus is bounded anteriorly by the pubic bones, laterally by the levator ani muscles and posteriorly by the perineal membrane and external anal sphincter (Dickinson, 1989). The basal tone of levator muscle keeps the hiatus closed, similar to the external anal sphincter closing the anus (Nichols et al., 1970). Pudendal nerve supplies the somatic innervation of the urethral rhabdosphincter and of some perineal muscles (compressor urethrae and urethrovaginal sphincter). These fibres originate from sphincter motor neurons in the ventral horn of the sacral spinal cord (S2-S4) in a region called Onuf's (Onufrowicz's) nucleus (Thor and Donatucci, 2004).

2.3 Functional considerations

The arrangement of the bladder detrusor smooth muscle fibres allows them to stretch to four times their length without increasing the linear tension, thus allowing the bladder to store a large volume of urine under low pressure (Tanagho, 1992). Unlike intestinal smooth muscle, bladder smooth muscle is not organized in distinct layers and at the bladder neck, the orientation of muscle fibres provide sphincteric closure of the proximal urethra. The proximal urethra, being the region of highest intraluminal pressure, contributes significantly towards providing continence at rest (Abrams, 2002a). The distinct inner longitudinal muscle fibres are found to be continuous above with the muscle at the bladder neck being surrounded by the sleeve of outer circular layer. In addition, the striated muscle fibres (rhabdosphincter) encircle the urethra at this point and provide the strength to maintain the basal tone for the urethral closure and thereby preventing leakage of urine with an increased intra-abdominal pressure (Hutch, 1972). Micturition is initiated by the opening up of the bladder neck and proximal urethra. It is still unclear from the studies so far, whether this opening up is primarily due to the contraction of longitudinal muscles at the bladder base going to urethra and thereby pulling apart the circular muscle fibres while the sphincter relaxes, or due to the reduction of basal tone of urethral muscle (Tanagho, 1992).

2.4 Innervation of bladder and urethra and normal micturition cycle

The alternating storage and voiding functions during the micturition cycle requires an adequate interplay between the reservoir and the outlet functions of the LUT, including the detrusor muscle, the urethral smooth muscle, the rhabdosphincter and the pelvic floor

muscle (Morrison et al., 2002). During voiding, detrusor contraction is preceded by relaxation of the outlet region, thereby facilitating the bladder emptying. Conversely, during the storage phase, the detrusor muscle is relaxed, and the outlet region is contracted to maintain continence (Tanagho and Miller, 1970). The control of these functions is complex and involves the central nervous system and both the afferent sensory and efferent somatic and autonomic divisions of the peripheral nervous system (De Groat, 1990). At each level of the neural control, different pathways and neurotransmitters are involved as described below (De Groat and Yoshimura, 2001). The peripheral motor innervation of the LUT involves parasympathetic, sympathetic, and somatic efferent nerves (Morrison et al., 2002). The bladder receives innervation from both the parasympathetic and sympathetic divisions of the nervous system. The parasympathetic system has been well characterized and functions to produce the intense contraction of the bladder wall during micturition, but the role of the sympathetic system in the bladder is less well understood (Gillespie, 2004a).

2.4.1 Parasympathetic pathway

Activation of the parasympathetic pathway results in contraction of the detrusor smooth muscle and relaxation of the outflow region, via the sacral parasympathetic nucleus in the spinal cord at the level of S2-S4 (Shefchyk, 2001). The bladder detrusor muscle coat is profusely supplied with autonomic nerve fibres and the majority of them contain acetyl-cholinesterase (McConnell et al., 1982). Daniel et al. (1983) demonstrated the presence of nerve varicosities in high frequency (9 to 15 profiles per 100 muscle cell profiles) in muscle bundles of urinary bladder. These nerve endings, mostly devoid of glial covering were in close contact with muscle. They were often present as single axons or in bundles

of only a few nerves. No myelinated nerves or nerve cell bodies were seen close to muscle bundles. It appears that the nerve distribution to bladder smooth muscle is designed primarily for neural control of contractile function with single nerves innervating discrete muscle regions (Daniel et al., 1983).

The axons, after leaving the spinal cord pass through the pelvic nerve and synapse with postganglionic nerves in the pelvic plexus in ganglia on the surface of the bladder (vesical ganglia), or within the walls of the bladder and urethra (intramural ganglia) (Lincoln and Burnstock, 1993). Small clusters of autonomic ganglion cells occur throughout all regions of the human bladder wall. The presence of two types of axosomatic terminal at the surface of the intramural ganglion cells has been confirmed by electron microscopy (Gilpin et al., 1983). Neurons in vesical parasympathetic ganglia receive excitatory and inhibitory inputs from both divisions of the autonomic nervous system and sacral parasympathetic pathways (cholinergic) provide the major excitatory input to these ganglia via activation of nicotinic receptors (De Groat and Booth, 1980) (Fig. 4). Although the preganglionic neurotransmission is mediated predominantly by acetylcholine acting on the nicotinic receptors in ganglia, the transmission can also be modulated by adrenergic, muscarinic, purinergic and peptidergic presynpatic receptors (De Groat et al., 1993). The postganglionic neurons supplying the human detrusor muscle were found to transmit the excitatory impulse by releasing acetylcholine from the nerve terminals. Acetylcholine acts on different types of muscarinic receptors on detrusor muscle cells initiating muscle contraction (Sigala et al., 2002). Apart from the cholinergic pathway, another contractile component was detected in bladders of most mammals, an atropine-resistant component (purinergic), mediated by the neurotransmitter ATP (Burnstock,

2001). Several studies have confirmed that this component may be responsible for up to 40-50% of contraction in human bladder in different conditions associated with detrusor overactivity (Bayliss et al., 1999; O'Reilly et al., 2002). Interestingly, the parasympathetic fibres to the outflow region and urethra were found to transmit an inhibitory impulse to the smooth muscle, by releasing nitric oxide (Andersson, 1993) and other neurotransmitters (Bridgewater and Brading, 1993).

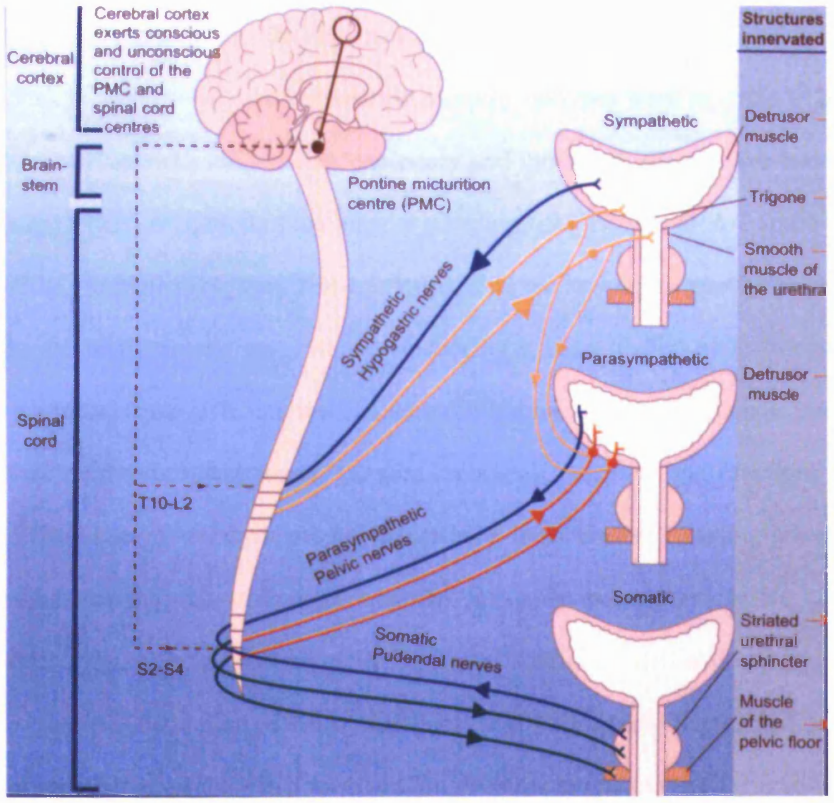


Fig. 4 *Innervation of lower urinary tract showing cortical and spinal levels, including parasympathetic, sympathetic and somatic pathways.*
(Reproduced from Clinical Manual of Incontinence in Women, based on the Reports of the 3rd International Consultation on Incontinence, 2005 , edited by Abrams P, Artibani W, Cardozo L, Khoury S and Wein A)

2.4.2 Sympathetic Pathway

The role of the sympathetic nervous system in human bladder function has always been an interesting point of discussion, partly because of the scarcity of the adrenergic innervation of human detrusor muscle (Gosling et al., 1999). However, sympathetic innervation was found to be quite intense in several animal species. Although major differences were observed in the responsiveness of the bladder of different species to sympathetic nerve activation, variations in the density of adrenergic nerves within the bladder wall and differences in the expression of adrenergic receptor subtypes were reported to be present (Vaughan and Satchell, 2003). Both excitatory and inhibitory effects have been reported with sympathetic stimulation of the bladder (Gosling, 1986; de Groat WC and Yoshimura, 2001). The mechanisms giving rise to sympathetic excitation are poorly understood. In contrast, the inhibitory elements in the bladder have been studied more intensively and appear to incorporate different mechanisms (Gillespie, 2004b). In the cat, one element resides within the parasympathetic ganglia, involves α receptors and functions to reduce the effectiveness of parasympathetic ganglionic transmission (Gosling et al., 1999). However, in the rat, α_{1A}-adrenergic receptors appear to be responsible for augmenting transmitter release from cholinergic presynaptic terminals and α_{1B}- or α_{1D}-receptors involved in direct activation of the smooth muscle (De Groat and Saum, 1972). A second inhibitory mechanism involves β receptors on the smooth muscle and is, in some species, responsible for direct bladder relaxation (Gosling et al., 1999; Szell et al., 2000).Two subtypes of β-adrenoceptors, β_1 and β_2 have been identified in the detrusor muscle of most species (Yamaguchi, 2002). Yamaguchi (2002) has mentioned in his study that although β_2 has an important role in muscle relaxation via activation of adenylate cyclase, evidence

suggests that a third subtype, β_3, which is implicated in metabolic functions of endogenous catecholamines, mediates relaxation of human detrusor muscle. He has also reported that there is a predominant expression of β_3 adrenoceptor messenger RNA (mRNA) in human bladder tissue, with 97% of total β adrenoceptor mRNA being represented by the β_3 subtype and only 1.5% and 1.4% by the β_1 and β_2 subtypes, respectively.

Generally, in the animal bladder, sympathetic innervation is most abundant in the urethra, and progressively decreases in the upward direction from the bladder base to the body and is virtually absent in the bladder dome. Animal studies with dogs and pigs have shown that the outer wall has greater sympathetic innervation than the inner wall in any region of the bladder and the adrenergic innervation of the bladder base is less prominent compared to that of rats (Elbadawi and Schenk, 1966, 1971, Elbadawi, 1982). Compared to other animals, the rat bladder has comparatively weaker innervation in the body of the bladder (Alm and Elmer, 1975). However, the base of the rat bladder is richly supplied by adrenergic nerves in its vertical, ventral midline bundle and dorsal walls (Elbadawi, 1982). Somogyi and DeGroat (1990) concluded that the mechanism of action of [^{3}H] norepinephrine released from the adrenergic nerve endings in electrically stimulated bladder strips was predominantly via activation of alpha-2 adrenergic presynaptic receptors. The sympathetic innervation of the cat detrusor muscle, acting as an inhibitory influence, was well documented in the studies of Wakabayashi et al (1994).

Studies in humans have suggested the possibility of an inhibitory effect of norepinephrine on detrusor function through prejunctional inhibition of parasympathetic activation, either at the level of intramural ganglia (Smet et al., 1996) or at the nerve terminal level (Mattiasson et al., 1987). In a comparative study of the autonomic innervation of the

human female and male bladder neck and proximal urethra Gosling et al. (1977) showed that bladder neck and female proximal urethra is mainly supplied with cholinergic nerves, although nerves of the noradrenergic type rarely are observed in these regions. In contrast the male proximal urethra is supplied richly with noradrenergic nerves, indicating that the region is under direct sympathetic control and functions to prevent vesical reflux of ejaculate. The predominant effect of the sympathetic system was described as contraction of the bladder base and the urethra (Ek et al, 1977).

The sympathetic innervation of the bladder and urethra originates from the mediolateral nuclei in the thoracolumber region (T10-L2) of the spinal cord (McConnell et al., 1982). Lincoln and Burnstock (1993) studied the autonomic innervations in rats extensively and commented that some axons, after leaving the spinal cord via splanchnic nerves, either synapse with post ganglionic cells in the inferior mesenteric ganglia and take the course of the hypogastric nerve, or synapse with cell bodies in the paravertibral sympathetic chain of ganglia (lumbosacral) and take the course of the pelvic nerve. These reports suggested that sympathetic signals are conveyed in both the hypogastric nerve and the pelvic nerve and both the nerves contain pre and postganglionic fibers (Lincoln and Burnstock, 1993). The main neurotransmitter mediating the preganglionic sympathetic transmission was found to be acetylcholine acting on nicotinic receptors (Snell, 2006), similar to parasympathetic preganglionic neurotransmission (De Groat et al., 1993). In humans, *in vitro* studies have shown that noradrenaline is the main neurotransmitter released by electrical stimulation of detrusor muscle (Mattiasson et al., 1987) and the normal response of detrusor muscle to released noradrenaline is relaxation (Perlberg and Caine, 1982). The relaxation effect was also reported by Gillespie (2004b), who showed that

sympathomimetic stimulation in the isolated whole bladder results in an inhibition of phasic activity, involving α_1 and β_3 receptors.

2.4.3 Sensory pathway

Most of the sensory nerves to the bladder and urethra reach the spinal cord (S2-S4) via the pelvic nerve and dorsal root ganglia, although some afferents travel in the hypogastric nerve (Andersson, 2006). The sensory nerves of the striated muscle in the rhabdosphincter travel in the pudendal nerve to the sacral region of the spinal cord (Lincoln and Burnstock, 1993). The afferents fibres for bladder emptying which carry the proprioception, the sensation of bladder distension, are mainly myelinated slowly conducting Aδ-fibres (Janig and Morrison, 1986). The sensation is transmitted via the dorsal column to the brain (pons), where it is integrated into processing centres supplying the cortex, bringing the awareness of bladder filling (Thor and Donatucci, 2004). The contribution of the brain in the control of micturition will be discussed separately below. Studies have shown that the unmyelinated C-fibres travelling via the pelvic nerve to the sacral spinal cord also convey information from receptors in the bladder wall to the spinal cord (Kuru, 1965). The former are excited by mechanoreceptors and convey information about bladder filling (Janig and Morrison, 1986), whereas 98% of afferent unmyelinated C-fibres are mechanically insensitive (i.e., so-called silent nociceptors), which are involved in abnormal afferent sensation under pathophysiological conditions (Habler et al., 1990). Normally, bladder sensation is not painful, it distinguishes warm from cold, and leads to an increasing conscious awareness of bladder filling which is related to intravesical volume, and not pressure (Andersson, 2006). Nociception normally transmits information about temperature and pain from the bladder (Andersson, 1993). The contribution of C-fibre

afferent pathways in urinary frequency and pain associated with painful bladder syndrome opens up a new area of research with a prospect of multiple targets for the treatment of this disease. This may be achieved by inhibiting the afferent transmission by C-fibre via one of the following mechanisms: (i) blocking specific sodium channels, (ii) elevating nitric oxide levels, or (iii) activating cGMP-dependent pathways (Yoshimura et al., 2002). Bladder sensation can be reduced in different disease conditions like neuropathy (e.g. diabetic) or subacute combined degeneration of spinal cord (Vit B12 and folate deficiency). Painful sensation is usually seen in patients with bladder stone, infection and interstitial cystitis.

The capsaicin receptor TRPV1 (Transient Receptor Potential) and the adenosine triphosphate gated purinergic receptor $P2X_3$ are known to be expressed by primary sensory neurons, and several studies support their role in sensory signal transduction in the normal animal bladder (Cockayne et al., 2000; Birder et al., 2002). Intravesical therapy with the C fibre toxin resiniferatoxin (RTX) resulted in a dramatic decrease of TRPV1 and $P2X_3$ expressing suburothelial fibres in patients with spinal neurogenic detrusor overactivity that responded to treatment (Brady et al., 2004), and also idiopathic detrusor overactivity (Silva et al., 2002), suggesting a role for the increased afferent suburothelial innervation in its pathophysiology. Apostolidis et al. (2005) suggested that decreased levels of sensory receptors $P2X_3$ and/or TRPV1 may contribute to the clinical effect of botulinum neurotoxin Type A in detrusor overactivity.

2.4.4 Somatic pathway

As mentioned earlier, the somatic innervations of the urethral rhabdosphincter muscle and some perineal muscles such as compressor urethrae are provided by the pudendal nerve

(DeLancey, 2006). Thor and Katofiasc (1995) have described that Onuf's nucleus, an area located in the ventral horn of the sacral spinal cord (S2-S4), is where the terminals of nerve tracts from higher centre in the central nervous system (the medulla and locus ceruleus) synapse with the cell bodies of pudendal motor neurons (Thor et al., 2002). The sacral nerve roots enter into the sacral plexus, from which the pudendal nerve diverges (S2-S4) and supplies the levator ani muscles. The pudendal nerve also receives postganglionic axons from the caudal sympathetic chain ganglia (Lincoln and Burnstock, 1993).

2.4.5 Brain

Since the 1920s, it has been known that motor control of the bladder arises within the pons, which is more specifically termed as the pontine micturition centre (Barrington's nucleus') (Barrington, 1925; Kuru, 1965; Griffiths et al., 1990). Holstege et al. (1979) refined the role of the brainstem in the functional anatomy of bladder related to storage and voiding in animals by demonstrating that stimulation of the medial part of the pons (termed as `M-region') causes a decrease in urethral pressure followed by a rise in detrusor pressure (Holstege et al., 1979). Further studies demonstrated direct connections between this M-region and different cell columns in sacral spinal cord; intermediolateral cell column of containing parasympathetic motor neurons to bladder and the intermediomedial cell group (Onuf's nucleus) containing inhibitory inter- neurons for the pelvic floor muscles (Holstege et al., 1986; Griffiths et al., 1990). The pontine centres also seem to receive afferent sensory information from the lower urinary tract (Blok and Holstege, 1999). Direct sacral projections to the mesencephalic periaqueductal grey matter (PAG) (Vanderhorst et al., 1996) and from the PAG to the M-region of the pontine micturition

complex (Noto et al., 1991) have been well documented in the literature.

The involvement of cerebral cortical and subcortical activity in micturition has been demonstrated in human functional brain imaging studies. Fukuyama et al. (1996) reported that upper pons, left sensorimotor and right frontal cortex, as well as bilaterally in supplementary motor areas are involved in micturition activity. Blok et al. (1998) demonstrated that changes in blood flow within specific areas in brain, such as pons, PAG, pre-optic area, hypothalamus and right anterior cingulate gyrus, resulted in alterations in voiding pattern in healthy right-handed females and males. Studies of Nour et al. (2000) included the additional activity in the globus pallidus and cerebellum. However, precise roles of these different areas in the cortico-subcortical network in micturition control are still unclear.

The inconsistent relationship between the amount of urine in bladder and the urge to void has been observed in different studies including functional imaging data to reflect this mismatch (Athwal et al., 2001). In their study, Athwal et al. (2001) demonstrated that regardless of the sensation of urge to void, an increase in the bladder volume activated a set of areas in brain responsible for control of micturition (PAG, pons, cingulate and frontal cortices). Where as changes in the degree of urgency may reduce activity in specific areas of brain such as right and left premotor cortex and cingulate cortex. The inconsistent relationship between the magnitude of bladder fullness and urgency (primarily high degree of urgency at low volume) were found to deactivate the somatosensory cortex bilaterally (Athwal et al., 2001).

2.5 Micturition cycle

2.5.1 The Storage phase

Urine storage in bladder is regulated by two different storage reflexes: sympathetic (autonomic) and somatic (Thor and Donatucci, 2004). When urine starts accumulating during bladder filling, distension of the bladder activates the stretch receptors in the bladder wall (A-δ fibres) and generates afferent sensory signals, which are transmitted to the spinal cord via the pelvic nerve (Lincoln and Burnstock, 1993) (Fig. 5a). These impulses cause reflex activation of the sympathetic nucleus in the thoraco-lumber spinal cord (Thor and Donatucci, 2004) which activates the efferent sympathetic fibres of the hypogastric nerve to release noradrenaline peripherally (Lincoln and Burnstock, 1993). At the neuroeffector junctions in the human detrusor and urethral smooth muscles, noradrenaline mediates urine storage through activation of β_3 adrenergic receptors to relax the detrusor smooth muscle (explained earlier in the chapter) (Yamaguchi, 2002) and stimulation of α_{1A} adrenergic receptors to induce contraction of the urethral sphincter smooth muscle (Walden et al., 1997). The main effect of sympathetic innervation has been found to involve the outlet region, where it mediates contraction of bladder neck and proximal urethra during the storage phase (Lincoln and Burnstock, 1993). During the emptying phase, this sympathetic pathway was found to be inhibited by the supraspinal mechanisms which resulted in detrusor contraction and relaxation of urethra (Thor and Donatucci, 2004). These Aδ-afferents from the stretched bladder wall and the sympathetic efferent fibres constitute a ***vesicospinovesical storage reflex arc*** which maintains the bladder in relaxed mode while the proximal urethra and bladder neck are contracted (Lincoln and Burnstock, 1993).

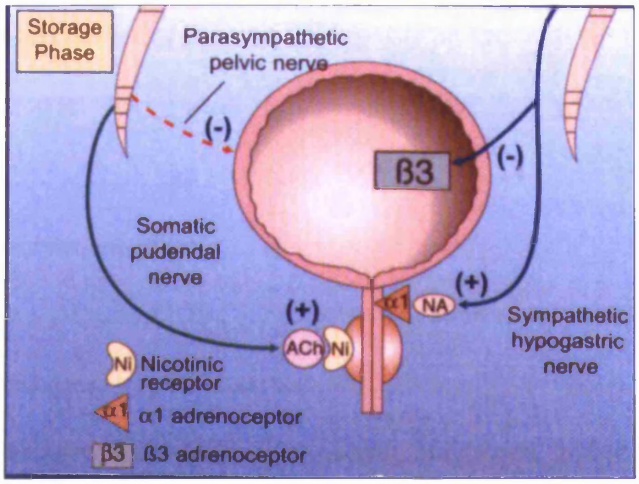


Fig. 5a *Peripheral action of neurotransmitters on micturition cycle: storage phase*

(Reproduced from Clinical Manual of Incontinence in Women, based on the Reports of the 3rd International Consultation on Incontinence, 2005 , edited by Abrams P, Artibani W, Cardozo L, Khoury S and Wein A)

A sudden increase in intra-abdominal pressure (on coughing, laughing or sneezing), initiates a more rapid somatic storage reflex (***pelvic-to-pudendal***), also called the guarding or continence reflex (DeLancey, 2006). The afferent impulse traverses through myelinated Aδ-fibres in the pelvic nerve to the sacral spinal cord and activates the efferent somatic urethral motor neurons, located in the nucleus of Onuf (Danuser and Thor, 1995; Thor and Donatucci, 2004). Activation of Onuf's nucleus augments release of acetylcholine from pudendal nerve terminal at the neuromuscular junction that acts on muscarinic receptors on the rhabdosphincter initiating muscle contraction (Danuser and Thor, 1996). This pathway is tonically active during the storage phase. Sudden increase in abdominal pressure makes this pathway 'dynamically active' (DeLancey, 2006) to contract rhabdosphincter muscle. Afferent impulse is also transmitted to the mesencephalic

periaqueductal grey matter (PAG) and lateral aspect of the pontine micturition centre (L-region). As mentioned earlier, a supraspinal input from the pons projecting directly to the nucleus of Onuf, was also found to be important for voluntary control of this rhabdosphincter muscle (Blok and Holstege, 1999).

2.5.2 The emptying phase

Proprioception, the sensation of bladder distension, is transmitted as an afferent impulse from the stretch receptors on the bladder wall; through the spinal tract neurons to the periaqueductal grey matter (PAG) in the rostral brainstem. The bladder sensation has already been discussed earlier in the chapter. PAG also receives afferent impulse from cerebral cortex and hypothalamus (Blok et al., 1998).This information is integrated in the PAG and the medial part of the pontine micturition centre (the M-region), that control the descending pathways in the micturition reflex (Sugaya et al., 2005; DeLancey, 2006). Thus the pontine micturition centre (PMC) acts like a 'switch' in the micturition reflex (De Groat, 1990; DeLancey, 2006), inhibiting the parasympathetic activity in the descending pathways when there is low activity in the afferent fibres, and activating the parasympathetic pathways when the afferent activity reaches a particular threshold (De Groat, 1993). The threshold is believed to be set by the inputs from more rostral areas in brain (Athwal et al., 2001). The cerebral cortex can consciously facilitate or inhibit this micturition reflex (***vesicobulbovesical reflex***). If it is decided to postpone micturition until a convenient time, the cerebral cortex suppresses the parasympathetic impulses from the PMC and sends signals to the lower urinary tract to contract the rhabdosphincter and the levator ani (Blok et al., 1998).

Thus when the desire to pass urine becomes very strong, the above mentioned 'threshold'

is reached and the activation of the parasympathetic nucleus in the spinal cord results in release of acetylcholine (ACh) from the pelvic parasympathetic efferent fibres (Lincoln and Burnstock, 1993). ACh stimulates the muscarinic (M_3) receptors of the detrusor smooth muscle to induce bladder contraction (Hegde and Eglen, 1999). Simultaneously the PMC sends impulses to the pudendal motor nucleus that inhibits the activity of the pudendal nerve, allowing the rhabdosphincter to relax (De Groat, 2006) (Fig. 5b).

Although the role of M_2 receptors in normal bladder contraction is less clear than those for M_3 receptors, it has been suggested that M_2 receptors may act by opposing sympathetically mediated (by activating β_3-adrenoreceptors) smooth muscle relaxation, via inhibition of adenylyl cyclase, resulting in decreasing level of cAMP (Hegde et al., 1997). M_2 receptor stimulation can also activate non-specific cation channels (Kotlikoff et al., 1999) and inhibit K_{ATP} channels through activation of PKC (Bonev and Nelson, 1993).

As mentioned earlier in the chapter, the possible role of α-adrenergic receptors in proximal urethral smooth muscle may explain the active relaxation of smooth muscle proximal urethra in initiation of micturition (Bennet et al., 1993).

A mechanical explanation of urethral opening as a primary factor was suggested by Tanagho (1992), who described that the opening was due to the contraction of longitudinal muscles at bladder base going to urethra and thereby pulling apart the circular muscle fibres while the sphincter relaxes, or due to the reduction of basal tone of urethral muscle. Moreover, relaxation of the basal tone of levator ani muscle, which normally keeps the urogenital hiatus closed, similar to the external anal sphincter closing the anus, contributes to the process of initiation of micturition (Nichols et al., 1970). These coordinated effects of detrusor contraction, relaxation of the urethral smooth muscle and rhabdosphincter and

relaxation of the levator ani muscle result in increased detrusor pressure and reduced urethral pressure and allow the bladder to empty.

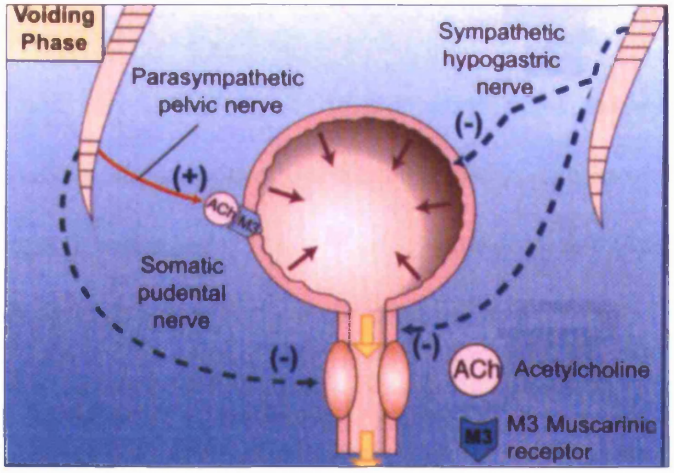


Fig. 5b *Peripheral action of neurotransmitters on micturition cycle: voiding phase (Reproduced from Clinical Manual of Incontinence in Women, based on the Reports of the 3rd International Consultation on Incontinence, 2005 , edited by Abrams P, Artibani W, Cardozo L, Khoury S and Wein A)*

2.6 Innervations of the mammalian urinary bladder

Various mammals (rat, dog, cat, swine, guinea-pig) are used for studying structure and function of the lower urinary tract and often, results that were found in one species are directly transferred to the human (Neuhaus et al., 2001). This study has been carried out using rat urinary bladder. Therefore a brief overview of innervation of mammalian bladder will be presented. Rats are popular animals used to study urinary bladder function (Steers, 1994). Several published studies have provided extensive information on normal micturition including *in vivo* and *in vitro* bladder contractile function, functional anatomy and biochemical pathways at cellular level (Levin et al., 1984; Steers, 1994). The

maximum contractile responses to different stimuli are found to be significantly greater in rat bladder compared to the rabbit bladder, which may be associated to some extent to a greater involvement of Ca^{2+} in the contractile responses of rat bladder compared to that of rabbit bladder (Damaser et al., 2000).

In spite of important species differences in the nature of excitatory innervation of the detrusor smooth muscle, the response to intramural nerve stimulation from all mammals studied is the same, i.e. contraction. This excitatory innervation to detrusor smooth muscle is via both cholinergic and non cholinergic mechanisms (Sibley, 1984). Although the evidence for cholinergic innervation is well established, it has also been recognised for a long time that in most mammalian bladders, cholinergic receptor blockers, such as atropine, fail to abolish the contractile response to pelvic nerve stimulation (atropine resistance). Atropine resistance was reported as early as 1895 by Langley and Anderson. This atropine resistant component is also 'nonadrenergic', as activation of β-adrenoceptors causes relaxation of detrusor smooth muscle, and although α-adrenoceptor agonists are excitatory in many species (Taira, 1972), the frequency response curves of the muscle strips to nerve stimulus remain unchanged in the presence of α-adrenoceptor antagonists like phentolamine (Sibley, 1984).

In his experiment, Sibley (1984) compared the spontaneous activity in bladder muscle strips from man, pig and rabbit. The nerve-mediated activity of detrusor muscle was studied by application of electrical field stimulation. It was concluded that human detrusor muscle contractile response evoked by electrical field stimulation was exclusively cholinergic, in contrast to the mammals studied in which a significant non-cholinergic component was demonstrated (Sibley, 1984). The widely accepted concept was that the

atropine resistant response was due to a 'nonadrenergic, noncholinergic' or NANC transmitter (Ambache and Zar, 1970). This transmitter was subsequently identified as ATP (Burnstock et al., 1972; Levin et al., 1980; Kasakov and Burnstock, 1982). It has been demonstrated that a specific antagonist for ATP receptors like Arylazido Aminopropionyl Adenosine Triphosphate or $ANAPP_3$ (Hogaboom et al., 1980) can block the effects of applied ATP and partially blocks the pelvic nerve-evoked contractile response without blocking cholinergic mechanism, (Theobald, 1982). Kasakov and Burnstock (1982) have described another analogue, α, β methylene ATP which desensitises P_2 purinoceptors after prolonged exposure (following initial excitation). Thus both acetylcholine and ATP have been suggested as co-transmitters in intrinsic parasympathetic neurons in the bladder (MacKenzie, 1982). The functional importance of these neurotransmitters for micturition contraction in normal unanaesthetized rat has been demonstrated (Igawa et al., 1993). Intra-arterial administration of ATP close to bladder resulted in a rapid, phasic and dose related increase in bladder pressure followed by bladder emptying. Pre-treatment with prolonged exposure to α, β-methylene ATP blocked the response to ATP. The administration of cholinergic receptor agonist like carbachol also produced similar excitatory effect. However, bladder emptying was demonstrated after blocking the micturition reflex by intrathecal administration of morphine suggesting that drug induced bladder emptying requires an intact micturition reflex (Igawa et al., 1993). This dual innervation is found in most animal species and purinergic mechanism is probably involved in behavioural activity, like scent marking, where complete emptying of bladder is not required (Brading and Inoue, 1991; Giglio et al., 2001). Chancellor et al. (1992) supported the fact that ATP may be important in the initiation of micturition, as ATP

generated pressure is more rapid than cholinergic stimulation alone.

2.7 Ultrastructure of smooth muscle cell

The orientation of and interaction between the smooth muscle cells in the bladder are both important in determining the functional nature of the bladder wall, specifically the impact of activity in the cells on its shape and intraluminal pressure (Andersson, 2004). The individual detrusor muscle cells are typical spindle-shaped smooth muscle cells with a central nucleus. In a fully relaxed state, the muscle cells are several hundred microns long, and the widest diameter is 5 – 6 μm (Gosling and Dixon, 1975). The detrusor smooth muscle cells contain smooth endoplasmic reticulum (SER) or sarcoplasmic reticulum (SR) around the nucleus and lots of mitochondria. The SER provides a site for intracellular calcium storage for utilization in muscle contraction and the mitochondria provides the ATP necessary for muscle contraction (Dixon and Gosling, 1990).

Bozler (1941) classified two types of smooth muscle on the basis of features associated with electrical coupling between fibres. The fibres of 'single-unit' smooth muscle are electrically and mechanically coupled to each other via several points of close contact, gap junctions. Gap junctions are essentially pathways with low-resistance that allow intercellular movement of ions, spreading an electrical signal rapidly throughout the tissue (Brink, 1998). This type is typical of visceral organs like gastrointestinal tract or urinary bladder. Multiunit smooth muscles are thought to be composed of discrete muscle fibres operating independently of each other. 'Multi-unit' smooth muscle contains relatively few gap junctions compared to single-unit, thus fewer number of adjacent fibres can act as a unit and are found in the iris of eye or airways of lung (Bozler, 1941).

2.7.1 Contractile proteins and filaments

The cytoplasm of smooth muscle is found to be packed with two types of contractile myofilaments, namely thick and thin. The thick filament is composed of the protein myosin and the thin filament is made up of the proteins, actin and tropomyosin (Andersson and Arner, 2004). Contraction of smooth muscle is a result of an interaction between the contractile myofilament proteins, actin and myosin, in a similar way to other muscle types. However, these myofilaments were not found to be arranged in discrete myofibrils in regular sarcomeric structures as in striated muscle. Instead, contractile units consist of parallel arrangements of thick and thin filaments crossing diagonally from one side of the fibre to the other (Dixon and Gosling, 1983). Within these units, myosin molecules are attached either to structures called 'dense bodies' (will be described later in the chapter) or to the cell membrane (Bond and Somlyo, 1982). Actin filaments have no constant relationship to myosin filaments (Dixon and Gosling, 1983).

There is minimal difference between the concentration of actin in smooth muscle and skeletal muscle (Cohen and Murphy, 1979). Thin filaments are composed of double helical strand of identical globular actin molecules resembling two strands of pearls twisted around each other (Nelson and Cox, 2005). The single globular molecule forming each strand is called G-actin. G-actin molecules (4nm in diameter, molecular weight of 50,000 daltons) are assembled into single stranded F-actin (about 1.0μm long). F-actin strands are twisted together to form the backbone of the thin filament (Nelson and Cox, 2005). In addition to actin, thin filaments also contain a regulatory protein, tropomyosin. Double helixes of tropomyosin lie on the F-actin strands. Each G-actin subunit has a single site where a myosin head can bind. As the F-actin strands are twisted together,

some of these myosin binding sites on G-actin molecules are not accessible to the myosin heads (Wu and Ma, 2004).

Four different isoforms of actin are expressed in smooth muscle, α-, β-, and two forms of γ-actin (Vandekerckhove and Weber, 1979). In bladder muscle, α-, β-, and γ-actin are present in relative proportions 33%:25%:42% for human (Malmqvist et al., 1991a), and 41%:19%:40% for rat (Malmqvist et al., 1991b).

Each thick filament is 1.5 μm long and 15 nm in diameter and is composed of several hundred myosin molecules, each with a molecular weight of about 500,000 daltons (Nelson and Cox, 2005). The backbone of the thick filament is a bundle of long chains of myosin molecules, the heads of which extend out to the sides. Myosin molecules have a polarity that affects how they associate with each other to form the thick filaments (Xu et al., 1996). Each myosin unit is a double structure composed of two heavy polypeptide chains that form a rod like tail and a double head (Geeves and Holmes, 1999). The heads, which bind to actin and engage in ATPase activity, are joined to the rest of the myosin molecule by peptide regions acting as hinges (Nelson and Cox, 2005). Three or four smaller (light) polypeptide chains are associated with the head and play a regulatory role, which will be discussed later in the chapter.

Smooth muscle contains a lower proportion of myosin (approximately three to five times less) than striated muscle (Malmqvist and Arner, 1991). The ratio of actin to myosin filaments is 15:1 in smooth muscle as compared to 2:1 in striated muscle (Somlyo, 1980). In the rat bladder tissue, the myosin concentration was found to be close to the above range (17mg/g smooth muscle cell) (Malmqvist and Arner, 1991).

Myosin present in smooth muscle belongs to the myosin II super family of filaments

(Sellers, 2000). Variants of the heavy chain structure allow functional differences of different muscle tissue to exist (Eddinger and Wolf, 1993). Gel electrophoresis identified two heavy chain variants, the SM1 (molecular mass ~ 204 kDa) and the SM2 (~ 200 kDa) (Eddinger and Murphy, 1988). The relative content of SM1 is about 70% in rats and 40% in humans (Malmqvist and Arner, 1991). The functional importance of any alteration in the SM1/SM2 ratio on the smooth muscle contraction is unclear. Some studies have reported an association between the maximal shortening velocity of muscle (V_{max}) and the SM1/SM2 expression in tissue (Hewett et al., 1993). Myosin heavy chains in mammals have also been classified into two other isoforms, SM-A and SM-B on the basis of an extra seven amino acid insert (SM-B) (Babij et al., 1991), providing four possible isoforms SM1-A, SM1-B, SM2-A and SM2-B in total (Babu et al., 2000). Myosin with the extra amino acid insert (SM-B) was found to drive actin at a higher velocity (V_{max}) than myosin without the insert (Kelly, 1993; Eddinger and Meer, 2001). Urinary bladder muscle belongs to a comparative fast smooth muscle type due to the high expression of the inserted myosin isoform (~80-90% SM-B) (Arafat, 2001).

Two types of myosin essential light chains were identified, the acidic LC_{17a} and the basic LC_{17b}, which combine randomly on the myosin heavy chain (Cavaille, 1986). A correlation has been suggested between the expression of essential light chain variants in smooth muscle and both V_{max} and the ATPase activity; high LC_{17b} content correlated with low shortening velocity and low ATPase activity (Malmqvist, 1991a). Comparative studies of smooth muscle also established an association between both the essential light chain and inserted heavy chain SM-B composition (Somlyo, 1993).

Besides the myosin heavy chains described above, smooth muscle can also express type II

filament forming non-muscle myosin isoforms (Giuriato, 1991): type A (NM-MHC-A, molecular mass 196 kDa) and type B (NM-MHC-B, molecular mass 198 kDa) (Katsuragawa et al., 1989). The expression of these non-muscle myosins was found to be low in urinary bladder, about 10% of total heavy chain in rat (Malmqvist and Arner, 1991). Morano et al. (2000) showed a contractile function of non-muscle myosin in smooth muscle, and suggested that this might be important during foetal life or in adult tissue with high content of nonmuscle myosins, e.g., large elastic arteries. Sjuve et al. (2001) reported that in hypertrophic rat bladders following obstruction, the number of NM-MHC-B immunoreactive cells was markedly increased. However, the function of the non-muscle myosins in the urinary bladder is still unclear and no data could be found on the non-muscle myosin from human bladder. As the concentration of non-muscle myosin in urinary bladder is quite low, it is improbable for this myosin to play any significant role in bladder contraction (Andersson, 2004).

2.7.2 Cytoskeleton and other structures

All individual cells in smooth muscle contain complex networks of protein fibers which constitute the cytoskeletal system of the cell. The cytoskeleton has been suggested to play an important role in maintaining the cellular structural framework and providing it with cell membrane attachment (Small and Gimona, 1998). The cytoskeleton is made up of three kinds of protein filaments, namely actin filaments (microfilaments), intermediate filaments and microtubules.

As mentioned earlier in the chapter, the contractile apparatus lacks a myofibrillar structure with visible cross-striations. The contractile units appear to consist of thin filaments attached to a cytoskeleton that overlap with much smaller numbers of myosin-containing

thick filaments. Instead of Z-lines that are present in skeletal muscles, specialized structural proteins called dense bodies are found in smooth muscle cell cytoplasm and cell membrane (Berne and Levy, 2003). The thin filaments are attached to these dense bodies and membrane dense areas (present in the cell membrane) to build up the contractile framework. The dense bodies and membrane dense areas are also linked by the cytoskeletal (10 nm) intermediate filaments (Mabuchi et al., 1997). Both cytoplasmic dense bodies and membrane dense areas (cell adhesion complexes) on cell membrane contain the protein α-actin (Janqueira and Carneiro, 2001). The membrane dense areas or focal densities also contain other proteins like filamin, calponin, vinculin and tensin (Small and Gimona, 1998).

The 'intermediate filaments' are mainly composed of the proteins desmin and vimentin (Osborne et al., 1987). Desmin is the most important protein of intermediate filament in all smooth muscles and vimentin is found predominantly in vascular smooth muscle (Janqueire and Carneiro, 2001). Studies have shown that desmin intermediate filaments are involved mainly in transmission of the active force in the smooth muscle, possibly by anchoring the intracellular contractile apparatus to the plasma membrane (Travo et al., 1982). Desmin is the predominant intermediate filament in urinary bladder, both in rat and human (Malmqvist et al., 1991a). Malmqvist et al. (1991b) demonstrated an up regulation of desmin concentration in bladder smooth muscle hypertrophy due to outlet obstruction, which returned to normal once the obstruction was removed.

Numerous invaginations of the plasma membrane were described at the periphery, called "caveolae", which were vesicular or saccular. These were believed to be involved in calcium regulation in muscle contraction, working with sarcoplasmic reticulum (SER) to

sequester calcium for storage in absence of its need (Small and Gimona, 1998). Finally, smooth muscle cells were found to be interconnected by the specialized communication channels, the 'gap junctions', enabling intercellular transfer of small molecules or ions via these junctions. These gap junctions provide communication links that regulate contraction of the entire bundle of smooth muscle (Dixon and Gosling, 1990).

2.7.3 Interplay of Actin-Myosin

In a muscle at resting phase length, some overlapping between the thick and thin filaments was noted. During contraction, the thick filaments pull the thin filaments and the pulling force is caused by the combined action of all the crossbridges formed when the myosin heads interact with actin binding sites (Guilford and Warshaw, 1998). In a contracting muscle, each myosin head undergoes a cycle of (a) adherence to an adjacent thin filament, followed by (b) a powerstroke that moves the head about 10nm relative to the site of attachment, followed by (c) detachment from the thin filament and then (d) beginning of a new cycle (Nelson and Cox, 2005). An individual myosin head can perform this cycle several times in a second. A myosin head that is ready to bind to actin during force generation and shortening of muscle has hydrolyzed a molecule of ATP to the products ADP and inorganic phosphate (Arner et al., 1998). ADP and inorganic phosphate remain bound to the myosin, forming a myosin-ADP complex. Myosin-ADP complex represents a 'high-energy' form of myosin that can bind to exposed actin sites in the presence of Ca^{2+}. Myosin heads are thought to have several sites that can interact with different binding sites on actin. Once the first site on the myosin head has bound to the first site on actin, the energy of the myosin-ADP complex is gradually transformed into mechanical energy by sequential binding with sites of greater affinity. This rotates the myosin head,

generating tension between thick and thin filaments. This is the cross-bridge power stroke, during which ADP and inorganic phosphates are released (Osterman and Arner, 1995). At the end of powerstroke, the myosin head is tightly bound to the thin filament in a low-energy form of actomyosin. In a smooth muscle, there is no practical limit to the distance that individual thick and thin filaments can slide relative to each other, which is very different from the striated muscle, where this sliding is limited only up to a short distance. The organization of smooth muscle is an adaptation for force development over a broad range of operating lengths. This adaptation is mainly for the muscle fibres in the walls of organs that accommodate large volumes like intestine and urinary bladder (Meiss and Pidaparti, 2005).

2.8 Mechanical properties of bladder smooth muscle

The bladder wall undergoes large changes in extension during normal filling and emptying. Several studies looked into the mechanical properties of intact bladders and also isolated bladder strips to determine the relationship between length and wall tension (Uvelius, 2001; Kuo and Seow, 2004; Meiss and Pidaparti, 2005). During normal bladder filling, intravesical pressure rises minimally with distension of the bladder wall. In humans, at filling rates up to about 100 ml/min, the pressure rises less than 5 cm/ 100 ml of water. Similar observations were noted in pigs (Sibley, 1985), and a similar volume-pressure relationship is found in rabbit bladder *in vitro* (Levin and Wein, 1982). *In vitro* studies subjecting detrusor smooth muscle to stretch and relaxation support the concept that bladder tonus and accommodation result from physical properties of the bladder wall and are not dependent upon neural activity (Finkbeiner, 1999). Longhurst et al. (1995)

demonstrated that the contractile responsiveness of rat urinary bladder strips was independent of the length of the detrusor muscle strips. Although there were some differences between the cholinergic responsiveness of strips from the ventral and dorsal surfaces of the bladder, the differences were so small that for most studies they would probably have no influence on data interpretation.

In order to interpret the mechanical behaviour, Uvelius and Gabella (1980) investigated the structural changes in rabbit and guinea pig bladder wall in passive distension. They reported that the length of the individual muscle cell increases linearly with bladder radius over normal functional volumes, the mean length of the cells in guinea pig bladder increasing nearly fourfold (from 160 to 580μm) during filling. In his earlier work, Uvelius (1976) showed that above a certain elongation, the passive tension was found to have a steep rise. Brading (2006) has highlighted another factor which is of importance in determining the mechanical behaviour of bladder muscle, i.e. spontaneous activity of the smooth muscle cells that occurs in the absence of any nervous stimulation. Gabella (1977) reported that the viscoelastic property of bladder smooth muscle perhaps implied the ability of individual muscle cells to generate force (5-6 mN which would be equivalent to 60 N/cm^2 cross-sectional area), independent of its length and far greater than that of striated muscle cell. The active force of the bladder muscle was found to be dependent on the extent of wall stretch (Uvelius and Gabella, 1980). They calculated the active force produced by the muscles in response to supramaximal pelvic nerve stimulation in the intact guinea pig bladder, by using volume- pressure relationship and muscle cross-sectional area. This active force was found to be nearly maximal over a wide range of cell lengths and was achieved at filling volumes of roughly 25% to 75% of bladder capacity.

At maximum capacity the active force was 75% of its highest value and about 25% when the bladder was empty (Uvelius and Gabella, 1980).

The cytoskeleton present in the smooth muscle cells may play a small role in contributing the passive tension generated in the bladder wall during stretching (Sjuve et al., 1998). The extracellular matrix present in between the muscle bundles is comprised mainly of elastic fibres and collagen fibrils (types I and III) and is primarily responsible for the passive viscoelasticity (Gabella, 1995; Kim et al., 2000).

The maximal rate at which the myosin propels the actin filaments denotes the maximal shortening velocity (V_{max}) of muscle. The bladder detrusor muscle has V_{max} about 10-times slower than that of the striated muscle (Cooke and Pate, 1985). The rate of the ADP release at cross-bridge dissociation during actin-myosin interaction is an important factor in determining the magnitude of V_{max} (Siemankowski et al., 1985). The ADP binding was found to be stronger in smooth compared to striated muscle (Lofgren et al., 2001). The relationship between active force and the maximal shortening velocity normally follows a hyperbolic curve (Hill, 1938). The kinetics of the detrusor muscle wall contraction during voiding was found to operate along this force-velocity relationship depending on intravesical pressure and bladder volume.

Another factor that plays an important role in the mechanical behaviour or the bladder is perhaps the spontaneous activity of the detrusor smooth muscle cells occurring in the absence of any nerve stimulation. Smooth muscles in both bladder and urethra show spontaneous contractile activity during the filling phase (Brading, 2006). The role of the spontaneous activity seems to be to allow the individual muscle bundles to adjust their length in response to filling. The normal bladder appears to maintain its shape throughout

filling, allowing micturition to be started rapidly whenever it is convenient for the bladder to be emptied.

Thus to summarize, during filling, the intravesical pressure remains stable without any significant increase and this passive nature of the bladder can be attributed to its viscoelasticity and plasticity. As the bladder fills, the muscle bundles undergo reorganization, and the muscle cells within the bundle get stretched up to four times their length in the empty bladder.

2.9 Excitation-contraction coupling

Smooth muscle contraction was found to be initiated by an increase in the intracellular Ca^{2+} concentration (Bozler, 1969; Ruegg, 1992), either by Ca^{2+} influx into the cell cytoplasm via Ca^{2+} channels or by release of Ca^{2+} from the sarcoplasmic reticulum (SR) (Maggi et al., 1989a; Kotlikoff et al., 1999; Brading, 2002; Ganitkevich and Isenberg, 2002). Bolton et al. (1999) suggested that the main contributors to increases in the intracellular Ca^{2+} concentration are Ca^{2+} influx through voltage-gated channels opened by depolarization or during action potential (AP) or slow-wave discharge, and Ca^{2+} release from store sites in the cell by the action of inositol trisphosphate (IP_3) or by Ca^{2+}-induced Ca^{2+}-release (CICR) via activation of RyRs (Xu et al., 1994; Lesh et al., 1998; Brading, 2002). The importance of Ca^{2+} release from the SR in detrusor muscle contraction was evident from studies carried out using blockers of SR function demonstrating that both nerve- and agonist- induced contractions were dependent on a functional SR (Damaser et al., 1997). Apart from contributing to the increase in intracellular Ca^{2+}, the SR can also influence bladder contractility by modulating the K channel activity causing relaxation

(Grant and Zuzack, 1991).

Activation of the contractile machinery involves a Ca^{2+}-dependent phosphorylation of the myosin heads and there are several regulatory processes which may be able to alter the relationship between free Ca^{2+} and the amount of tension generated (Horowitz et al., 1996; Gallagher et al., 1997). Key elements for calcium regulation of smooth muscle contraction involve (a) phosphorylated form of myosin light chain binds to actin to stimulate the MgATPase, which supplies energy for the contractile process; (b) myosin regulatory light chains are phosphorylated (on serine at position 19) by a myosin light chain kinase (MLCK); (c) the MLCK is activated by a Ca^{2+} -calmodulin complex; (d) the formation of the Ca^{2+} -calmodulin complex is dependent on the concentration of intracellular Ca^{2+} (Horowitz et al., 1996; Stull et al., 1996); (e) muscle contraction is terminated on dephosphorylation of the regulatory light chain by myosin light-chain phosphatase (MLCP). Several subtypes of the protein phosphatase were identified in different types of muscles. Protein phosphatase 1 (PP1) and protein phosphatase 2 A (PP2A) were demonstrated in smooth muscles (Cohen, 1989); PP1 was found to be present in bladder smooth muscle (Shirazi et al., 1994). Sellers (1985) suggested that release of phosphate ion was controlled by phosphorylation of the myosin light-chain. Kitazawa and Somlyo (1990) reported on a possible correlation between the myosin light chain phosphorylation and the concentration of Ca^{2+}. Different regulatory mechanisms affecting functions of MLCK and MLCP ultimately influence Ca^{2+} sensitivity in smooth muscle cells (Stull et al., 1990; Somlyo et al., 1999; Somlyo and Somlyo, 2003).

Pfitzer et al. (1984) showed that relaxation of smooth muscle in presence of cGMP and the cGMP-dependent kinase (PKG) was due to inhibition of Ca^{2+} movement and Ca^{2+}

sensitization. Studies also highlighted the role of protein kinase C (PKC) in modulating Ca^{2+} sensitivity to smooth muscle by affecting MLCP (Jensen et al., 1996). A specific kinase (*Rho*-associated kinase), activated via G proteins was found to play an important role in inhibition of MLCP and Ca^{2+} sensitization (Somlyo and Somlyo, 2000). Studies have also demonstrated the presence of two different types of *Rho*-kinase (ROCK I and ROCK II) (Wibberley et al., 2003), the concentrations of which may be affected by different kinds of bladder pathology (Bing et al., 2003).

Caldesmon, a calmodulin-and actin-binding protein, present in the smooth muscle was found to play an inhibitory role in muscle contraction by binding to the thin filaments interacting with tropomyosin and myosin (Furst, 1986; Marston and Redwood, 1993) or that by influencing actin myosin interaction (Chalovich et al., 1987).

2.10 Electrical properties of bladder smooth muscle

2.10.1 Resting membrane potential and action potential

Various investigators have looked into the electrophysiological properties of detrusor muscle from different animals, including humans (Ursillo, 1961; Creed, 1971; Creed et al., 1983; Brading and Mostwin, 1989; Brading, 1992; Hashitani et al., 2000). The resting membrane potential (conventionally measured inside the membrane and is negative in the resting state) depends on the relative permeability of the membrane to ions, and the concentration gradients of ions across the membrane (electrochemical potential gradient). The flow of each ion across the plasma membrane tends to bring the resting membrane potential (RMP) towards equilibrium potential for that ion. The ability of an ion to bring the membrane potential towards its equilibrium potential is higher if the plasma membrane

is more conductive to that particular ion (Berne and Levy, 2003).

The Na^+ - K^+ -ATPase, located in the plasma membrane, expels Na^+ actively out from the cell and take K^+ actively into the cell. The Na^+-K^+ pump is responsible for the high intracellular K^+ concentration and low intracellular Na^+ concentration. As the pump helps shifting a larger number of Na^+ ions out than K^+ ions in (3 Na^+ to 2 K^+), it causes a net transfer of positive charge out of the cell resulting in a negative equilibrium potential, which contributes to the generation of RMP. However, the direct contribution of the Na^+-K^+ pump to the RMP is usually small – less than 5mV. The major process that contributes to the generation of RMP is the ionic diffusion, particularly that of K^+, which has the largest resting conductance (about 10 times higher compared to Na^+) and thus has the largest impact on the RMP (Hille, 1992; Berne and Levy, 2003). For this reason, changes occur in the concentration of K^+ in a patient's extracellular fluid will affect the RMP of all cells. Published studies showed that bladder smooth muscle has a relatively low RMP of -47 mV in rat detrusor muscle (Creed, 1993) and in isolated human detrusor muscle it was found to be between -47 and -55 mV (Montgomery and Fry, 1992).

The mechanisms underlying action potential generation in all excitable tissues involve a sequence of voltage-dependent changes in membrane permeability, leading to ion flow down pre-existing electrochemical gradients (Mostwin, 1986). The initial influx of ions generates an inwardly directed current which depolarizes the cell membrane (Na^+ and Ca^{2+} ions). This is followed by a switching off of the inward current and the activation of an outward repolarizing current (K^+ ions). In fast conducting tissues like nerve and striated muscle, the inward current is carried by Na^+ ions; and as the extracellular sodium concentration is large, the current flow per unit area of cell membrane can be high (Creed,

1971). This leads to a rapid upstroke (up to 100 V/s), and fast propagation of the signal. Smooth muscles do not require such rapid propagation of action potentials, and the upstroke of action potential is caused by inflow of calcium ions (10 V/s). Electrical connections between the smooth muscle cells in muscle bundles allow the bundles to behave as cables along which action potentials can propagate. Intracellular recorded action potentials depolarize the cells by about 50 mV, and have duration at half amplitude of 10-15 ms (Mostwin, 1986).

The spontaneous action potentials generated by slow waves of depolarization were found in detrusor muscle strips from different animals including human (Ursillo, 1961; Mostwin, 1986; Visser and van Mastrigt, 2000a). Investigations in guinea pig bladder smooth muscle showed that the rising phase of the action potential was generated mainly by Ca^{2+} and not by Na^{+} influx, as tetrodotoxin (TTX), a Na^{+} channel blocking agent, failed to block elicited action potentials (Creed, 1971). The significant role of Ca^{2+} influx in depolarization was also confirmed by other researchers working with different animals including human (Mostwin, 1986; Montgomery and Fry, 1992; Bramich and Brading, 1996; Hashitani et al., 2000). In studies on guinea pigs, it was suggested that the repolarization phase of action potential was likely to be due to inactivation of the inward Ca^{2+} current and activation of an outward K^{+} current that was partially Ca^{2+} dependent (Imaizumi et al., 1998). Studies on guinea pigs have also shown that ATP, by activation of purinoceptors, initiated excitatory junction potentials (EJPs), which in turn triggered action potentials and the influx of Ca^{2+} through L-type Ca^{2+} channels, and also Ca^{2+} release from intracellular Ca^{2+} stores (Hoyle and Burnstock, 1985; Fujii, 1988; Collier et al., 2000; Hasitani et al., 2000). Gallegos and Fry (1994) demonstrated that the action

potential configuration in isolated human detrusor smooth muscle cells was altered in different pathological conditions due to alterations in the inward current of Ca^{2+}.

2.10.2 Role of ion channels in increasing free intracellular calcium

An increase in intracellular calcium concentration is usually achieved by activating release from internal stores (SER) or by increasing the permeability of the membrane to calcium (Brading and Inoue, 1991). Ion channels present on the cell membrane have been shown to be the key regulators for maintaining membrane potential by controlling permeability of different ions with the two most extensively investigated ion channels being calcium and potassium (Andersson, 2004).

2.10.2.1 Ca^{2+} channels

The key process needed for the activation of smooth muscle contraction is definitely an increase of intracellular Ca^{2+} concentration. This happens either by Ca^{2+} release from intracellular store or by Ca^{2+} influx from extracellular space (Andersson and Arner, 2004). Although several types of Ca^{2+} channels have been demonstrated in smooth muscle (Berridge et al., 2003), but in detrusor muscle the involvement of two main types of calcium channels have been proven, namely the voltage-sensitive channels and receptor operated channels. Activation of either will allow calcium influx down the electrochemical gradient (Brading, 1992; Berridge et al., 2003).

a. Voltage-sensitive L-type calcium channels

Different types of Ca^{2+} channels are present in smooth muscle (Berridge et al., 2003), but for detrusor smooth muscle, most of the information are based on the voltage-operated Ca^{2+} channels (Andersson and Wein, 2004). The Ca^{2+} influx into the cell cytoplasm through these voltage-operated L-type Ca^{2+} channels depends on the membrane potential,

as depolarization of the cell membrane augments opening of these calcium channels resulting in calcium influx (Ganitkevich and Isenberg, 1992). The Ca^{2+} influx can in turn induce release of Ca^{2+} from intracellular stores, which was responsible primarily for the phasic rise in intracellular Ca^{2+} concentration. The repolarization is most likely due to inactivation of the Ca^{2+} current and potentiation of an outward K^+ current (Montgomery and Fry, 1992). When depolarized by high K^+ solutions or by prolonged application of agonists, the Ca^{2+} channels can be closed even at persistent depolarization (Brading, 1992) resulting in fading of the contractile response. The Ca^{2+}-induced inactivation of the channels may also be a contributing factor to the inability to sustain the muscle tone (Nakayama and Brading, 1993).

Although cholinergic stimulation primarily results in bladder muscle contraction, it has been shown that muscarinic receptor agonist (carbachol) can suppress the L-type Ca^{2+} channel inward current in pig and human detrusor cells (Yoshino and Yabu, 1995). However, the significance of this muscarinic receptor-mediated inhibitory action on L-type Ca^{2+} channels in normal detrusor contraction is still unclear (Kajioka et al., 2002).

In human detrusor, the extracellular Ca^{2+} influx seemed to play a major role in the activation of muscle contraction (Fovaeus et al., 1987). Several studies have demonstrated that the most common route of entry for the extracellular Ca^{2+} during both muscarinic and or purinergic stimulation was through dihydropyridine-sensitive Ca^{2+} channels (Harriss et al., 1995). However, therapeutically, there is no evidence that calcium antagonists will treat detrusor overactivity (Andersson, 2004). Elliott et al. (1994) studied the effect of treatment with calcium antagonists *in vitro* and *in vivo* on the contractile response of isolated rat and human detrusor muscle. Their results suggested that nimodipine, a calcium

channel blocker had a significant inhibitory effect on rat detrusor muscle *in vitro* and after a single dose *in vivo*, but had no significant effect after 8 days' treatment in vivo. The possible up-regulation of 1, 4-dihydropyridine-sensitive calcium channels may explain the lack of clinical effect of chronic treatment with calcium antagonists in patients with detrusor overactivity.

b. Receptor-operated and store operated calcium channels

Apart from the voltage-gated channels, other types of calcium-permeable channels, non-voltage gated, are also present in smooth muscle cells, which include the so-called receptor-operated calcium channels (ROCCs) and store-operated calcium channels (SOCCs) (Parekh and Putney, 2005). Receptor-operated channels (ROCC) are activated in response to interaction of neurotransmitters such as acetylcholine, or agonist drugs with specific receptors on the cell membrane, the G-protein-coupled receptors (GPCR) (Haugaard et al., 1996). Some agonists cause depolarization of the cell membrane by opening up of channels for intracellular sodium influx and subsequent opening up of voltage-sensitive calcium channels. The store-operated calcium channels (SOCCs) are activated following depletion of the calcium stores within the sarcoplasmic reticulum. The knowledge of the functional roles of ROCCs and SOCCs in smooth muscle excitation-contraction coupling is much less advanced compared to the voltage-gated calcium channels. However, in a recent review of the electrophysiological, functional and pharmacological properties of ROCCs and SOCCs in smooth muscle by McFadzean and Gibson (2002), it was suggested that the two channel types may be closely related, being formed from proteins of the Transient Receptor Potential Channel (TRPC) family, and

their diversity provides a possible explanation for the wide variety of receptor-operated and store-operated responses recorded in smooth muscle.

2.10.2.2 K^+ channels

Potassium channels are the other important ion channels involved in smooth muscle contraction. Normally, the resting membrane potential in smooth muscle cells is determined mainly by the membrane permeability for the potassium ions. An increase in the permeability will lower the membrane potential by augmenting potassium efflux from the cell resulting in opening up of the voltage-operated calcium channels and initiate muscle contraction (Kurihara and Creed, 1972; Andersson, 1992; 2004).

Different types of potassium channels (voltage gated, calcium activated, and ATP sensitive) have been shown to modulate spontaneous electrical and mechanical activity in the detrusor smooth muscle (Brading, 2006).

Among these channels, the importance of two main subtypes found in human detrusor was highlighted, ATP-sensitive potassium channels and Ca^{2+}-activated potassium channels (Ashcroft and Ashcroft, 1990; Bonev and Nelson, 1993; Wickenden, 2002).

a. ATP-sensitive K^+ channels

ATP-sensitive specific K^+ channels (K_{ATP}) are found in urinary bladder smooth muscle which play an important role in regulation of bladder contraction (Andersson, 1992). Petkov et al. (2001) demonstrated that activation of <1% of K_{ATP} channels was sufficient to significantly inhibit action potentials and the related phasic contraction (Petkov, 2001). In smooth muscles, including those from the urinary bladder, potassium channel openers activate K_{ATP} channels, leading to membrane hyperpolarization, reduction in cellular calcium entry, and inhibition of muscle contractility. Electrophysiological studies have

demonstrated the presence of K_{ATP} channels in guinea pig urinary bladder, the modulation of which may regulate bladder contractility (Bonev and Nelson, 1993). The pharmacological properties and the molecular composition of these channels were later examined in detail in the studies of Gopalkrishnan et al. (1999). In this study, they characterized the pharmacology of K_{ATP} channels expressed in cultured guinea pig urinary bladder cells by fluorescence-based membrane potential techniques. It was found that K_{ATP} channels could be activated by metabolic inhibition or by diverse potassium channel openers with rank order potency consistent with those typical of smooth muscle K_{ATP} channels.

Andersson (1992) reported that that K_{ATP} channel openers can inhibit spontaneous bladder contractions, agonist (carbachol)-induced contractions and contractions induced by electrical field stimulation.

b. Ca^{2+}-activated K^+ channels

Small-conductance (SK_{Ca}) and large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) are key regulators of excitability of detrusor smooth muscle (Herrera and Nelson, 2002). Grant and Zuzack (1981) suggested that the large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) are involved in the control of basal tension and possibly the membrane potential, which was later confirmed by other investigators (Heppner et al, 1997; Karicheti and Christ, 2001). The repolarizing phase of action potential was found to be mediated mainly via these large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) along with other types of K^+ channels which are voltage dependant (Klockner and Isenberg, 1985; Fujii et al., 1990; Heppner et al., 1997). After the spike and repolarization, the action potential in detrusor smooth muscle displays a prolonged after-hyperpolarization phase, when the

membrane potential is even more negative than resting membrane potential. SK_{Ca} channels appear to be responsible for this after-hyperpolarization phase (Creed et al., 1983; Brading, 1992). Exploring the relationships between voltage sensitive Ca^{2+} channels, ryanodine receptors (RyRs) and BK_{Ca}/SK_{Ca}, Herrera and Nelson (2002) have shown in their experiments that activation of SK_{Ca} channels depends absolutely on the Ca^{2+} influx through the voltage sensitive Ca^{2+} channels and not on the Ca^{2+} release by RyRs. On the contrary, activation of BK_{Ca} channels depends on both Ca^{2+} influx through the voltage sensitive Ca^{2+} channels and the Ca^{2+} release by RyRs.

Some investigators have suggested that these channels may play an important role in affecting detrusor function in pathological bladder; though specific interactions of ion transport mechanisms through these channels modulating detrusor contraction in vivo needs further evaluation (Davies, 2002; Thorneloe and Nelson, 2003). Several studies on isolated human detrusor and on bladder tissue from other animal species have demonstrated that drugs acting via opening up of potassium channels, reduce both spontaneous contractions and contractions induced by muscarinic receptor agonist (carbachol) and electrical field stimulations (Malmgren et al., 1989; Andersson et al., 2002; Wickenden, 2002; Andersson and Wein, 2004). However, like calcium channel blocking agents, evidence based treatment option with potassium channel openers for overactive bladder is limited (Andersson et al., 2002). However, because these compounds also activate vascular smooth muscle channels causing vasodilatation, the clinical efficacy has been severely limited by hemodynamic side effects including hypotension and tachycardia (Nurse et al., 1991). Wojdan et al. (1999) compared the effects of the ATP-dependent potassium channel agonists ZD6169, celikalim, and WAY-133537 on bladder

contractile function in vitro on isolated bladder strips and in vivo on spontaneous bladder contractions. They reported that the antihypertensive agent celikalim exerted effects on the bladder at doses that significantly reduced systemic blood pressure; whereas both WAY-133537 and ZD6169 inhibited bladder hyperactivity at doses that produced minimal changes in both mean arterial blood pressure and heart rate. They therefore suggested that both WAY-133537 and ZD6169 may have a role in the treatment of bladder instability at doses with minimal hemodynamic side effects. A recent randomized, double-blind, placebo-controlled phase II study that evaluated the efficacy and safety of an ATP-sensitive potassium channel opener- ZD0947 (25 mg/day for 12 weeks) in patients with overactive bladder, published the results to be quite disappointing (Chapple et al., 2006). This may be due to ineffective receptor selectivity.

2.10.3 Stretch-activated channels

Mechanical stretching of cell membrane can activate non-specific cation channels present on detrusor smooth muscle plasma membrane showing similar permeability to Na^+, K^+ and Ca^{2+} (Wellner and Isenberg, 1993). This mechanical stretch of human detrusor muscle cells can also stimulate intracellular Ca^{2+} release, although this can vary between the detrusor muscle cells obtained from stable and unstable (overactive) bladders (Chambers, 1997).

2.10.4 Ligand - activated channels

Ligand-gated ion channels are present on cell membranes that are targets for various neurotransmitters or modulators. Acetylcholine and ATP are of specific interest in detrusor smooth muscle. Activation of purinoceptors (P2X) by ATP opens channels on plasma membrane, which are relatively nonselective to cations (North, 2002). The cation

influx results in membrane depolarization that may be adequate to activate L-type Ca^{2+} channels and generate an action potential (Inoue and Brading, 1991) (Fig. 7). No significant difference was observed between the mechanism of action of the two purinoceptors, $P2X_1$ and $P2X_2$ with respect to their effect on the permeability of the cell membrane to monovalent organic ions, although the calcium permeability due to $P2X_1$ receptor activation was found to be greater than that of $P2X_2$ receptors (Evans et al., 1996; Rogers, 1997; Khakh et al., 2001).

2.11 Neural and Hormonal Control

In most animal species, contraction of urinary bladder muscle is mediated by both cholinergic (Sjogren et al., 1982; Chess-Williams, 2002; O'Reilly et al., 2002) and nonadrenergic, noncholinergic (NANC) mechanisms (Ambache and Zar, 1970; Taira, 1972; Kinder and Mundy, 1985, Palea et al., 1993, Tagliani et al., 1997). In human, the bladder muscle contractions for normal voiding in vivo and the contraction induced by electrical field stimulation in vitro, are mediated mainly, if not exclusively, via stimulation of cholinergic pathway, as these responses are almost completely blocked by atropine (Bayliss et al., 1999; O'Reilly et al., 2001).

2.11.1 Cholinergic mechanisms (muscarinic receptors)

In isolated detrusor muscle, acetylcholine produced slight depolarization to generate the action potential resulting in contraction of the muscle (Creed et al., 1983). These contractions were inhibited by cholinesterase inhibitors and abolished by atropine, demonstrating that they were mediated by muscarinic receptors (Bayliss et al., 1999). Five subtypes of muscarinic receptors have been cloned (M_1–M_5) and pharmacologically

defined (Bonner, 1989; Poli et al., 1992; Andersson, 1993; Eglen et al., 1994 & 1996; Caulfield and Birdsall, 1998). Detrusor smooth muscles from various species were found to contain muscarinic receptors of the M_2 and M_3 subtypes (Eglen et al, 1996; Hegde and Eglen, 1999; Chess-Williams et al., 2001). Human bladder was found to contain all muscarinic receptor subtypes with a predominance of M_2 and M_3 subtypes that were functionally coupled to G proteins (Tobin and Sjogren, 1995; Yamaguchi et al., 1996; Nilvebrant et al., 1997; Sigala et al., 2002). M_1, M_3 and M_5 receptors coupled preferentially to $G_{q/11}$, activating phosphoinositide hydrolysis, in turn leading to calcium release into cell cytoplasm from smooth endoplasmic reticulum (SER) (Bonner et al., 1987). In cat detrusor muscle, contraction induced by acetylcholine is mediated via M_3 receptor-mediated activation of $G_{q/11}$ and phospholipase C (PLC)-β_1 and inositol trisphosphate (IP_3)-dependent intracellular Ca^{2+} release (Somlyo et al., 1988; An et al., 2002). Importantly, when the contribution of IP_3 production to muscarinic receptor-mediated contractions was investigated, it has been observed that most studies have used high concentrations of muscarinic receptor agonists like carbachol (Mostwin, 1985; Iacovou et al., 1990; Andersson et al., 1991). There is a possibility that the concentration of ACh released by the nerve endings may not be sufficient enough to trigger IP_3 production (M_3 muscarinic receptor activation), whereas M_2 muscarinic receptors, which does not involve production of IP_3, may be activated by lower concentrations of acetylcholine (Hirst et al., 1996; Hegde and Eglen, 1999). In normal rat bladders, the M_2 receptor appears to provide a contractile function by inhibition of β-adrenergic receptor-induced relaxation, which is only displayed in the presence of an inactivated M_3 receptor population (Hegde et al., 1997). Braverman et al. (1998) have demonstrated a selective

increase in the density of M_2 receptors resulting in a significant change in the M_2-to-M_3 ratio from ~5.4 in normal to ~9.5 in denervated rat bladders. Prejunctional inhibitory M2-receptors or M4-receptors and prejunctional facilitatory M1-muscarinic receptors in the bladder have also been reported, but their relevance to the clinical effectiveness of muscarinic antagonists is unknown (Chapple et al., 2002).

Although M_2 receptors are the predominant cholinergic receptors present in human urinary bladder (Hegde, 2006), the smaller population of M_3 receptors appears to be the most functionally important and mediates direct contraction of the detrusor muscle (Tobin and Sjogren, 1995; Yamaguchi et al., 1996; Chapple et al., 2002; Chess-Williams et al., 2002; Schneider et al., 2004). M_2-receptors modulate detrusor contraction by several mechanisms and may contribute more to contraction of the bladder in pathologic states, such as bladder denervation or spinal cord injury (Chapple et al., 2002). The majority of muscarinic receptors in the bladder from all species tested are of the M_2 subtype (Wang et al., 1995), the predominant (if not exclusive) receptor subtype found in cardiac tissue. If the M_2 receptor in the rat bladder is associated with postsynaptic mechanisms in a manner similar to cardiac M_2 receptors, then stimulation of these receptors is predicted to cause inhibition of muscle contractility (Wang et al., 1995). It has been reported that muscarinic agonists, acting through presynaptic receptors, can induce a decrease in the release of acetylcholine in rat bladder (D'Agostino et al., 1986). Thus the M_2 receptors of rat bladder are more likely to be located on the presynaptic cholinergic nerve terminals and, upon stimulation, may induce inhibition of acetylcholine release (Wang et al., 1995).

The role of receptor subtypes in disease is unclear, but an enhancement of M_2 receptor mediated responses has been reported to occur in diabetes (Uchiyama and Chess-

Williams, 2004). Hinata et al. (2004) investigated the distribution of muscarinic receptor subtypes with mRNA and protein expressions in patients with and without OAB, and investigated both the dome and trigone area. The M_2 receptor was predominant, but there was no significant difference in the level of M_2 expression between the groups in the dome area. M_5 expression in the dome area was significantly higher in the OAB group than in the non-OAB group. In the trigone area, the level of M_2 mRNA expression was the highest in the non-OAB group, and was significantly lower in the OAB group. This confirmed the multiformity of muscarinic receptor subtypes in human bladder. Pontari et al. (2004) demonstrated that the contractile response of isolated human detrusor muscle strips from patients with neurogenic bladder dysfunction can be mediated by the M_2 receptors. Braverman and Ruggieri (2003) suggested that detrusor hypertrophy in obstructed bladders alters the muscarinic receptor subtype mediating bladder contraction from M_3 toward M_2.

Jezior et al. (2001) showed that muscarinic receptor stimulation activates Rho-kinase and non selective cation channels. Activation of Rho-kinase triggers inhibition of myosin light chain phosphatase (MLCP) and thereby results in an increased sensitivity of the contractile system to calcium (Fig. 6). Schneider et al. (2004) reported that carbachol-induced contraction of human detrusor muscle is mediated via M_3 receptors and mainly depends on calcium influx through nifedipine-sensitive calcium channels and activation of Rho-kinase pathway. Andersson (2004) confirmed that the main pathway for muscarinic receptor activation of detrusor (via M_3) involves calcium influx through L-type calcium channels and activation of Rho-kinase (Harriss et al., 1995; Fetscher et al., 2002; Wibberley et al., 2003).

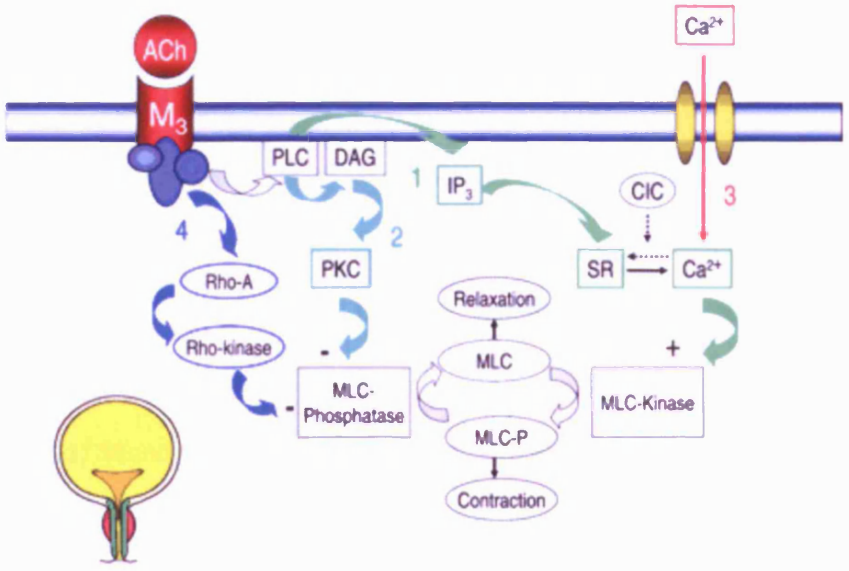


Fig. 6 *Transmitter signal pathways (1–4) involved in activation of detrusor contraction via muscarinic M3 receptors. Ach, acetylcholine; PLC, phospholipase C; DAG, diacylglycerol; PKC, protein kinase C; MLC, myosin light chain; SR, sarcoplasmic reticulum; CIC, calcium-induced calcium release. There seem to be differences between species in the contribution of the different pathways in contractile activation. In human detrusor, Ca^{2+} influx (3) is of major importance. (Andersson and Wein, 2004)*

However, the mechanism of action of M_2 receptors is different from that of M_3 receptors. M_2 receptors were found to act by suppressing sympathetically mediated (β-adrenoreceptors) smooth muscle relaxation, via inhibition of adenylyl cyclase and decreasing the cAMP levels (Matsui et al., 1996; Hegde et al., 1997; Giglio et al., 2001). It was also reported that mechanism of muscle contraction mediated by M_2 receptor stimulation involved opening of non-specific cation channels causing depolarization (Kotlikoff et al., 1999) and inhibition of K_{ATP} channels by activation of PKC (protein kinase C) (Bonev and Nelson, 1993). Nakamura et al. (2002) demonstrated the muscarinic

M_2 receptors inhibited Ca^{2+}-activated K^+ channels in rat bladder smooth muscle. This M_2 receptor-mediated pathway perhaps enhanced contraction, which was initiated by M_3 stimulation in rat bladder smooth muscle.

D'Agostino et al. (2000) have demonstrated the presence of a muscarinic negative feedback mechanism controlling acetylcholine release from the nerve terminals at the presynaptic level. They described the prejunctional muscarinic receptors to be inhibitory (M_4) or facilitatory (M_1).

Kinder and Mundy (1987) investigated detrusor muscle strips from normal and pathological human bladders in an organ bath preparation and did not find any significant differences in the degree of inhibition of either agonist-induced muscle contractions (acetylcholine) or electrically induced contractions in presence of TTX or atropine. They concluded that the pathophysiology of the involuntary detrusor contraction is common to both idiopathic detrusor instability and detrusor hyper-reflexia, and related to a disorder of an intrinsic neuromodulatory mechanism within the detrusor muscle.

Changes in the cholinergic pathway in neurogenic bladders have not been reported consistently in the literature (Latifpour et al., 1989; Pontari et al., 2004). A change in muscarinic receptor mechanism has also been highlighted in bladder outlet obstruction or detrusor overactivity (Braverman and Ruggieri, 2003). German et al. (1995) reported that the contractions of bladder muscle obtained from patients with neurogenic detrusor overactivity showed an enhanced response in presence of muscarinic receptor agonist (acetylcholine) and potassium, but the contractile response to electrical field stimulation was similar compared to the control. The results suggested that the postjunctional modulation was the most likely mechanism of enhanced detrusor contractions, secondary

to a partial parasympathetic denervation (German et al., 1995). However, Gup et al. (1989) did not find any such change in the mechanism of action via muscarinic receptor stimulation in patients with congenital neurogenic bladders (e.g., myelomeningocele). The presence of an atropine-resistant component in the contraction of the human neurogenic bladder has been reported by some investigators (Saito et al., 1993), but not by others (Bayliss et al., 1999). An increased density of muscarinic receptors and an enhanced muscarinic receptor-mediated contractile response was found in bladders from diabetic animals (Latifpour et al., 1989; Mimata et al., 1995). Immunoblotting using monoclonal antibodies has shown that the density of M_3 receptor protein (M_3-mAChR) to be significantly increased in diabetic bladders by 70% (Tong and Cheng, 2002) and by 55% (Hashitani and Suzuki, 1996) compared to the controls. This up regulation of M_3-mAChR biosynthesis may explain the enhanced contractile response to muscarinic stimulation in diabetic animals. In clinical setting, detrusor overactivity (DO) has been demonstrated in the urodynamic tests of diabetic patients (Kaplan et al., 1995).

Denervation supersensitivity of detrusor muscle resulted from outflow tract obstruction has been suggested to play an important role in the pathogenesis of detrusor overactivity in several animals including humans (Gosling, 1986, Nilvebrant et al., 1986; Gunasena, 1995; Braverman et al., 1998). The obstructed human bladder often shows an increased (up to 50%) atropine-resistant contractile component (Sjogren et al., 1982). This may well be a reflection of a change in cholinergic and purinergic functions of the bladder, because in normal bladder, the atropine-resistant component is almost negligible (Bayliss et al., 1999).

2.11.2 Adrenergic mechanisms

The sympathetic innervation has already been discussed earlier in this chapter (pp 32); further details of specific receptors in the adrenergic system will be described here.

a. α-adrenoceptors (α-ARs)

The functional role of the α_1-adrenoceptors in the human bladder function has been investigated by many investigators, and an overall low expression of adrenergic innervations was suggested (Goepel et al., 1997; Malloy et al., 1998). Various researchers have looked into the relative proportions of α_1-AR in the urinary bladder muscle of different animals. For example, Malloy et al. (1998) found two-thirds of the α_1-AR mRNA expressed in human bladder to be α_{1D}. Hampel et al. (2002), while investigating rat bladder, found the predominant α_1-AR to be α_{1A}. These proportions have also been reported to change in certain pathological conditions, such as in obstructed rat bladders, where a shift in predominance from α_{1A} to α_{1D} was noted (Hampel et al., 2002).

Studies have demonstrated an up-regulation of α_1-AR function and an increase in the density of α_1-ARs in human detrusor muscle strips from patients with bladder outlet obstruction (Restorick and Mundy, 1989; Bouchelouche et al., 2005). Thus, although the functional importance of α-ARs in the normal human bladder is still unclear, there is a possibility that this may play a role in pathological bladders such as outflow obstruction (Perlberg and Caine, 1982) and neurogenic damage (Sundin and Dahlstrom, 1973) by altering the balance between the α_1-ARs mediated contraction and β-ARs mediated relaxation (Rohner et al., 1978).

b. β-adrenoceptors (β-ARs)

Both functional and receptor binding studies have demonstrated the presence of β-

adrenoceptors in the detrusor muscle of several mammals, including human (Morita et al., 1990; Morrison et al., 1996). In most species, β_2-adrenoceptor seems to predominate, playing an important role in relaxation of detrusor muscle via activation of adenylyl cyclase with species differences (Morita et al., 1993; Longhurst and Levindusky, 1999). However, in humans, the β -adrenoceptors of detrusor muscle were shown to have functional characteristics of neither β_1 nor β_2-adrenoceptors because they could be blocked by propranolol but not by practolol or butoxamine (Nergårdah et al., 1977; Larsen, 1979). Takeda et al. (1999) have looked into the evidence for β_3-adrenoceptor subtypes in relaxation of the human urinary bladder detrusor by reverse transcription-polymerase chain reaction (PCR) and the results suggested that β_3-adrenoceptor may have some role in storage of urine. In the human bladder, it is now accepted that the most important β-AR for bladder relaxation is the β_3-AR (Igawa et al., 1999; Yamaguchi, 2002). However, Hudman et al. (2001) have shown that the β_2-AR agonist, clenbuterol, inhibited electrically evoked contractions in human 'unstable' bladder, but not in the normal detrusor. This reconfirmed the results of previous studies in humans showing inhibition of detrusor overactivity with β_2-AR agonist such as terbutaline (Lindholm and Lose, 1986). Badawi et al. (2005) also demonstrated the β_2-AR mediated relaxation of porcine detrusor muscle. Although the effects were smaller compared to the pig, similar relaxant effect was observed in the human detrusor in presence of selective β_2-and β_3-AR-agonists.

Alteration in the receptor types and subtypes has always intrigued researchers in this field. Nomiya et al. (2002) compared the expression level of α_1-AR subtype mRNA to that of β-AR subtype mRNA in control and obstructed human bladders by a real-time quantitative

reverse transcriptase-polymerase chain reaction. They also examined whether α_1-AR mediated contraction and β-AR mediated relaxation of human detrusor muscle are altered by bladder outlet obstruction. These findings indicate that neither up-regulation of α_1-AR nor down-regulation of β-AR occurs. It also indicates that relaxation mediating β_3-ARs are by far the most predominant in the human obstructed bladder. Therefore, it is unlikely for α_1-ARs to be responsible for detrusor overactivity and storage symptoms in patients with benign prostatic obstruction.

2.11.3 Non-adrenergic Non-cholinergic (NANC) mechanisms

In most mammalian species, an atropine resistant component of the neuronally induced bladder contraction has been well recognised for a long time that (Andersson, 1993). Two different components of the contractile response to electrical stimulation were demonstrated by *in vitro* (Maggi et al., 1985; Levin et al., 1986; Parija et al., 1991) and *in vivo* (Brading and Inoue, 1991; Igawa et al., 1993) studies; a cholinergic component and a non-adrenergic non-cholinergic component (NANC). The fast response was elicited by a NANC transmitter (Lluel et al., 2002).

The contribution of NANC pathway-mediated response in the total contraction depends on the frequency of electrical field stimulation and also on the animals. Atropine was found to inhibit the contractile response to the electrical field stimulation by 25%, 40% and 75% in bladder strips from rats, rabbits and pigs respectively (Brading and Inoue, 1991). In the nonhuman bladders, atropine resistance was greatest at low frequencies of stimulation (Brading and Williams, 1990).

The role of a NANC mechanism in the normal human bladder contraction was disputable and inconsistent findings have been reported in different studies (Andersson et al., 1991).

In normal human bladders atropine completely abolished the nerve mediated contractions indicating that nerve mediated contraction in human bladder to be exclusively cholinergic (Sibley, 1984; Kinder and Mundy, 1985). Comparative studies on stable, unstable and obstructed human bladders also did not show any significant alteration in the atropine resistant component of muscle contraction to electrical field stimulus (Tagliani et al., 1997; Bayliss et al. 1999; Fry et al., 2002; Harvey et al., 2002). On the other hand, some researchers have suggested that the NANC component perhaps play an important role in normal bladder contraction (Cowan and Daniel, 1983; Luheshi and Zar, 1990; Bayliss et al., 1999).

Yoshida et al. (2001) found significant positive and negative correlations between age and the purinergic or cholinergic neurotransmissions in human isolated bladder smooth muscles. The age-related changes in neurotransmissions may contribute to the changes in bladder function in the elderly. An atropine-resistant component up to 50% of the electrically induced contraction in detrusor muscle was demonstrated from male patients with detrusor overactivity (Sjogren et al., 1982) and outflow tract obstruction (Nergardh and Kinn, 1983). The contribution of the atropine-resistant component up to 50% of bladder contractile response in different pathological conditions associated with detrusor overactivity has also been reported by other investigators (Kinder and Mundy, 1985; Palea et al., 1993; Bayliss et al., 1999). However, in normal human detrusor, this component proved to be very small (Sjogren et al., 1982; Brading, 1987; Andersson, 1993). Thus, the significance of the NANC component for normal bladder contraction and in different micturition disorders remains to be established.

a. ATP

Levin et al. (1986) suggested that the purinergic transmitter ATP (adenosine triphosphate) may be responsible for the atropine-insensitive portion of the contraction. Several investigators supported that the NANC component of contractile response by nerve stimulation in mammalian bladder was actually mediated by the excitatory transmitter ATP (Andersson, 1993; Theobald, 1995; Burnstock, 2001).

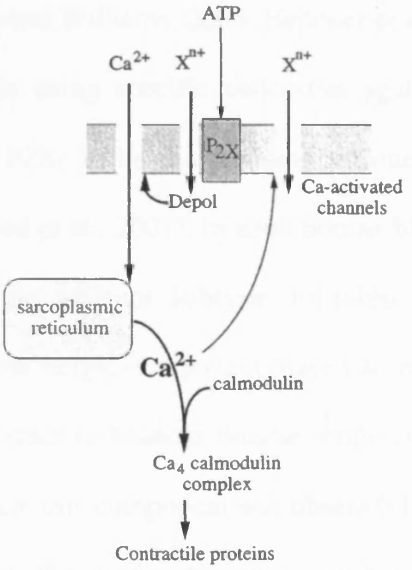


Fig. 7 *Illustration showing the origin of the purinergic Ca^{2+} transients mediated by P2X receptors: ATP released from nerve endings activates P2X receptors located on bladder smooth muscle cell membrane. Ca^{2+} influx through the P2X receptors underlies the purinergic Ca^{2+} transients. The membrane potential depolarization arising from Ca^{2+} and Na^{+} influx through P2X receptors would open voltage-gated calcium channels (VDCCs) allowing significant Ca^{2+} influx into the cell. (Wu et al., 1999)*

ATP was found to act on two types of purinergic receptors: ion channel (P2X) and a G protein-coupled receptor (P2Y) (Abbracchio and Burnstock G, 1994). Seven P2X

subtypes and eight P2Y subtypes have been identified (Valera et al., 1994; Zhou and Galligan, 1996; Ralevic and Burnstock, 1998; Khakh et al., 2001; North, 2002). Bo and Burnstock (1993) demonstrated that the phosphate side chain in molecules of ATP and its analogues is the key structure responsible for the binding to P2X receptors. The contractile response of detrusor muscle to ATP seemed to be mediated by activation of a ligand-gated cation channel (P2X receptor) resulting in extracellular Ca^{2+} influx (Dubyak and El Moatassim, 1993; Burnstock and Williams, 2000; Heppner et al., 2005) (Fig. 7).

Immunohistochemical studies using specific antibodies against different types of P2X receptors demonstrated that $P2X_1$ to be the predominant one in rat and mouse detrusor muscle (Lee et al., 2000; Elneil et al., 2001). In adult human bladder, $P2X_1$ was also found to be the principal purinergic receptor subtype, followed by $P2X_4$, $P2X_7$ and $P2X_5$ (O'Reilly et al., 2001). The purinergic component played an active part (~ 50%) in nerve-mediated contractions in overactive bladder muscle strips compared to normal bladder muscle of control group, where this component was absent (O'Reilly et al., 2002). Calvert et al. (2001) reported that the purinergic component of nerve-mediated detrusor contraction in rabbits was enhanced while the cholinergic component was decreased in early stages of bladder obstruction. The enhanced purinergic response may be due to changes in P2X receptor subtypes in bladders (Moore et al., 2001; O'Reilly et al., 2002). Selective absence of $P2X_3$ and $P2X_5$ receptors in detrusor muscle from patients with overactive bladder was demonstrated by Moore et al. (2001) and this was suggested to be a contributory factor in the pathophysiology of urge incontinence. The activated purinergic response contributing to abnormal bladder contractions was explained as a result of reduced hydrolysis of ATP rather than an increased sensitivity of ATP to bladder muscle

(Harvey et al., 2002; Wu et al., 1999)

b. Nitric oxide

The inhibitory NANC response in the lower urinary tract depends mainly on nitric oxide (NO) derived from L-Arginine (Andersson and Persson, 1995; Burnett, 1995; Mumtaz et al., 2000). NO was found to relax smooth muscle from the urinary outflow region of various animals, including humans, by activating guanylate cyclase and thereby increasing cGMP levels (Murad, 1994; Persson and Andersson, 1994). Pharmacological studies have provided evidence that NO is a transmitter that relaxes the urethral smooth muscle resulting in a decrease the intraurethral pressure at the beginning of normal micturition (Lee et al., 1994). Although NO-guanylate cyclase-cGMP system seems play the key role in NANC nerve-mediated relaxation of urethral smooth muscle, the adenylyl cyclase-cAMP system is more important for relaxation of detrusor smooth muscle (Morita et al., 1992; Truss et al., 1996). Inhibition of the NO-cGMP pathway in the lower urinary tract can interfere with bladder outlet relaxation causing micturition disorders. Contractions of detrusor muscle against a partially closed urethra can result in incomplete emptying of the bladder. Detrusor overactivity may be a result of this incoordination between the bladder and its outlet (Persson et al., 2000). Detrusor smooth muscle has a low sensitivity to NO compared to the outflow region (Andersson and Persson, 1995). However it has been suggested that production of NO perhaps play a role in the NANC-mediated detrusor relaxation in response to electrical stimulation (Klarskov, 1987; James et al. 1993; Theobald, 1996), possibly by altering the blood flow to bladder wall during the filling phase (Azadzoi et al., 1996; Kozlowski et al, 1999). Although, the available data failed to establish a definite role of NO as a neurotransmitter causing direct relaxation of the

detrusor smooth muscle, this does not exclude the possibility of NO modulating the effects of other neurotransmitters, or its role in afferent neurotransmission (Felsen et al., 2003).

c. Neuropeptides

In addition to the "classical" neurotransmitters, noradrenaline and acetylcholine, the urinary bladder contains several neuropeptides. These include vasoactive intestinal polypeptide (VIP), substance P (SP), somatostatin (SOM), enkephalin (ENK), neuropeptide Y (NPY) and calcitonin gene-related peptide (CGRP) which has been demonstrated in the lower urinary tract of various mammals, in cultures of guinea-pig bladder and in man (Gu et al., 1984; Mattiasson et al., 1985; Crowe et al., 1991). The functional role of these neuropeptides in the human lower urinary tract has not been fully established. Their contribution in the pathophysiology of overactive bladder is at present unclear (Maggi, 1991). Uckert et al. (2002) evaluated the functional effects of different peptides, including atrial natriuretic peptide (ANP), calcitonin gene related peptide (CGRP), endothelin 1 (ET-1), substance P (SP) and vasoactive intestinal polypeptide (VIP) on isolated strip preparations of human detrusor smooth muscle and determined the presence of those peptides in the human detrusor by means of immunohistochemistry. Their results showed that ET-1 and SP elicited dose-dependent contractions of the tissue and VIP caused relaxation accompanied by an increase in cGMP levels.

d. Vasoactive Intestinal Polypeptide

Vasoactive intestinal polypeptide (VIP) is known to bind to two types of receptor, $VPAC_1$ and $VPAC_2$, in several types of smooth muscle (Harmar et al., 1998), including the human urinary bladder (Reubi, 2000). Although the peptide inhibited the spontaneous contractile activity of isolated human detrusor muscle, it had little effect on agonist-induced

contractions or contractions evoked by electrical field stimulation (Kinder et al., 1985; Sjogren et al., 1985). Uckert et al. (2002) demonstrated a concentration-dependent relaxation of human detrusor strips in response to carbachol in the presence of VIP. VIP was proved to be one of the non-cholinergic and non-adrenergic neurotransmitter in the pelvic nerve system, and was thought to play an important role in the muscle relaxation of the bladder neck and the posterior urethra (Hosokawa and Kaseda, 1993). The effect of vasoactive intestinal polypeptide (VIP) on the contractile activity of the urinary bladder was investigated in the rat by Erol et al. (1992). They demonstrated that VIP caused a weak contraction and a small potentiation of carbachol- and acetylcholine-induced contractions. VIP levels were found to be significantly reduced in patients with idiopathic detrusor overactivity (Gu et al., 1983; Chapple et al., 1992) or neurogenic detrusor overactivity (Kinder et al., 1985), which might play a role in the pathophysiology of some forms of detrusor overactivity.

e. Endothelins

Endothelins are found to be present in rabbit and human detrusor smooth muscle (Saenz et al., 1992). ET-1, as well as ET-3, can produce slow contractions in both human and animal detrusor muscle (Maggi et al., 1989b; Garcia-Pascual et al., 1990; Andersson and Persson, 1993). Traish et al. (1992) demonstrated that the threshold concentration of ET-3 required initiating a contractile response was higher than that of ET-1 and ET-2. ET receptors were also classified into two other subtypes, ET_A, bound to ET-1 and ET-2; and ET_B, bound to ET-3 (Traish et al., 1992). The probable role of ET_A in detrusor contraction both in animal and human bladders was defined by few other investigators (Donoso et al., 1994; Latifpour et al., 1995; Okamoto-Koizumi et al., 1999; Calvert et al., 2002). The

mechanism of action of ETs involves activation of L-type Ca^{2+} channels and phospholipase C (Maggi et al, 1989b; Persson et al., 1992; Garcia-Pascual et al., 1990 and 1993). However the functional role of ETs in bladder contraction has yet to be properly defined. The slow onset of the contractile effects of ET probably suggests that it does not play any direct role in bladder emptying (Andersson and Arner, 2004). However, an increased expression of ET_A receptors has been reported in rabbit bladder with outflow obstruction (Khan et al., 1999).

g. Prostanoids

Prostanoids (prostaglandins and thromboxanes) are synthesized by cyclooxygenase (COX) and are found in the bladder muscle and mucosa (Maggi, 1992; Khan et al., 1998). Their synthesis was found to be initiated by different physiological stimuli like stretch of detrusor muscle or pathological stimuli like injury to the vesical mucosa or inflammatory agents such as bradykinin (Jeremy et al., 1987; Khan et al., 1998). Andersson (1992) has shown that $PGF_{2\alpha}$, PGE_1, and PGE_2 contract isolated human and animal detrusor muscle, and suggested that prostaglandins may play a role in the maintenance of detrusor tone. Creed and Callahan (1989) suggested that the prostanoids may have an effect on the excitation-contraction coupling in the bladder smooth muscle, directly or indirectly via effects on neurotransmission, possibly by mobilizing Ca^{2+} from the sarcoplasmic reticulum.

2.12 Summary

The ultrastructure of urinary bladder plays an important role in its functional anatomy which includes interplay between autonomic system, smooth muscle, connective tissue

and epithelial elements. Extensive research in this field has demonstrated the presence of numerous neurotransmitters or modulators and their involvement in smooth muscle contraction, although the contribution of different receptors to muscle contraction was found to vary amongst different species. The two most important receptors involved in muscle contraction are muscarinic (M_3) and purinergic ($P2X_1$) (Fig 6 & Fig 7). In rat urinary bladder, both cholinergic and purinergic pathways are active. The muscarinic pathway is mainly responsible for normal human bladder contraction; however, this contribution may alter in some pathophysiological conditions. The main pathway for detrusor muscle relaxation is via activation of adenylyl cyclase-cAMP by adrenergic β_3-receptors, although other pathways involving NO also may contribute.

As with all smooth muscles, detrusor contraction involves the interaction of actin and myosin, via phosphorylation of myosin light chains by myosin light-chain kinase (MLCK) (Hathaway et al., 1985; Kamm and Stull, 1989). In turn, phosphorylation of myosin filaments depends upon a phasic increase in cytoplasmic calcium concentration either by extracellular Ca^{2+} influx into cell cytoplasm or by release of vesicle-bound or stored Ca^{2+} from the sarcoplasmic reticulum (SR) (Stull et al., 1988). Generation of detrusor pressure depends on the interaction of actin and myosin with Ca^{2+}, mediated by MLCK, and the breakdown of cytosolic ATP (Stull et al., 1988; Kamm and Stull, 1989; Chacko and Longhurst, 1994).

CHAPTER 3

HISTORICAL BACKGROUND

OF

URINARY INCONTINENCE

3.1 Introduction

Urinary incontinence (UI) is a very common problem, affecting up to two-thirds of all women (Smith et al., 2006). It is more prevalent in elderly populations and is associated with social isolation and stigmatization, decreased quality of life (Burgio and Ouslander, 1999), depression (Dugan et al., 2000; Wong et al., 2006), and the end of independent living for some elderly women (Jackson et al., 2005). The prevalence is definitely underestimated in the clinical setting, as a substantial number of patients avoid any medical help; it is estimated that only 1 in 4 symptomatic women seek help for this problem (Nygaard and Heit, 2004). UI is also associated with skin problems, sleep disturbances, urinary tract infection, falls, fractures and institutionalization (Brown et al., 2000; Le Lievre S, 2001; Coyne et al., 2003; Heidrich and Wells, 2004; Teo et al., 2006). In a recent report, Abrams et al. (2002b) estimated that 200 million adults worldwide are incontinent. It has large resource implications for the health service and the individual (Dallosso et al., 2003a). The cost in the American elderly population alone has been estimated to exceed $19.5 billion yearly (Hu et al., 2004).

3.2 Evolution of definitions

The *International Continence Society* (ICS) is an international organisation, which tries to provide useful and standard definitions. Bates et al. (1979) defined urinary incontinence as an involuntary loss of urine, which is objectively demonstrable and causes a social or hygenic problem, but recently the ICS updated the definitions (2002b). The new definitions are compatible with the WHO publication ICIDH-2 (International Classification of Functioning, Disability and Health) published in 2001 and ICD10, the

International Classification of Diseases. 'As far as possible, the definitions are descriptive of observations, without implying underlying assumptions that may later prove to be incorrect or incomplete'. By following this principle of objective assessment, the International Continence Society (ICS) aims to compare of the outcome and enable effective communication by investigators who use urodynamic methods.

The three most common types of urinary incontinence were described as a symptom and sign or urodynamic observation. Symptoms are the subjective indicator of a disease or change in condition as perceived by the patient, carer or partner and may lead him or her to seek help from health care proffesionals. Signs are observed by the physician including simple means, to verify symptoms and quantify them and urodynamic observations are observations made during urodynamic studies.

3.3 Stress Urinary Incontinence

- as a symptom it is defined as the complaint of involuntary leakage on effort or exertion, or on sneezing or coughing.
- as a sign it is defined as observation of involuntary leakage from the urethra, synchronous with exertion/effort, or sneezing, or coughing. Stress leakage is presumed to be due to raised abdominal pressure.
- As a urodynamic observation it has been defined as **urodynamic stress incontinence** – the involuntary leakage of urine during increased abdominal pressure in the absence of detrusor contraction.

3.4 Urge Urinary Incontinence:

- as a symptom it is defined as a complaint of involuntary leakage accompanied by or immediately preceded by urgency, which is a sudden compelling desire to pass urine that may be difficult to defer.
- as a urodynamic observation it has been defined as **detrusor overactivity (DO) incontinence** - leakage due to involuntary detrusor contractions during the filling phase which may be spontaneous or provoked.

3.5 Mixed Urinary Incontinence:

- as a symtom it is defined as a complaint of involuntary leakage associated with urgency and also with effort or exertion, or on sneezing or coughing
- as a urodynamic observation it has both the features of urodynamic stress incontinence (USI) and detrusor overactivity (DO)'' (Abrams et al., 2002b).

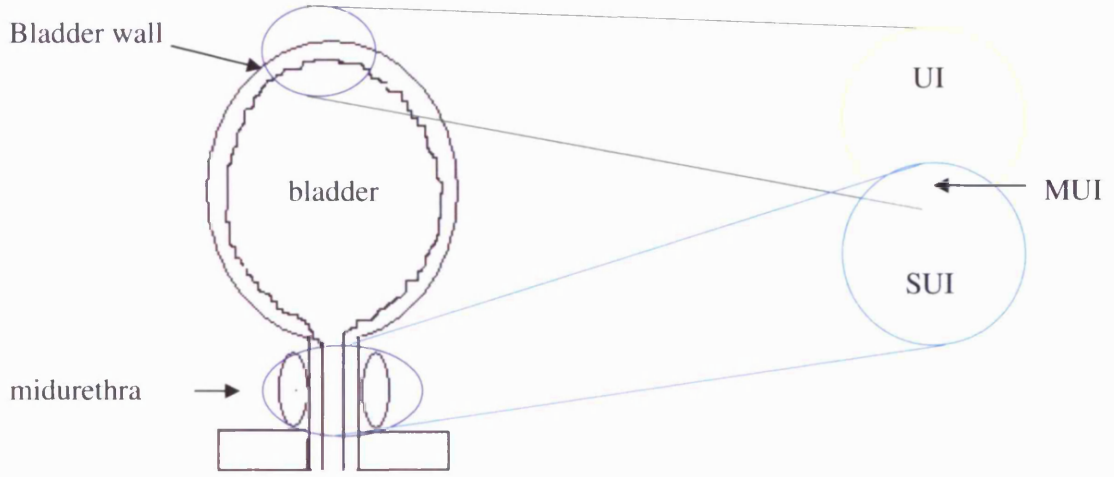


Fig. 8 *Shematic diagram showing the relationships between different types of urinary incontinence*

3.6 Lower Urinary Tract Symptoms (LUTS)

The report from the standardization sub-committee of the international continence society (2002) classified the LUTS into three main groups: ***storage***, ***voiding*** and ***post micturition*** symptoms. Storage symptoms are experienced during the storage phase of the bladder cycle, which include urinary incontinence of any nature (Abrams et al., 2002b), increased daytime frequency, urgency and nocturia. Increased daytime frequency is the complaint by the patient who considers that he/she voids too often by day. Nocturia is the complaint that the individual has to wake at night one or more times to void. Urgency is the complaint of a sudden compelling desire to pass urine, which is difficult to defer.

3.7 Overactive Bladder (OAB)

While redefining the terminology of lower urinary tract function, the ICS introduced a new term - a symptom complex, Overactive Bladder (OAB), that includes most of the components of storage symptoms excluding stress urinary incontinence (Abrams et al, 2002b). Overactive Bladder, according to the ICS updated standardization, is characterized by urinary urgency with or without urge incontinence, usually with frequency and nocturia (Abrams, 2002b). Urodynamic test demonstrates that detrusor overactivity (DO) is characterized by involuntary and unpredictable contractions of the detrusor muscle during filling cystometry, which causes increased urgency (an uncontrollable desire to void) and, for the most part, maps onto the clinically defined syndrome (Wagg et al., 2007).

Although, it is widely believed that OAB symptoms are due to involuntary contractions of the detrusor muscle during bladder filling, other factors like increased sensitivity of the

detrusor muscle or abnormalities of the urothelium may also play significant roles (Abrams and Wein, 2000). If the urge cannot be suppressed, incontinence occurs. Urgency is one of the cardinal symptoms and the only sensory symptom of OAB (Abrams and Wein, 2000). Only about a third of patients with OAB experience UI (Wein and Rackley, 2006). The relevance of storage symptoms in the OAB syndrome was confirmed by Milsom et al. (2001), who found that urinary frequency and urgency were the most frequent symptoms (85% and 54% respectively), while urge incontinence was found in 36% only. If incontinence is associated with overactive bladder symptoms then it is classified as 'wet' OAB (about 33% of OAB cases) and absence of any leakage problem is termed as 'dry' OAB (66%) (van derVaart et al., 2002). It is interesting to note that patients complaining of OAB symptoms do not always have urodynamic proven detrusor overactivity (DO) and vice versa. This has been described in detail later in the chapter (pp 101).

Millions of people of both sexes and all age groups suffer from this highly prevalent and disabling bladder condition, and although it is not restricted to the elderly population, the prevalence of OAB increases with age (Stewart et al., 2003). OAB carries significant social impact for an individual patient, for the community and public healthcare systems (Hu et al., 2004). The unpredictable nature of OAB has a detrimental affect on quality of life (Stewart et al., 2003) and it can affect individual mental health and social well being (Abrams et al., 2000).

OAB diagnosis may require clinical and instrumental evaluations (urological or gynaecological) and long-term treatment. Disease management includes physiotherapy, drugs treatment and continence devices. Significant economic damage results from the

indirect costs secondary to lost wages and productivity (Debruyne and Heesakkers, 2004). The combined community and institutional costs of OAB in the US have been estimated to be around US $ 12.6 billion. Wet OAB is associated with higher costs compared to the dry syndrome (Hu and Wagner, 2000). Considering the current underestimation of OAB prevalence, due to reluctance to seek medical assistance, particularly in some social groups, the costs of managing the condition will probably increase in the future (Tubaro and Palleschi, 2005).

3.8 Epidemiology of urinary incontinence and overactive bladder

3.8.1 Prevalence

Prevalence is the probability of suffering from a pathologic condition within a defined population and at a defined time point; it expresses the distribution of a specific disease in a defined population. The estimation of disease prevalence is essential to evaluate its relevance as a medical problem and to plan adequate intervention (Tubaro and Palleschi, 2005). As mentioned previously, urinary incontinence affects mainly the elderly population; about 200 million adults worldwide are incontinent (Abrams, 2002b).

3.8.2 Prevalence of urinary incontinence

Over the past decades, various studies have been carried out to assess the prevalence of UI in the general population. In a recent study, Anger et al. (2006a) have reported an estimated overall prevalence of urinary incontinence in women to be 38%. Throughout the years, wide ranges of prevalence rates (5% to 54%) have been reported in several studies with a mean prevalence of 28% (Diokno et al., 1986; Holtedahl and Hunskaar, 1998; Hannestad et al., 2003a, Minassian et al., 2003). This is due to lack of uniformity in

definitions, populations studied and study designs (Hunskaar et al., 2003, Nygaard and Heit, 2004). For example in a cross-sectional survey conducted by Asian Society for Female Urology (ASFU) of 5,506 Asian women to determine the prevalence of incontinence (Lapitan and Chye, 2001) the question used to establish the prevalence of incontinence was different from the American and European surveys. The question asked in the Asian study seemed more focused on urge incontinence than any incontinence ("Have you ever leaked urine inappropriately before reaching the toilet in the past 6 months?"). In a large population based study to measure the prevalence of incontinence in women in the community Anger et al. (2006a) analyzed data from women responding to the National Health and Nutrition Examination Survey (1999-2000). The question asked was "In the past 12 months, have you had difficulty controlling your bladder, including leaking small amounts of urine when you cough or sneeze (exclusive of pregnancy or recovery from childbirth)?" Whereas, in the study by Jackson et al. (2004), urinary incontinence was classified clearly as stress, urge, or other by asking "In the past 12 months, when does your leakage of urine usually occur?" The response options were (a) "With an activity like coughing, lifting, standing up or exercise" (stress); (b) "When you have the urge to urinate and can't get to a toilet fast enough" (urge); or (c) "You leak urine unrelated to coughing, sneezing, lifting or urge" (other). The overall urinary incontinence was reported to be 21% at least weekly; of which 42% and 40% being urge and stress incontinence respectively. It becomes obvious that the outcome of different epidemiological studies performed in different countries at different times were often not comparable as they failed to specify target groups with specific validated symptom-based criteria. The Leicestershire MRC study group has worked on a validated questionnaire

targeted to a specific age group in the community after the introduction of standardization in the terminology of lower urinary tract symptoms by the ICS in 2002 (Dallosso et al., 2003a).

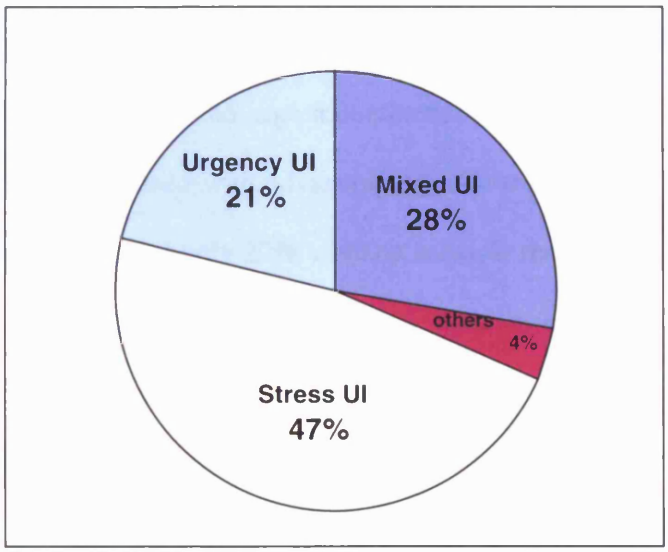


Fig. 9 *Prevalence of patients having different types of urinary incontinence (Abrams et al., 2005)*

3.8.3 Prevalence of overactive bladder

Overactive bladder is the commonest bladder problem in late life, affecting up to 41% of men and 31% of women over 75 and the elderly experience more severe disease (Chapple et al., 2004).

The most important recent data on OAB prevalence came from the National Overactive Bladder Evaluation (NOBLE) program. This was the first epidemiologic analysis reporting OAB prevalence in both sexes, in a population of 18 years of age or older, using validated symptom-based criteria. In this report, estimation of prevalence in the

United States was 16% in men and 16.9% in women, with a global number of 33 million citizens suffering from symptoms of OAB (Stewart et al., 2003). In Europe, a recent study conducted by Milsom et al. (2001) estimated an overall OAB prevalence of 16.6% (15.6 in men and 17.4% in women), in a population-based survey in individuals over 40 years of age. In this study, frequency (85%) was the most commonly reported symptom, followed by urgency (54%) and urge incontinence (36%). The prevalence of overactive bladder symptoms increased with advancing age. Overall, 60% of symptomatic patients had consulted a doctor and only 27% were on medical treatment. The overall prevalence of frequency and urgency were similar in both men and women, whereas urge incontinence was more prevalent among women than men.

In an Asian study from eleven countries, the overall prevalence of OAB was found to be 53.1% (Lapitan and Chye, 2001). The most common presenting symptom was urgency (65.4%). Twenty-one per cent of the OAB population presented with incontinence, giving an overall prevalence of 11.4% for urge incontinence among Asian females (Lapitan and Chye, 2001). Another epidemiological survey in Japan determined the prevalence of overactive bladder (OAB) symptoms to be 12.4% (men 14%, women 11%), where OAB was defined as a symptom complex of daily urinary frequency of eight or more times and urgency once or more per week (Homma et al., 2005). An Austrian study (Temml et al., 2005) has reported the prevalence of overactive bladder to be 10.2% in men (dry- 8.4%, wet-1.8%) and 16.8% in women (dry-10.3%, wet-6.5%). Teloken et al. (2006) in Brazil has found the prevalence of OAB to be 18.9%. In the Spanish study, the overall prevalence of symptoms suggestive of OAB according to the OAB definition from ICS report 2002 was 21.5%, significantly higher in women (25.6%) than men (17.4%)

($p<0.05$) (Castro, 2005). A Canadian study based on a validated, computer-assisted telephone interview (CATI) system, reported the prevalence of overactive bladder (OAB) to be 18.1% (men 14.8% and women 21.2%). Dry OAB was assessed to be the highest sub-type, with a rate of 13.6% (11.7% in men and 15.6% in women). The prevalence of wet OAB was estimated to be 2.3%, 2% in men and 2.6% in women (Corcos and Schick, 2004). van der Vaart et al. (2002) reported that 66% of the patients with OAB were found to be dry (without urinary incontinence) and about 33% present wet OAB (with urinary incontinence). In the NOBLE study, the overall prevalence of dry and wet OAB was 10.3% and 6.1%, respectively, with a clear gender difference. In women, the prevalence of wet and dry OAB was similar (9.3% and 7.6%, respectively), while in men, dry OAB was more common -13.4% versus 2.6% (Stewart et al., 2003). Thus it is clearly evident from most of these studies that dry OAB is more common than wet and the later is found more often in women. Patients, irrespective of the nature of OAB – wet or dry, are strongly conditioned by OAB symptoms in their social, psychological (Corna and Cairney, 2005; Vigod and Stewart, 2006), occupational (Fultz et al., 2005; Han et al., 2006) and sexual activities (Lam et al., 1992; Patel et al., 2006; Coyne et al., 2007; Sen et al., 2007). Wet OAB is associated with a higher degree of bother and higher costs compared with the dry syndrome (Tubaro and Palleschi, 2005).

The report of the recent EPIC study has been published in December 2006 (Irwin et al., 2006). To date, this is the largest population-based cross-sectional survey to assess prevalence rates of OAB, UI, and other LUTS among men and women in five countries (Canada, Germany, Italy, Sweden, and the United Kingdom) and also to evaluate these symptoms simultaneously using the 2002 ICS definitions. The overall prevalence of OAB

was 11.8%; rates were similar in men and women and increased with age. OAB was more prevalent than all types of UI combined (9.4%). At least one LUTS was reported to be present in 64.3%. Nocturia was the most prevalent LUTS (men, 48.6%; women, 54.5%). The prevalence of storage LUTS (men, 51.3%; women, 59.2%) was greater than that for voiding (men, 25.7%; women, 19.5%) and postmicturition (men, 16.9%; women, 14.2%) symptoms combined.

Table 1 *Prevalence of overactive bladder (OAB) in different studies (different countries)*

Authors		**Country**	**Prevalence**
Tikkinen et al	2007	Finland	6.5% (men: 9.3%, women: 10.6%)
Irwin et al	2006	Eur,UK,Canada	11.8% (men and women equal)
Teloken et al	2006	Brazil	18.9%
Temml et al	2005	Austria	13.5% (men: 10.2%, women: 16.8%)
Castro et al	2005	Spain	21.5% (men: 17.4%, women: 25.6%)
Homma et al	2005	Japan	12.4% (men: 14%, women: 11%)
Coros et al	2004	Canada	18.1% (men: 14.8%, women: 12.2%)
Stewart et al	2003	U.S.A	16.4% (men: 16%, women: 16.9%)
Milsom et al	2001	Europe	16.6% (men: 15.6%, women: 17.4%)
Lapitan et al	2001	Asia	11.4% (women)

3.9 Natural history and relationship between OAB and detrusor overactivity

The overactive bladder was probably first described 100 years ago by Dudley who distinguished between active and passive incontinence based mainly on sphincter weakness (Dudley, 1905). However, this distinction failed to incorporate the full meaning of the recent terminology as it refers purely to detrusor overactivity incontinence. Following Dudley's description, several other terms were introduced including uninhibited or hypertonic bladder and bladder dyssynergia. The emphasis on 'urgency' was highlighted by Taylor and Watt (1917) to distinguish incontinence preceded by urgency from simple incontinence without urgency. The term 'unstable bladder' was introduced by Bates et al. (1970) while describing the investigation of urge incontinence using cinecysturethrography, which is now known as videourodynamics. Moreover, the unstable bladder was sub classified into detrusor hyperreflexia (in the presence of a neurological lesion) and detrusor instability (without any obvious neurological cause) (Bates et al, 1970). These were generic terms used in the English speaking world and Scandinavia before the first ICS terminology report in 1976, which did not have exact definitions and were used as general terms for detrusor overactivity (Garnett and Abrams, 2003). As each term was more popular than the other in different parts of the world each was allocated a definition as a compromise but there is no real logic or meaning to the terms. In view of this, the ICS decided to standardize the relevant terminologies (Abrams and Wein, 2000). The term overactive bladder was introduced for better understanding by patients and detrusor overactivity was defined as an urodynamic observation characterized by involuntary detrusor contractions during the filling phase, which may be spontaneous or provoked (Abrams et al., 1988). The involuntary bladder contractions

may be symptomatic or not. When the spontaneous bladder contractions occur at volumes below the patient's functional bladder capacity, the overactive bladder becomes symptomatic and this usually occur under circumstances of normal daily activity. The exact relationship between the sensation of urgency and increased detrusor pressure with muscle contraction is still unclear (Wyndaele, 2001). Haylen et al. (2007) has commented that sensory urgency and detrusor overactivity appear to be part of the same spectrum of bladder dysfunction.

Amongst patients complaining of symptoms of the overactive bladder, the commonest cause is detrusor overactivity, which is a diagnosis made on urodynamic study. Detrusor overactivity might not be detected on conventional urodynamic study in presence of overactive bladder symptoms. A role for ambulatory urodynamics was highlighted by Radley et al. (2001), where ambulatory urodynamics was compared with the conventional video cystometry findings in women with symptoms of detrusor overactivity. Objective evidence of clinically important detrusor overactivity was obtained to a greater extent by ambulatory urodynamics in the majority of women with symptoms suggestive of detrusor overactivity. The correlation of symptoms with the findings of ambulatory urodynamic study suggested that probably clinical symptoms are more reliable for diagnosing detrusor overactivity. Normal cystometrogram showing stable bladder in symptomatic women should be interpreted with caution.

Detrusor overactivity may be neurogenic or idiopathic in origin. A subset of patients with an overactive bladder will also complain of urge urinary incontinence, which may prove to be detrusor overactivity incontinence on urodynamic investigation (Garnett and Abrams, 2003).

3.10 Risk factors for urinary incontinence and overactive bladder

Primary prevention of any prevalent health problem is based on identifying populations at risk of developing the condition, the factors associated with those problems, and strategies that help individuals to alter the modifiable risk factors in their lives. This includes a focus on early treatment to manage and resolve symptoms or disease progression. The populations at risk for urinary incontinence and overactive bladder symptoms have been identified: women in selected occupations (Jorgensen et al., 1994), with higher education (Anger et al., 2006a), childbearing women, older adults with lifestyle risk factors (Kuh et al., 1999), older adults with co morbidity (Rohr et al., 2005), and nursing home residents (Sgadari et al., 1997). Lifestyle factors such as smoking (Bump and McClish, 1992; Hannestad et al., 2003b) or caffeine consumption (Arya et al., 2000; Freeman and Adekanmi, 2005) and modifiable risk factors such as diet (Dallosso et al., 2004a, b) or physical activities (Danforth et al., 2007) are also considered. The details of the studies describing these associations are discussed below.

3.10.1 Age

Increasing age was highly associated with both forms of incontinence (Rohr et al., 2005). A recent review of the literature showed that the prevalence of significant urinary incontinence increases from the second to the eighth decades with two distinct peaks, one in the fifth decade of life, and the second in the eighth decade (Minassian et al., 2003). A similar trend was also reported by Hunskaar et al. (2003), who mentioned that the prevalence of urinary incontinence in women increases along the lifespan, stabilizing at age 50 years (at around 30%) and then increasing at age 70 years to approximately 40%. Stress incontinence was found to be much higher than urge (12.4% and 1.6%

respectively) in a population with mean age of 40 years (Peyrat et al., 2002). More severe and troublesome incontinence probably occurs with increasing age, especially age older than 70 years (Hunskaar et al., 2000).

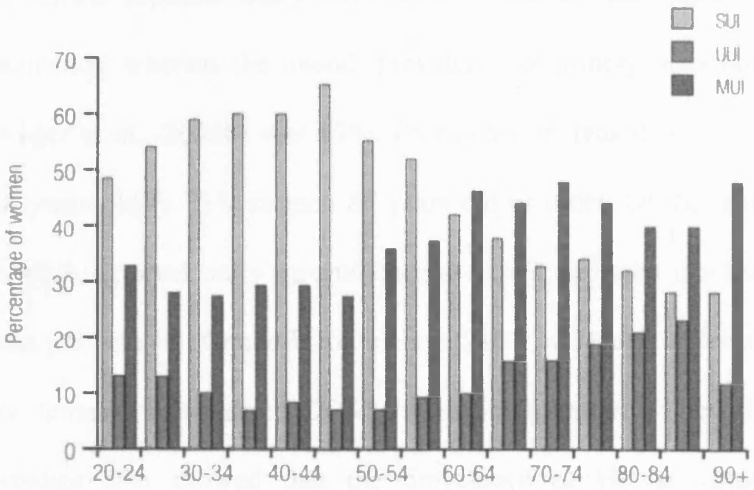


Fig. 10 *Prevalence of the three most common types of incontinence by age. (Hannestad et al., 2000)*

The prevalence of overactive bladder symptoms increased with advancing age (Milsom et al., 2001). The prevalence of wet OAB presents a steeper increase with age in the female population while the opposite occurs for dry OAB (Stewart et al., 2003). The relation between OAB prevalence and age was confirmed in studies conducted in Europe (Milsom et al., 2001).

3.10.2 Sex

UI was found to affect 3 in 10 women and the prevalence of UI in men was reported to be half that of women, although maintaining the increasing trend with age (Hannestad et al., 2002). As mentioned earlier in the chapter, Anger et al (2006a) in U.S in their study based on the data of National Health and Nutrition Examination Survey (1999-2000), has

reported the overall prevalence of urinary incontinence in women to be 38%. The prevalence of daily incontinence increased with age, ranging from 12.2% in women 60 to 64 years old to 20.9% in women 85 years old or older. Of women reporting any incontinence, 13.7% reported daily incontinence, and an additional 10.3% reported weekly incontinence, whereas the overall prevalence of urinary incontinence in men in that study (Anger et al., 2006b) was 17%. Prevalence increased with age from 11% in men 60 to 64 years old to 31% in men 85 years old or older. Of the men reporting any incontinence, 42% reported daily incontinence and 24% reported it weekly. Black men had the highest prevalence of male incontinence (21%) and black women had the lowest prevalence of female incontinence (20%). A recent literature review including many worldwide studies also showed that the prevalence of UI in women was 27.6% (Minassian et al., 2003). It affects up to 30% of those living in the community and 50% of those living in institutions (Fantl et al., 1996; Hu et al., 2004). Importantly, prevalence in long-term care facility residents is higher; approximately 50% of both sexes are incontinent of urine (Kinchen, 2002). The most common type of female urinary incontinence is stress urinary incontinence, affecting 50% of all incontinent women followed by mixed incontinence (32%) and urge urinary incontinence (14%) (Abrams et al, 2002a). Among older, non-institutionalized women with incontinence evaluated in referral centres, stress incontinence is found less often, and detrusor abnormalities and mixed disorders are more common than in younger ambulatory women. This could have been explained by the fact that female bladder and urethral function appear to deteriorate throughout adult life (specifically, detrusor contractility, bladder sensation, and urethral pressure) whether detrusor overactivity is present or not (Pfisterer et al., 2006). The

overall prevalence of urinary incontinence was 20% among women less than 60 years of age and 44% among those older than 80 years (Rohr et al., 2005).

3.10.3 Pregnancy and Childbirth

Childbirth was found to be an important risk factor in developing urinary incontinence (MacLennan et al., 2000; Rortveit et al, 2003). Almost 32% of women report stress urinary incontinence during pregnancy and most tends to be new onset (Viktrup et al., 1992). It has been shown that women who report stress urinary incontinence during their first pregnancy and those who develop stress incontinence during the immediate postpartum period are at high risk for persistent stress urinary incontinence 5 years later (Holroyd-Ledue and Straus, 2004). An Italian study also reported that female urinary incontinence often manifests itself for the first time during pregnancy in a great number of women (23-50%), mostly during the third trimester (43%). This could be attributed to the stress induced by the pregnancy on the pelvic floor and in most cases it is transitory in nature (DiStefano et al., 2000). Other independent obstetric risk factors of urinary incontinence are multiparity and vaginal delivery (Holroyd-Ledue and Straus, 2004, Minassian et al., 2003). Forceps-assisted vaginal delivery may be an additional risk (Arya et al., 2001). Electrophysiological research suggests that vaginal delivery may be responsible for development of urinary incontinence by causing pudendal nerve damage and subsequent pelvic floor damage (Snooks et al., 1986) and denervation of urethral striated muscle (Hale et al., 1999). A recent study by Glazener et al. (2006) has analyzed the obstetric and other risk factors in development of new postnatal urinary incontinence in three months following delivery. The prevalence of urinary incontinence reported in this study was 29%. New incontinence first beginning after delivery was associated with

older maternal age (oldest versus youngest group, OR 2.02, 95% CI 1.35-3.02) and method of delivery (caesarean section versus spontaneous vaginal delivery, OR 0.28, 95% CI 0.19-0.41). Instrumental vaginal delivery and vaginal breech delivery were not found to be associated with higher incidence of incontinence compared to simple vaginal delivery. However, in women whose incontinence first occurred during pregnancy, their body mass index (BMI) and the weight of the baby were the significant risk factors. The prevalence of incontinence was found to be 29% in primiparae (Glazener et al., 2006). Data regarding the impact of the size of the baby, fetal head, and the length of second stage of labour on the development of urinary incontinence are controversial (Holroyd-Ledue and Straus, 2004). However, a survey of nulliparous, postmenopausal women with regard to the prevalence of urinary incontinence (Buchsbaum et al., 2002) showed a surprisingly high prevalence of symptoms of urinary incontinence among post-menopausal, nulliparous nuns with a significant negative impact on quality of life. These findings appear to be contrary to the conventional belief that nulliparity protects against stress urinary incontinence.

3.10.4 Body mass index (BMI)

Increased body mass index (BMI) has been established as an independent risk factor for the development of urinary incontinence (Jackson et al., 2004). It has been shown that with each unit increase in BMI, there is a 5% increase in the odds of having urinary leakage (Sampselle et al., 2002). In the NOBLE study, the prevalence of wet OAB was found to be increased with increasing body mass index (BMI) in women but not in men, and a BMI of 30 or higher was considered to be a risk factor (Stewart et al., 2003).

3.10.5 Race, ethnicity and country

Race and ethnicity were found to be associated with development of urinary incontinence. Several studies have demonstrated that white race is a significant risk factor for the development of stress urinary incontinence (Minassian et al., 2003; Holroyd-Ledue and Straus, 2004). However, Jackson et al. (2004) reported that both stress and urge incontinence are associated with white race. Fultz et al. (1999) reported a lower prevalence of urinary incontinence in older African American women compared to older Caucasian women. An American study compared urodynamic findings and the rate of incontinence among various ethnic groups (Duong and Korn, 2001). African American women with urinary incontinence were found to have different urodynamic diagnoses than Hispanic, white, or Asian women. The rates of genuine stress incontinence were reported to be similar in Hispanic, white, and Asian women. Although African American women had lower rates of genuine stress incontinence than Hispanic and white women, they had higher rates of detrusor instability than all 3 groups. The study conducted by Graham and Mallett (2001) confirmed the higher incidence of urge incontinence in black population (odds ratio [OR], 2.6; 95% confidence interval [CI], 1.45–4.80).

Anger et al. (2006a, b) found that black men had the highest prevalence of male incontinence (21%) and black women had the lowest prevalence of female incontinence (20%). The prevalence, type and management of women with urinary incontinence in four European countries were determined in a population based survey (Hunskaar et al., 2004), which showed stress urinary incontinence to be the most prevalent type. The lowest prevalence was in Spain (23%), while the prevalence was 44%, 41% and 42% for France, Germany and the UK, respectively. About a quarter of women with urinary

incontinence in Spain and the UK had consulted a doctor for the condition; in France (33%) and Germany (40%) the percentages were higher. Overall, less than 5% of the women had ever undergone surgery for their condition. Incontinence pads were used overall by 50% of the women with some differences among the countries (Hunskaar et al., 2004). The Asian Society for Female Urology (ASFU) reported the prevalence of incontinence in eleven Asian countries; ranging from 17% for Thailand and 13% for the Philippines, to 4% for Singapore and China (Lapitan and Chye, 2001). As explained earlier, the discrepancy between the prevalence of incontinence in Asia, Europe, and America may be attributable in part to the study methods and questions used or due to the diversity in socio-cultural background, race and ethnicity (Sampselle et al., 2002). African-American women were reported to be at lower risk for incontinence (Bump, 1993) which may be explained by urodynamic studies, showing higher urethral pressure, larger urethral volume, and greater vesical mobility in African-American women compared to Caucasian women (Sampselle et al., 2002; Fultz et al., 1999).

3.10.6 Miscellaneous factors

Other factors reported to modify the risk of developing urinary incontinence and overactive bladder symptoms include lung disease (Sampselle et al., 2002; Jackson et al., 2004), menopause (Hannestad et al., 2003), hysterectomy (Brown et al., 2003), urinary tract infection and constipation (Aggazzotti et al., 2000). In women, abdominal hysterectomy was found to be associated with the development of urinary incontinence and especially urge incontinence (van der Vaart et al., 2002). However, De Tayrac et al. (2004) did not find any evidence of a cause–effect relation between vaginal hysterectomy and urge or stress urinary incontinence, although patients undergoing vaginal

hysterectomy were found to have a trend for developing urge symptoms (62.4% versus 54.3%). Current oral oestrogen use was reported to be associated with development of both types of urinary incontinence (Jackson et al., 2004). In men, surgical treatment for prostate cancer was reported to be associated with incontinence (Palmer, 2003). The identified associations for frail men and women are functional impairment like restricted mobility (Wetle et al., 1995), altered cognitive status (Palmer et al., 2002), depression (Jackson et al., 2004), diabetes (Jackson et al., 2004; Moorthy et al., 2004), and neurological conditions such as multiple sclerosis, Parkinson's disease, or cerebrovascular accidents (Madersbacher et al., 2002). Women with less than a high school education were less likely to report incontinence than were those with at least a high school education (Anger et al., 2006a).

3.10.7 Diet and lifestyle

Several studies have assessed the association of incontinence with lifestyle factors such as diet (Dallosso et al., 2004a, b), cigarette smoking (Bump & McClish, 1992), caffeine consumption (Bryant et al., 2002), and high-impact sports or exercise (Thyssen et al., 2002). Physical activity was associated with a significant reduction in urinary incontinence, particularly stress incontinence compared to urge incontinence (Danforth et al., 2007). However, it becomes difficult to obtain a definite cause and effect relationship, as most of these studies are cross-sectional population surveys or case-control studies.

3.10.7.1 Physical exercise

Jiang et al. (2004) summarized that current data indicates that most types of exercise are not a risk factor for the development of urinary incontinence. However, certain extreme high-impact sports such as parachute jumping may cause pelvic organ support defects

that result in stress urinary incontinence. Eating disorders also increase the risk of urinary incontinence in athletes. Overall, women should be encouraged to pursue physical activity that will benefit their general health without the risk of development of urinary incontinence later in life. Women athletes should be counselled about the increased risk of urinary incontinence with ultra high-impact sports and eating disorders (Jiang et al., 2004).

3.10.7.2 Psychological symptoms

Individuals with depression, anxiety, and attention deficit disorder may experience symptoms of OAB more often than the general population (Steers, 2002). Wolfe and colleagues (1997) suggested that depression, anxiety, feeding disturbances, pain, irritable bowel syndrome, fibromyalgia, and changes in voiding are associated with disturbances of specific neurotransmitters in brain, e.g. serotonin (5-hydroxytryptamine, or 5-HT). Steers (2002) speculated that as 5-HT influences both emotional states and bladder function, there might be a connection between these shared conditions in which the monoamine level is altered. Zorn and colleagues (1999) confirmed the association of depression with mixed or urge incontinence, but with stress urinary incontinence.

3.10.7.3 Caffeine, alcohol and smoking

High caffeine intake has also been associated with a higher risk of urge urinary incontinence (OR, 2.4; 95% CI, 1.1–6.5) (Arya et al., 2000, Holroyd-Ledue and Straus, 2004). Caffeine reduction is an internationally accepted treatment strategy for patients with urinary symptoms and in a randomized clinical trial, Bryant et al. (2002) demonstrated that when caffeine intake was reduced in the experimental group, urgency and frequency outcomes were significantly improved. The association between caffeine

and overactive bladder symptoms were also described by other workers (Creighton and Stanton, 1990; Tomlinson et al., 1999; Gray, 2001).

Minassian et al. (2003) did not find any statistically significant correlation between urinary incontinence and alcohol intake. However, significant negative association was found between beer intake and subsequent onset of overactive bladder by Dallosso (Dallosso, 2004b).

Bump and McClish (1992) demonstrated a strong statistical relationship between current and former cigarette smoking and both stress (OR 2.48 in current and 2.2 in former) and urge (OR 1.89 in current and 2.92 in former) urinary incontinence in women. According to Tampakoudis et al. (1995), women who smoke are more likely to develop incontinence, especially urge incontinence, than non-smokers. The association between smoking and urinary incontinence was also found in other epidemiological studies (Arya et al., 2000; Hannestad et al., 2003b; Danforth et al., 2006). Dallasso et al. (2003a) in their large longitudinal study, which is described in detail below, has shown that current smokers were 1.44 times more likely to develop overactive bladder than non-smokers (95% CI, 1.05-1.98) and the increased risk for ex-smokers was also found to be significant (OR, 1.24; CI, 0.97-1.58).

3.10.7.4 Diet

Various food and drinks are believed to have adverse or beneficial effects on urinary symptoms, although there is little scientific evidence. The published evidence on total diet and urinary symptoms is from studies assessing prostatic enlargement in men, defined either clinically (Araki et al., 1983; Lagiou et al., 1999) or by symptom scores which included urgency and urge incontinence (Koskimaki et al., 2000; Suzuki et al.,

2002). Two of the studies reported an increased risk of symptoms with red meat (Chyou and Nomura, 1993; Koskimaki et al., 2000) and reduced risks were reported with vegetables (Araki et al., 1983; Koskimaki et al., 2000) and fruit (Lagiou et al., 1999). Suzuki et al (2002) reported a positive association with intakes of total energy, protein and specific long-chain polyunsaturated fatty acids (Suzuki et al., 2002). One study has shown that eating disorders such as anorexia nervosa were associated with increased risk of all urinary symptoms, including urgency, frequency, nocturia, stress and urge urinary incontinence (Boos et al., 1999). This observation has also been reported by Bo and Borgen (2001), who noted an increased prevalence of stress urinary incontinence in athletes with eating disorders.

3.10.7.5 Leicestershire MRC Incontinence Study

In the large longitudinal prospective study on women over 40 years of age, the Leicestershire MRC Incontinence Study Group investigated the possible role of diet and other lifestyle factors in the onset of the clinically recognized main subgroups of incontinence, the overactive bladder and stress incontinence (Dallosso et al., 2003a). This study showed that increased consumption of vegetables, chicken and bread was associated with a reduced risk of onset of overactive bladder, and bread was also associated with a reduced risk of the onset of stress incontinence. The consumption of carbonated drinks and obesity were positive risk factors for the onset of both the conditions (Dallosso et al., 2003a). This was large well-designed prospective epidemiological study, which used a validated questionnaire targeted to a specific age group in the community after the introduction of standardization in the terminology of lower urinary tract symptoms by the ICS in 2002. The association between carbonated

soft drinks and both urinary incontinence and overactive bladder was demonstrated for the first time in this epidemiological study. Simple modifications in diet and lifestyle may prove to be beneficial in managing urinary incontinence and improving the quality of life. Since this study generated the idea behind this thesis, it is worth describing the study in detail.

A baseline postal questionnaire was initially mailed to 20,244 women at the end of October 1998 and two reminders were sent at 4- week intervals to those who did not respond. The questionnaire collected information on lower urinary tract symptoms, lifestyle factors, general health, co-morbidities, activities of daily living and socio-demographic variables. A food-frequency questionnaire (FFQ), which was developed and validated for use by the UK arm of EPIC study (described earlier), was sent to responders to the first and second mailings of the baseline questionnaire, except for those of South Asian origin, who comprised 5.3% of the Leicestershire population aged over 40 years, as they have very different dietary habits to the population in which the questionnaire was validated. In October 1999 a follow-up postal questionnaire on urinary symptoms was sent to all responding to the FFQ and two reminders were sent at 4-week intervals to those not responding. Statistical analysis was based on logistic regression models to examine the association of food groups, drinks and lifestyle factors with the onset of stress urinary incontinence and overactive bladder after one year. Two multivariate logistic regression models were used to determine the incidence of new onset stress urinary incontinence and overactive bladder between baseline and follow-up. All factors associated ($p < 0.10$) with the dependent variable in the univariate analyses were entered into the multivariate models. Backward stepwise techniques were used to build the final

models, with likelihood ratio tests used to determine significance. Carbonated drinks were the only fluid independently associated with both conditions. Those who consumed carbonated drinks daily or more often were significantly more likely to have onset of stress urinary incontinence (OR, 1.62; 95% CI, 1.18–2.22,) and overactive bladder (OR, 1.41; 95% CI, 1.02-1.95) than those who did so less than weekly. Eating bread more than daily was found to be protective for development of both the conditions (stress incontinence- OR, 0.76; 95% CI, 0.61-0.96 and overactive bladder- OR, 0.68, 95% CI, 0.55-0.86), whereas consumption of chicken twice or more per week was found to be protective for overactive bladder (OR, 0.64; CI, 0.48-0.87). The consumption of total vegetables was associated with reduced risk of onset of overactive bladder (OR, 0.69; CI, 0.48-0.98).

In the univariate analysis, although beer and coffee appeared to be associated with a reduced risk of developing stress urinary incontinence, this association was not confirmed when adjusted for other confounding lifestyle factors in multivariate analysis. The association with carbonated drinks cannot be explained by a high fluid intake, as in the multivariate model there was no association between total fluid intake and either stress urinary incontinence or overactive bladder. Tomlinson et al. (1999) also showed a similar finding that an increase in the average amount of fluid intake was significantly related to an increase in the average volume of urine voided, but not to urinary incontinence. About half of the carbonated drinks consumed nationally are 'cola drinks' (URL 1) and contain significant amounts of caffeine, which has been proved to be a diuretic and has excitatory effects on the detrusor smooth muscle (Arya et al., 2000; Holroyd-Ledue and Straus, 2004). Surprisingly, in this study, no clear association was found between overactive

bladder with caffeine. The frequently reported clinical association of incontinence with coffee (Gray, 2001; Tomlinson et al., 1999) could be a result of the triggering of episodes of leakage in patients, because of the diuretic and stimulant properties of caffeine (Bryant et al., 2002). Many carbonated drinks are rich sources of sugar, and others have high levels of artificial sweeteners. Additives used in the manufacture of carbonated soft drinks include colorants, antioxidants and preservatives, such as citric acid, ascorbic acid and sodium benzoate. Avoiding acidic foods, e.g. fruit juice and carbonated drinks, is often part of the clinical advice given to patients with certain urinary symptoms (Dallosso et al., 2003a).

The strength of the results from any longitudinal study depends on the extent of failure to respond and loss of subjects from the cohort group during follow-up. The response rates to both the baseline questionnaire and the food frequency questionnaire were 65%, a rate comparable to other recent large postal surveys in this country that used similar wide sampling frames. On detailed investigations into failure to respond at baseline (Dallosso et al., 2003b), little evidence of nonresponse bias in the reporting of urinary symptoms was detected. Moreover, about 91% of the baseline responders returned the questionnaire at the end of one year. Considering this evidence we regard the analysis of the data in this study as more robust and reliable than other cross-sectional surveys and therefore have used this to generate the hypothesis of our laboratory work.

Vegetables and bread were found to provide 26% and 30% of fibre in the UK diet (Gregory et al., 1990). A low fibre intake predisposes an individual to chronic constipation and bowel straining, and in this way may affect pelvic floor neurological function (Snooks et al., 1985). Vegetables are important sources of many vitamins and

minerals, and bread is a valuable source of the vitamin B complex. Dallosso et al. (2003a) speculated that the reported reduced risks could be related to one of the essential biochemical functions that these micronutrients have in the body. Vegetables, and fruit, which were negatively associated with stress incontinence in the univariate analysis of Dallosso et al. (2003a), are important sources of vitamin C. This is essential for collagen production, and studies have suggested that changes in collagen metabolism contribute to defects in the paraurethral connective tissue of women with stress urinary incontinence (Falconer et al., 1994).

The reduced risk with vegetables might be associated with a decrease in serum oestrogen levels. The role of oestrogen replacement therapy in the prevention of ischaemic heart disease has been assessed in a 4-year randomised trial, the Heart and Oestrogen/progestin Replacement Study (HERS); the overall combined hormone replacement therapy was associated with worsening stress and urge urinary incontinence (Grady et al., 2001). Jackson et al. (2004) also showed an association of oral oestrogen intake and development of urinary incontinence. The lower fat content of a vegetarian diet appears to influence the excretion of oestrogens in women (Goldin, 1986; Gorbach and Goldin, 1987), and the amount and type of plant foods in the diet determine its phytoestrogen content, which can exert both oestrogenic and antioestrogenic effects on metabolism (Tham et al., 1998). Koskimaki et al. (2000) reported an increased risk of LUTS in men with a lower vegetable intake, and suggested that 'vegetables may contain natural ingredients with preventative or curative effects on diseases which cause LUTS'. The food additives in carbonated soft drinks may act adversely, individually or in

combination (antioxidants, artificial sweeteners and preservatives) on different systems including urinary system.

To conclude, several risk factors for urinary incontinence and overactive bladder are described in the literature; mainly age, smoking, chronic bronchitis and asthma, ethnicity, obesity, pregnancy (mode of delivery), hysterectomy, dementia, stroke and Parkinson's disease, medication, physical activities and diet. However, at present, for the majority of them there are no controlled trials demonstrating that intervention reduces the incidence, prevalence or degree of severity of urinary incontinence.

CHAPTER 4

REVIEW OF LITERATURE

CARBONATED SOFT DRINKS

4.1 Introduction

There has been an overwhelming rise in the consumption of soft drinks, in particular carbonated beverages, over the past two or three decades. They are now the most popular refreshments among much of the world's population, which includes people from various age groups (Jurgens et al., 2005). BBC Health News Report commented that more than 5,560 million litres of carbonated soft drinks are consumed every year in the UK (URL 2). Carbonated (fizzy) soft drinks now represent about 8% and non carbonated around 27% of total drinks consumption. Diet and low calories drinks now make up around 28% of carbonated soft drinks consumption in the UK, with many people actively switching to diet carbonated soft drinks as part of weight control (URL 3). Various studies published so far have explored the possibility of potential health risks like obesity, dental health and carcinogenicity with consumption of carbonated soft drinks. However most of the studies are observational cross-sectional population surveys or case-control studies, making it difficult to generate a confident conclusion. According to Cunningham and Marshall (2003), the adverse oral and systemic effects attributed to the consumption of soft drinks are associated with individual ingredients used to formulate the product: soft drinks are merely a vehicle for their delivery. The ingredients of concern are sweeteners and acids. It is very likely that these components working together may well modify their individual effects or become important risk factor for developing some health problem (Fried and Nestle, 2002).

Forshee and Storey (2003) looked into the patterns of beverage consumption among children and adolescents related to age, race and gender. They showed that boys, particularly white adolescent boys consumed beverages more than girls and carbonated

beverages were found to be more popular drink among the older teenagers than milk or any fruit juice. The relationship between body mass index (BMI) and beverage consumption was described by Forshee and Storey (2003). Although no association was found between BMI and consumption of regular carbonated beverages, a positive association was demonstrated between BMI and diet carbonated drinks (Forshee and Storey, 2003). Kvaavik et al. (2005) investigated the association between carbonated soft drinks consumption from age 15 to 33 years, lifestyle factors and body weight. In this study, soft drink intake from age 25 to 33 years was found to be associated with smoking and physical inactivity, but not with body weight.

The consumption of soft drinks is of particular concern as they are rich in sugars and sweeteners but few essential nutrients. Around 25% of adolescents drink more than 26 oz/day (about 300 kcal), providing approximately 12% to 15% of their daily calorific need (Fried and Nestle, 2002). Children taking soft drinks regularly perhaps consume more calories than that of required nutrients and are more at risk to be overweight or obese after adjustment for variables such as anthropometric, demographic, dietary, and lifestyle factors (Ludwig et al., 2001). Furthermore, a marked preference for soft drinks and snack foods over milk and vegetables has been shown by students who had access to these compared to students who did not have such access (Cullen et al., 2000).

4.2 Impact on human body

4.2.1 Effect on bone

Intake of carbonated beverages has been associated with reduced bone mass or increased fracture risk in observational studies, both later in life (Wyshak et al., 1989)

and in children and adolescents (Wyshak and Frisch, 1994; Petridou et al., 1997). In most reports, cola drinks were more strongly associated than were other carbonated beverages. Spencer et al. (1978) suggested that consumption of carbonated soft drinks that use phosphoric acid as the acidulants, increase the phosphorus or the net acid load and consumption of caffeinated beverages increase the caffeine load (Smith et al., 1989). The increase in net acid load perhaps results in an increase of urinary calcium excretion which is responsible for the association between consumption of carbonated soft drinks and reduced bone mass. Individually, phosphorus and caffeine were shown to have little or no net effect (Spencer et al., 1965; Spencer et al., 1978; Smith et al., 1989; Kynast-Gales and Massey, 1994; Barger-Lux and Heaney, 1995; Wise et al., 1996) but concern remains about the total acid load (Brazel, 1995). More recently, reduced bone mass was reported to be associated with fructose, found in beverages which use natural sweeteners, and this was attributed to an increased loss of urinary calcium (Milne and Nielsen, 2000). Increased calcium excretion was more marked when high fructose diet contained less dietary magnesium. High dietary fructose also significantly decreased phosphorus balance and increased the concentration of serum alkaline phosphatase. The effect of all these factors, as would be found in many cola drinks, has not been directly investigated. Heaney and Raferty (2001) have investigated the effects of carbonated beverages on urinary calcium excretion. They reported that the association of calciuria with intake of carbonated beverages was mainly related to caffeinated beverages.

McGartland et al. (2003) have observed an inverse association between carbonated soft drink consumption and bone mineral density (BMD) and interestingly it was shown to

be confined to girls. Tucker et al. (2006) reported in their recent study that consumption of cola drink particularly in women was associated with significantly lower bone mineral density (BMD) at each hip site, but not the spine. The mean BMD at the femoral neck and at Ward's area were found to be 3.7% and 5.4% lower respectively in people who were drinking regular cola daily compared to those who had cola once in a month. Although similar findings were observed for diet and decaffeinated cola, no such association was observed between intake of non-cola carbonated drinks and reduction in BMD. Although there was no significant difference in total phosphorus intake in daily cola consumers than in non-consumers; the calcium-to-phosphorus ratio was found to be lower in cola drinkers (Tucker et al., 2006).

In children and adolescents the most important source of calcium is milk. It accounts for roughly two thirds or more of total calcium intake for infants and toddlers and more than half of total calcium intake for adolescent girls (Fleming and Heimbach, 1994; Albertson et al., 1997). Harnack et al. (1999) found that preschool, school-age and adolescent children who consumed carbonated soft drink of over 266 mL per day were 2.9–3.9 times more likely to consume less than 237 mL of milk per day, which showed that carbonated soft drink displaced milk from the diet. Heaney and Raferty (2001) suggested that the skeletal effects of carbonated beverage consumption were likely due primarily to milk displacement and indirectly related to reducing calcium levels in the skeletal system. McGartland et al. (2003) supported the hypothesis of the possibility of apparent association resulting from the displacement of more nutritious beverages from the diet.

4.2.2 Effect on dental health

Dental caries is an infectious disease involving all age groups. Diet, especially high consumption of sugars was described as a significant factor in developing dental caries. While the association between the consumption of sugars (all mono-and disaccharides) and dental caries in permanent teeth has been well-documented (Ismail et al., 1984; Rugg-Gunn, 1996; Jones et al., 1999), the association between carbonated soft drink consumption and dental caries in primary dentition is less clear. Some studies have reported significant associations (Grindefjord et al., 1995; Moynihan and Holt, 1996; Levy et al., 2003), while others did not find any (Heller et al., 2001; Sayegh et al., 2002). It was also reported that exposure to the carbonated beverage accelerated the enamel wear (al-Hiyasat et al., 1998).

Carbonated beverages are rich sources of sugars and significant amounts of caffeine, which may be responsible for development of dental caries in adolescents (Majewski, 2001). However, Forshee and Storey (2004) did not find any association between the carbonated soft drinks and poor dental health.

4.2.3 Effect on the upper gastrointestinal system

Kapicioglu et al. (1999) reported that cola drinks have proliferative and regenerative effects on the rat oesophageal mucosa, which might be as a result of an irritant effect. Although carbonated soft drinks have been associated with gastro oesophageal reflux (Hamoui et al., 2006), which is an established risk factor for oesophageal adenocarcinoma, Mayne et al. (2006) reported that high consumption of carbonated beverages did not increase risk of any oesophageal or gastric cancer subtype in men or women. Their findings indicated that consumption of carbonated beverages (mainly diet

drinks) is inversely associated with risk of oesophageal adenocarcinoma, and thus it is not likely to have contributed to the rising incidence rates (Mayne et al., 2006). Lagergren et al. (2006) also did not find any association between consumption of carbonated soft drinks including carbonated beer and oesophageal adenocarcinoma. Cuomo et al. (2002) showed that in patients complaining of functional dyspepsia and constipation, carbonated water decreases satiety and improves dyspepsia, constipation and gallbladder emptying.

4.2.4 Effect on the urinary system

Rodgers (1999) highlighted the fact that consumption of carbonated soft drinks can potentiate the risk factors associated with calcium oxalate stone formation by reducing the urinary pH and increasing excretion of oxalate while decreasing magnesium excretion (Rodgers, 1999). The risk factors for urinary calcium stone formation include low urine volume, hypercalciuria, hyperoxaluria, hyperuricosuria and increased sodium excretion (Finkielstein and Goldfarb, 2006).

Carbonated soft drinks contain several food additives including artificial sweeteners, preservatives, antioxidants, colorants, caffeine and acids. Different individual ingredients were investigated for their effect on different systems of the human body. When taken together in a carbonated soft drink, they may act differently, either reducing or enhancing some specific systemic effects. On the basis of preliminary laboratory work in the form of a screening test in this research, it was decided to focus on a few specific food additives for detailed investigation. Some of the ingredients of a carbonated soft drink are described below.

4.3 Individual components of carbonated soft drinks

4.3.1 Caffeine

The effects of caffeine on health and wellbeing have been studied and debated extensively. Caffeine consumption has been linked to many human diseases in epidemiologic studies (Chou and Benowitz, 1994). Caffeine is a methylxanthine and an adenosine-receptor antagonist, and all tissues containing adenosine receptors may be affected by caffeine exposure (Van Soeren and Graham, 1998). Caffeine acts as competitive antagonist at the A_{2a} adenosine receptor on the same postsynaptic neurons as the D_2 dopamine receptor. Thus by blocking this receptor, caffeine enhances the functional capacity of dopamine as a neurotransmitter (Ferre et al., 1992).

Reduced consumption of caffeinated beverages has been shown to improve urinary incontinence (Creighton & Stanton, 1990). On multivariate analysis of data from a case-control study, Arya et al. (2000) found a statistically significant association between high caffeine intake (>400mg/day) and detrusor instability, after controlling for other compounding factors such as age and smoking. However, this study did not determine whether a decrease in caffeine intake would be associated with improvement in detrusor instability. The theory that caffeine might cause detrusor instability is biologically plausible because caffeine has been shown to have an excitatory effect on detrusor smooth muscle and could cause an increased rise of bladder pressure during filling (Creighton and Stanton, 1990). *In vitro* animal studies have shown that contraction of detrusor smooth muscle is mediated by an increase in the concentration of cytoplasmic free calcium (Shenfeld et al., 1998). Caffeine has been shown to induce transient

contraction of smooth muscle through the release of intracellular calcium from intracellular storage sites (Ganitkevich and Isenberg, 1992; Lee et al., 1993; Sugita et al., 1998).

Benowitz et al. (1982) have described caffeine as a methylxanthine, that being clinically similar to theophylline with its sympathomimetic effects, causes the cardiac manifestations of caffeine overdose due to increased release of catecholamines (Strubelt and Diederich, 1999). Caffeine was found to increase the blood pressure in short-term studies, although this effect decreased on long term use time (Jee et al., 1999). This increase in blood pressure can be explained by caffeine induced increase in the plasma levels of several stress hormones such as cortisol, epinephrine and norepinephrine (Robertson et al., 1978; Lovallo et al., 1989; Lane et al., 1990). Interestingly, Winkelmayer et al. (2005) reported that the risk of hypertension was associated with regular consumption of cola beverages (both diet and regular) rather than regular coffee consumption. Thus they speculated that it was not caffeine but perhaps some other component present in the beverages that was responsible for the increased risk of hypertension. Caffeine toxicity by self-intended poisoning was found to produce tachyarrhythmia such as supraventricular tachycardia, atrial fibrillation, ventricular tachycardia, and ventricular fibrillation (Zimmerman et al., 1985; Price and Flinger, 1990; Chopra and Morrison, 1995; Frost and Vestergaard, 2005).

Coffee drinking has been examined in several epidemiological studies as a possible risk factor for bladder cancer (Cole, 1971; Fraumeni et al., 1971; Howe et al., 1980; Hartage et al., 1983), but the results have not been consistent. Kantor et al. (1988) found no association between coffee drinking and squamous cell carcinoma. However, an

association of adenocarcinoma with caffeine was observed in a small number of patients. The trend associated with transitional cell carcinoma has been previously analyzed and appears to be a noncausal relationship (Hartage et al., 1983).

4.3.2 Artificial sweeteners

Artificial sweeteners are added to a wide variety of food, drinks, drugs and hygiene products. The term "sugars" was conventionally used to describe the mono- and disaccharides (Food and Nutrition Board, 2005); the monosaccharides containing one sugar unit, which includes glucose, galactose and fructose, whereas the disaccharides such as sucrose, lactose and trehalose are formed of two sugar units. Many commonly used sweeteners also contain trisaccharides and higher saccharides, such as corn-derived sweeteners. The carbohydrates in some corn syrups were found to contain primarily trisaccharides and higher saccharides (67% or more), and the remaining 33% were mono- and disaccharides (Glinsmann et al., 1986). Among the artificial sweeteners, saccharin, cyclamate and aspartame were developed first and were described as the 'first generation' sweeteners. These were followed by the 'new generation' sweeteners which included acesulfame-K, sucralose, alitame and neotame (Ludwig et al., 2001). The most commonly used artificial sweeteners in soft drinks are aspartame, acesulfame K and sodium saccharine, each of which may be used individually or blended with sugars or one or more of the others (Frazier et al., 2000).

Human beings have a natural preference for sweet foods; studies have shown this tendency to be present even in newborn babies (Maone, 1990). Honey was perhaps the first sweetener that was used in the ancient cultures of Greece and China (Bright, 1999). Common sugar, which was originally obtained from sugar cane, became more popular

eventually. The first artificial sweetener was saccharin, which was synthesized in 1879 by Remsen and Fahlberg. It was used widely during World Wars I and II in view of its low production costs and the scarcity of regular sugar (Bright, 1999). With post war economic improvement of living standards, sugar became readily affordable. Later on saccharin was primarily used for its low calorific value rather than low cost. Thus a wide range of low- calorie 'diet products' evolved, in which sugar was substituted or supplemented with artificial sweeteners. As saccharin had a bitter aftertaste (Kuhn et al., 2004), there arose a need for an ideal sweetener with improved taste and low calorie. Cyclamate came into the market in the 1950s, which provided a better taste than saccharin (Weihrauch and Diehl, 2004). This difference in taste sensation can possibly be explained by the difference in mechanism of action of individual sweetening agents mediated by different receptors.

The growing trend of regular use of artificial sweeteners raised the concern of its safety in daily life. The European Scientific Committee on Food, after review of 500 reports (2002) from biochemical, clinical and behavioural research, concluded that the acceptable and safe daily intake of one of the sweetening agents, aspartame to be 40 mg/kg/day, except for people with phenylketonuria (Navia, 1994). Moreover, as the quantity of different sweeteners used in soft drink is so small, that it is practically very difficult to cross the limit of acceptable daily intake of any particular sweetener (Lean and Hankey, 2004). Even when sweeteners are used in combination, high level consumers rarely exceed 10 mg/day. Although daily intake of aspartame over 1g was found to change the normal milieu of brain neurotransmitters and provoke seizures in monkeys, no such neurological or behavioural changes were detected in humans

(Wolraich et al., 1994; Butchko and Stargel, 2001). A study which has estimated the consumption of the artificial sweeteners acesulfame-K, aspartame, cyclamate and saccharin in group of Swedish diabetics, revealed that some children had a daily intake up to and sometimes above the acceptable daily intake (ADI) for aspartame and acesulfame K (Ilback et al., 2003).

Canty and Chan (1991) reported that consumption of non-caloric sweet drinks (containing artificial sweeteners) did not increase hunger or subsequent food consumption. However these results were found to be different from those of Blundell and Hill (1986) and Rogers et al. (1988), which suggested that non-caloric sweet drinks stimulated hunger although not necessarily food intake. In a review of the effects of intense sweeteners on hunger, food intake, and body weight, Rolls (1991) suggested that although saccharin consumption may be followed by increased food intake both in rats and humans, aspartame has not been found to increase food intake.

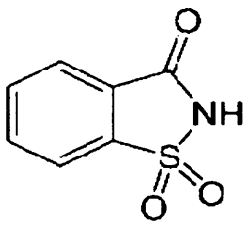
Ludwig et al. (2001) examined the association between consumption of sugar containing drinks and childhood obesity. Sugar-sweetened drinks, such as soft drinks, have been suggested to encourage obesity because compensation at later meals for energy consumed in the form of a liquid could be less complete than for energy consumed in the form of solid food (Mattes, 1996). Bray et al. (2004) also reported that the over consumption of the intake of high fructose corn syrup in sweetened beverages may play a role in the epidemic of obesity.

Martin-Villa and colleagues (1981) conducted an elaborate study to analyze sixteen different soft drinks for their soluble carbohydrate contents both qualitatively and quantitatively. They demonstrated that the total carbohydrate content in the respective

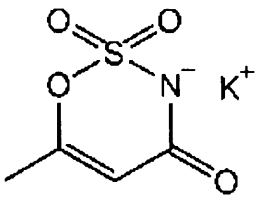
drinks was constant throughout the four analyses: 10 gm /100ml in Coke, 11 gm/100ml in Pepsi, 11gm/100ml in Fanta Lemon and 12 gm/100ml in Fanta Orange. The carbohydrate content was found to be low in some drinks, such as orange, lemon, and cola Casera, (5 gm/ 100 ml), and Schweppes tonic water was in an intermediate position with 8 gm/ l00 ml. Accordingly, the elevated consumption of these drinks among the population, implies a considerable energy contribution to the individual diets (Martin-Villa, 1981).

Since their introduction, there was always a concern of potential carcinogenic risk associated with artificial sweeteners (Taylor et al., 1980; Gurney et al., 1997; Takayama et al., 2000). In 1970, the Food and Drug Administration (FDA) banned cyclamate from all dietary foods and fruits in the USA in view of its cancer-inducing effect in experimental animals (Lindley, 1999). Epidemiological studies in humans did not confirm the carcinogenic effects of saccharin and cyclamate on the bladder which had been reported previously from animal studies in rats (Cohen and Lawson, 1995; Weihrauch and Diehl, 2004). Kantor et al. (1988) reported in their epidemiological study that the use of artificial sweeteners was not consistently related to any cell type of bladder cancer. Although Olney et al. (1996) suggested an association between aspartame and brain cancer in humans, this hypothesis was not supported by any biologic or experimental evidence (Gurney et al., 1997; Ross, 1998). After tobacco, saccharin may be the substance that has been most studied epidemiologically (Morgan and Wong, 1985). Several studies failed to demonstrate any significant association between saccharin and bladder cancer (Morgan and Wong, 1985; Nomura et al., 1991; Elcock and Morgan, 1993).

Saccharin, cyclamate and aspartame were followed by new generation of sweeteners such as acesulfame-K and sucralose. However, even the new sweeteners are not completely devoid of bitter and metallic aftertaste (Weihrauch and Diehl, 2004).



sodium saccharin



acesulfame K

Fig. 11 *Chemical structures of sodium saccharin and acesulfame K (URL 4 & 5)*

Saccharin is the oldest and the most extensively researched of all artificial sweeteners. The increased incidence of bladder cancers was reported by Fukushima et al. (1983). However this report was criticised later as the rats used in this trial were frequently infected with the bladder parasite *Trichosomoides crassivanda*, which made them susceptible to saccharin-induced bladder cell proliferation (Whysner and Williams, 1996). Bladder cancer was also reported in other studies (Taylor et al., 1980; Squire, 1985) which resulted in prohibition of saccharin in Canada and in the USA, and since 1981; warning labels were being attached to saccharin-containing products that it can cause cancer in laboratory animals. However, the National Institute for Environmental Health Sciences removed saccharin as a potential cancer-causing agent, as no studies were able to establish the cancer-inducing mechanisms in humans that were detected in earlier studies in rats (Weihrauch and Diehl, 2004). Studies have shown that sodium ascorbate or L-ascorbic acid, when fed in similar high doses to saccharin, could also

cause superficial urothelial cytotoxicity and bladder cancer in rats (Fukushima et al., 1983; Iwata et al., 1997). However, rodent bladder tumours do not predict a similar outcome for humans (Cohen et al. 1998). Studies did not find an increase in bladder cancer in humans, when saccharin consumption was high (Armstrong and Doll, 1975; Jensen and Kamby, 1982). The cytotoxicity in rodents was explained due to the high urine osmolarity in rodents enhancing precipitation of calcium phosphate-containing crystals, which are cytotoxic to the superficial layer of the bladder epithelium, leading to regenerative hyperplasia and tumours (Cohen et al., 1998).

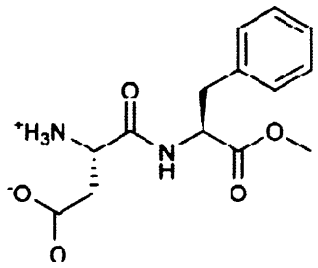


Fig. 12 *Chemical structure of aspartame (URL 6)*

Aspartame was introduced in the market in 1981 as the third artificial sweetener, and was not related to any worries about carcinogenicity (Mazur, 1984). Animal studies did not show aspartame to have any cancer-inducing effects, even in very high doses (Ishii, 1981; Hagiwara et al., 1984; Jeffrey and Williams, 2000). The carcinogenic effect was also not detected in humans (Gurney et al, 1997; Schwartz, 1999).

Aspartame has been considered a possible cause of hyperactivity and other behaviour problems in children (Wurtman, 1983). The presumed reaction to aspartame has been

attributed to the possibility that its metabolism results in elevated plasma phenylalanine concentrations, which in turn may alter the transport of essential amino acids to the brain (Elsas and Trotter, 1988).

After the introduction of cyclamate and aspartame, it became unreasonable to link bladder cancer solely to the intake of saccharin, as most of the time a combination of different artificial sweeteners was used in variety of food products to improve the taste. Therefore, it is reasonable to accept that most epidemiological studies in humans are related to overall sweetener consumption, and not to a specific one (Weihrauch and Diehl, 2004). Many of these studies (case-control) were conducted in a small number of patients with bladder cancer (1965 – 1986), and failed to show any increased risk of bladder cancer associated with the use of artificial sweeteners (Wynder and Goldsmith, 1977; Morrison and Burning, 1980; Wynder and Stellman 1980; Piper et al., 1986). However a case–control study published by Sturgeon et al. (1994) has shown heavy sweetener and coffee consumption to be associated with an increase risk of bladder cancer. The authors reported that heavy artificial sweetener use was associated with higher grade, poorly differentiated tumours.

4.3.3 Acidulants, preservatives and antioxidants

4.3.3.1 General overview

Acidulants help in beverage preservation for long-term storage by reducing the pH. Acidulants can also be used as chelating agents, buffers, coagulants, and flavouring agents, imparting a tart taste (Kuntz, 1993). The pH of a cola drink is between 2.8 to 3.2. However, the human kidney can excrete urine with a minimum pH of 5. Therefore the

excretion to happen, the acidity of the soft drink needs to be buffered. Acidulants help in this buffering action (Barzel and Massey, 1998).

The most common acidulants used in soft drinks are phosphoric and citric acids. The common preservatives used in carbonated soft drinks include sodium benzoate, benzoic acid, potassium sorbate, ascorbic acid, citric acid and sodium citrate (Lambert and Stratford, 1999).

Phosphoric acid is more effective in reducing the pH than organic acids and is predominantly found in cola drinks. Citric acid, on the other hand is typically added to citrus beverages to add a strong tartness (Kuntz, 1993). However, these acids are often blended together to produce a more distinctive taste (Dionex Corporation Application 169). The low pH value of soft drinks helps to control the bacterial growth. Carbon dioxide and the preservatives present in the carbonated soft drinks also add to the protective effect. Sodium benzoate is used as a broad spectrum antimicrobial, inhibiting bacteria, moulds, and yeasts (Sagoo et al., 2002).

The role of ascorbic acid is described primarily as an anti-oxidant (May, 1999). Antioxidants used as food additive are substances which prevent chemical reactions responsible for destruction of aromatic substances present in soft drinks. Aromatic substances add a pleasant taste to the soft drinks. Natural aromatic substances are those that are found in nature and are transferred to soft drinks through the natural juices or other plant parts like peel of orange. Caffeine in cola-drinks is also an aromatic substance (URL7); and although it is present in very small quantity, it contributes largely to the drink's taste (Griffiths and Vernotica, 2000).

Antioxidants, such as α-tocopherol and ascorbic acid, have been used as food additives as they play an important role in preventing or delaying oxidation (Madhavi et al., 1996). Alpha-tocopherol is a primary antioxidant which acts mainly by donating hydrogen or electrons to free radicals and converting them to more stable compounds (Frankel, 1998). Ascorbic acid is a secondary antioxidant that acts as an oxygen scavenger, reacting with free oxygen and removing it in a closed system (Madhavi et al., 1996).

The two preservatives ascorbic acid and citric acid will be described here, as they were studied in detail in our study. As this thesis involves the physiology of smooth muscle contraction, the following review of literature will be focussed on studies that explored the effects of ascorbic acid and citric acid on smooth muscle contraction.

4.3.3.2 Ascorbic acid

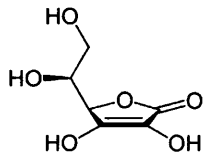


Fig. 13 *Chemical structure of ascorbic acid (URL8)*

As mentioned earlier, ascorbic acid (chemical formula: $C_6H_8O_6$) used in carbonated soft drink functions as an antioxidant to prevent reactions which destroy aromatic substances thus protecting the flavours, colour, and taste. Ascorbic acid was isolated from cabbage, lemon juice and adrenal glands by Szent-Gyorgy in 1928, and identified as the antiscorbutic factor by Waugh and King in 1932. Thus the name "ascorbic" comes from its property of preventing and curing scurvy (Davies et al., 1991). Its structure was established by Haworth and co-workers in 1933, and the same year Haworth in

Birmingham and Reichstein, in Switzerland, succeeded in synthesizing the vitamin (Bender, 2003). It was also described that of the four stereoisomers (L-ascorbic acid, D-ascorbic acid, L-isoascorbic acid and D-isoascorbic acid or erythrobiotic acid) of ascorbic acid, only L-ascorbic acid has significant vitamin C activity. They are isomers differing in orientation of the hydrogen and hydroxyl ion on Carbon 5. The stereoisomers have no biological activity other than small amount for isoascorbic acid (2.5-5% compared to L-ascorbic acid) (Eitenmiller and Landen, 1999). Ascorbic acid is available commercially as a food additive L-ascorbic acid and as its sodium salt. The commercially available form of vitamin C supplements for food in U.S.A also includes ascorbyl palmitate and calcium ascorbate (Burdock, 1999).

The functional importance of ascorbic acid in human has been described as the main water-soluble antioxidant organic acid in human plasma with protective effects on coronary heart diseases (Frei et al., 1988). It protects lipids against peroxidative damage by scavenging superoxide and other reactive oxygen species (Bendich et al., 1986). Kaufmann et al. (2000) has reported that ascorbic acid can restore the coronary microcirculation function in smokers.

Metabolism: Ascorbic acid is absorbed in the intestine by sodium dependent active transport at the brush border membrane and sodium independent transport at the basolateral membrane, throughout the intestinal tract (Malo and Wilson, 2000). Ascorbic acid is reversibly oxidised to dehydroascorbic acid in the body and both forms, ascorbate and dehydroascorbate, are physiologically active. This reaction, which proceeds by removal of the hydrogen from the enediol group of ascorbic acid, is part of the hydrogen transfer system forming a monodehydroascorbate radical which rapidly dissociates into

ascorbate and dehydroascorbate (Dollery, 1991). Most tissues have both nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione-dependant monodehydroascorbate reductase enzymes, which reduces the radical back to ascorbate (Burns, 1975).

Elimination: The major fate of ascorbic acid in human metabolism is excretion in the urine, either unchanged or as dehydroascorbate and diketogluconate (McEvoy, 1993; Bender, 2003). The latter is produced by non-enzymatic hydration in tissues. Both ascorbate and dehydroascorbate are filtered at the glomerulus and then reabsorbed, by a sodium-independent process. Reabsorbed dehydroascorbate is reduced to ascorbate in kidneys.

The plasma concentration of ascorbate shows a sigmoidal relationship with intake (Friedman et al., 1940). Below 30 mg per day, the plasma concentration is extremely low and there is little or no urinary excretion of ascorbic acid (Bender, 2003). With an increase in intake above 30 mg per day, the plasma concentration rises sharply reaching a plateau of 70 to 85μmol/L. At intake between 70 and 100 mg per day, when the renal threshold is reached, ascorbic acid is excreted quantitatively with increasing intake (Levine et al., 1996).

Effect on smooth muscle: Dillon and co-workers studied the effect of ascorbic acid on smooth muscle. In 2000, they demonstrated that norepinephrine (NE) and ascorbate bind to each other with a dissociation constant of 37 μM. In their later study, Dillon et al. (2004) reported that ascorbic acid has a profound excitatory effect on catecholamine-induced contractions of rabbit aortic smooth muscle, which is independent of the direct antioxidant effect of ascorbate on norepinephrine and epinephrine. They described (i) the

effect of ascorbate on the number of available adrenergic receptors using radio-labelled and immunological agents, (ii) the effect of ascorbate on the second messenger activity of the adrenergic receptor and the second messenger effects on Ca^{2+} release from the sarcoplasmic reticulum and the opening of Ca^{2+} channels, and (iii) the binding of adrenergic compounds to their receptor when there is an adrenergic-ascorbate complex. In this aspect, they highlighted the possible role of ascorbic acid in enhancement of muscle contraction by modulating the state of adrenergic receptors, independent of the reduction of oxidation. The adrenergic receptors have been shown to exist in two states with a fivefold difference in sensitivity to catecholamines, with the basic state more sensitive than the acidic state (Rubenstein and Lanzara, 1998). Dillon et al. (2004) suggested that ascorbic acid modulates the state of adrenergic receptors in a way that favours enhancement of the muscle contraction.

The mechanism of this enhancement of catecholamine induced contractions was explained decades ago by Green and Richter (1937). Maxwell et al. (1983) described that the cytochrome-indophenol-oxidase system, which catalyzes the oxidation of epinephrine to adrenochrome, was detected at a higher concentration in cardiac muscle than other tissues. Inactivation of these enzyme systems should delay oxidation of epinephrine resulting in potentiation of its action (Verly, 1948; Kitto and Bhor, 1953).

The mechanism of actions of L-ascorbic acid on the guinea pig isolated ileum was studied by Terada et al. (1980). Ascorbic acid (10^{-3} M) was found to produce a stimulated motility (A effect) and a enhancement of acetylcholine (ACh)-induced contraction (B effect). The A effect showed a tachyphylaxis, and was inhibited by tetrodotoxin, atropine, adenosine, morphine, strychnine, Mg^{2+}, papaverine and glucose- and Ca^{2+}-free medium,

but was stimulated by eserine. Thus, ascorbic acid appears to have not only a direct myogenic effect but also a neurogenic effect by stimulating ACh release from intramural cholinergic nerves (Terada et al., 1980; Joiner, 1973).

Interestingly, although several of the above mentioned studies suggested ascorbic acid contracts cardiac muscle, vascular or gut smooth muscles, Powiardowska and Puglisi, (1980) reported that on isolated tracheal smooth muscle, L-ascorbic acid alone induced a dose-related relaxation. They demonstrated that L-ascorbic acid increased the relaxing effect of β agonist like isoprenaline on the tracheal smooth muscle and this relaxation effect was abolished by a β blocker, propranolol, suggesting that the effect of L-ascorbic acid may be related to stimulation of beta adrenergic receptors. Thus ascorbic acid showed opposite effects on muscle contraction in different tissues, probably due to its diverse modes of action. The diversity may be due to that fact that ascorbic acid activates different receptors in different tissues, like parasympathetic stimulation in gut and sympathetic activation in tracheal smooth muscle.

Beneficial and adverse effects: In 1991, COMA (Committee on Medical Aspects of Food and Nutrition Policy) recommended the daily intake of ascorbic acid to be 40 mg/day for adults, with an increase in pregnancy to 50 mg/day, and during lactation to 70 mg/day (COMA, 1991: URL9). A large number of people habitually take between 1 to 5 gm per day of ascorbic acid supplements. Excessive consumption of carbonated soft drink increases this load considerably more. There is little evidence of any significant toxicity from these high intakes, although several potential problems have been highlighted (Rivers, 1989). Several studies have suggested that a high intake of ascorbic acid is associated with increased excretion of oxalate (Reznik et al., 1980; Swartz et al.,

1984); however, much of the oxalate may be the result of nonenzymatic formation from ascorbate under alkaline conditions, occurring either in the urinary bladder or after collection, and thus not a risk factor for renal stone formation (Chalmers et al., 1986). Ascorbate may also increase the urinary excretion of uric acid, and possibly be protective against the development of gout, but may increase the urinary concentration of above the solubility threshold, thus increasing the chance of developing urate renal stones (Bender, 2003).

Some epidemiologic studies suggested a protective effect of ascorbic acid against cancer (Henson et al., 1991; World Cancer Research Fund, 1997; Khaw et al., 2001; Vecchia, 2001; Lee et al., 2003). World Cancer Research Fund (1997) has suggested that the chemopreventive effects not to be associated with any increased adverse effect even at doses higher than the recommended dietary allowance of 60 mg/d. However, this protective effect was not observed in clinical trials with high dose of dietary ascorbic acid supplement (Blot et al., 1993; Yong et al., 1997). Lee et al. (2001) reported that daily dietary ascorbic acid of 200 mg can enhance production of endogenous genotoxins by degradation of lipid hydroperoxides, and this may act against cancer chemoprevention. Although, later in their study, Lee et al. (2003) has also highlighted that in reality, the risk of such adverse effects related to ascorbic acid would be very minimal due to the presence of intracellular endogenous antioxidants such as glutathione peroxidase and catalase. Production of glutathione in human lymphocytes was reported to be enhanced by daily intake of ascorbic acid (500–1000 mg) which may be responsible for inhibition of lipid peroxidation (Lenton et al., 2003).

Overall, the majority of studies have found that ascorbic acid to have more beneficial than harmful effects.

4.3.3.3 Citric acid

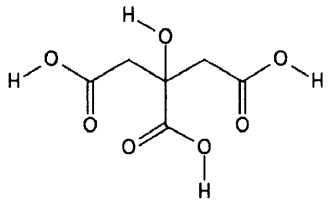


Fig. 14 *Chemical structure of citric acid (URL 10)*

Citric acid or 2-hydroxy-1,2,3-propanetricarboxylic acid (HO_2 CCH_2 $C(OH)(CO_2H)CH_2$ CO_2 H), is a weak organic carboxylic acid containing three carboxyl groups and is responsible for the tart taste of various fruits. Although it is found in a variety of fruits and vegetables, it is most concentrated in lemons and limes comprising up to 8% of the dry weight of the fruit. Citric acid has been used as an important food additive/ preservative for long time since its extraction from lemon juice for the first time in 1784 by Carl Wilhelm Scheele, a Swedish chemist. It is a natural preservative and hence has been used in soft drinks, laxatives and cathartics (The Columbia Encyclopaedia, 2006). A wide range of food products containing citric acid includes non-alcoholic drinks, beer, wine, bakery products, cheese, ice cream, jams, packet soups, sweets, tinned fruits, sauces and vegetables. (Bizri and Wahem, 1994)

The acidity of citric acid results from the three carboxyl groups COOH, each of which can lose a proton in solution, providing its excellent buffering action for controlling the pH of acidic solutions. Citrates can chelate or form salts with many metal ions, such as

calcium citrate or "sour salt", which is commonly used as a preservative and flavouring agent giving a tart taste to the food product (Phillips, 1999). The role of citric acid as a food additive has been shown to be versatile and it was also found to enhance the efficacy of other antioxidants (Hazan et al., 2004). Its use in wine production is well known, where besides being a flavouring agent and preservative, it also prevents formation of iron-tannin precipitates which cause cloudiness in wine (FDA, 1988; FDA/IFIC, 1992). In brewing it is used to produce an acidic milieu to reduce the growth of certain bacteria, yeasts and moulds (Phillips, 1999). This acidic milieu also helps in faster enzyme activity which is required in processing of cheese (Branen et al., 1990).

Metabolism: Gonce and Templeton (1930) observed no increase in the citric acid output of four normal children when 0.6 g of citric acid per kg of body weight was given daily for 3 days. Ostberg (1931) reported that out of five normal individuals ingesting 10 to 40 g of citric acid every day, only one exhibited any considerable increase in urinary citric acid excretion. He concluded that citric acid appeared to be generally distributed in body, although its metabolism was not clearly defined.

Citric acid is rapidly but not completely oxidized in humans. When given in doses of 2 to 20 g, 1.5 to 2.5 % escaped oxidation and was excreted in the urine (Kuyper and Mathill, 1933). Analyses of rabbit blood drawn from various parts of the body failed to demonstrate a relationship between any particular tissue and the oxidation of citric acid.

Sherman et al. (1935) investigated the effect of the ingestion of large doses of citric acid by dogs on the amounts of citric acid present in the urine, blood, and faeces, and on the urinary pH, organic acid, and nitrogen excretion. In some instances, blood and urine citrate level measurements were made at frequent intervals in an attempt to establish the

relationship between the concentration of citrate in the blood and its rate of excretion by the kidney. Following the oral administration of 0.5 to 2.0 g of citric acid per kg of body weight, an average of 0.7% of the acid given escaped oxidation and appeared in the urine; a rise in the blood citrate level was maintained for 3.5- 7.5 hours; and no extra citric acid appeared in the faeces. Apparent renal threshold values were found to be 2.2 mg to 6.0 mg. of citric acid per 100 ml of whole blood. Ingestion of citric acid in addition to a constant diet did not affect the pH or the total nitrogen of the 24 hour urine collection (Sherman et al., 1935).

Beneficial and detrimental effects

Various investigators have tried to establish the effects of citric acid on human physiology, exploring its beneficial and detrimental effects. The majority of work confirms the beneficial effect in prevention of urinary stone formation, although citric acid can be a local irritant and in large amounts can cause teeth erosion.

Some studies have shown that citrate inhibits stone formation by preventing crystal growth, aggregation and nucleation (Parks et al., 1996; Laube et al., 2002). Deficient urinary excretion of citric acid has often been associated with urinary stone disease (Nicar et al., 1983; Whalley et al., 1996). Moreover, successful correction of hypocitruria, which has been documented largely in adults, positively correlates with a decreased stone recurrence rate. Citrate is a strong chelator of calcium (Parks et al., 1996) and in view of its ability to alkalinize urine and decrease urinary supersaturation of calcium oxalate, it has been used as one of the most common therapeutic agents to prevent calcium oxalate, uric acid or cystine stones (Pak et al., 1983; Ettinger et al., 1997).

Since citrate is involved in maintaining cellular endogenous antioxidant defences by increasing formation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) (Mallet and Sun, 2003), Byer and Khan (2005) hypothesized that administration of exogenous citrate may augment defence mechanisms and decrease the cellular injury inflicted by oxidative stress produced by exposure to increased oxalate crystals. It has been speculated that citrate combined with methionine may lower lipid peroxidation by preventing free radical production. (Selvam and Kurien, 1992). Citrate is the most commonly prescribed medicine for kidney stone disease based on the theory that it inhibits the formation and retention of crystals in the kidneys (Ashbey and Sleet, 1992; Rodgers et al., 2006).

Slanina et al. (1986) reported that dietary citric acid enhanced absorption of aluminium in antacids. Thus people who take regular high doses of antacids along with concomitant regular high intake of citric acid (in diet) may be at risk of developing aluminium toxicity. Hallberg and Rossander (1984) studied the comparative impact of soy protein, ascorbic acid and citric acid on iron absorption from a simple Latin American-type of meal to determine if adding a specific substance like ascorbic acid would improve iron nutrition. Their finding showed that addition of a piece of boiled cauliflower containing 65 mg ascorbic acid increased the bioavailability of iron in the present type of meal three to four times and that 50 mg pure ascorbic acid increased it three times. In contrast, the addition of citric acid actually significantly decreased the absorption of iron, although no explanation was given (Hallberg and Rossander, 1984).

Citric acid toxicity is unknown, apart from after intravenous administration (Bunker et al., 1962; Lee et al., 1986). Citrate toxicity has recently been reported during

haemodialysis by using citrate as an anticoagulant (Meier-Kriesche et al., 1999). Fukushima et al (1986) reported that sodium citrate promotes bladder carcinogenesis in rats.

Amato et al. (1997) reviewed the data from 99 publications reporting health related harmful and beneficial effects of soft drink consumption from 1970 to 1997 (Medline). They concluded that although many of these studies were lacking proper methodological design, yet it would be prudent to comment that high consumption of soft drinks may not be entirely safe as generally believed and in a broader perspective it represents a public health problem.

CHAPTER 5

MATERIALS & METHODS

5.1 Introduction

The experimental set up was based on the traditional organ bath that has been used extensively in the past to investigate the physiology and pharmacology of *in vitro* tissue preparations such as isolated rat detrusor muscle strips. Bladder muscle contraction is known to occur primarily in two ways – firstly, involving the presynaptic release of neurotransmitters (acetylcholine-parasympathetic and ATP-purinergic) from nerve terminals and secondly, through post synaptic receptor activation (cholinergic and purinergic). Calcium influx through L-type voltage-gated calcium channels independent of any receptor activation is another important mechanism for muscle contraction. The presynaptic mechanism of muscle contraction was elicited by stimulating the intrinsic nerves during electrical field stimulation (EFS). The receptor activation mechanism was investigated by using agonists such as carbachol and ATP. Calcium influx was investigated by using potassium chloride, which depolarized the cell membrane opening calcium channels. The effects of individual carbonated soft drink components and the whole soft drink itself on these mechanisms were investigated.

5.2 Physiological background for methodology

Although it has been described in detail in the review of literature chapter, a brief summary of the physiological background is helpful in explaining the methodology.

5.2.1 Neurotransmitters and receptors

In vivo rat detrusor muscle contracts in response to activity in neurons, which releases neurotransmitters. With regard to the excitatory innervations of urinary bladder,

several studies have shown that two different neurotransmitters exist in mammalian detrusor muscle. Acetylcholine (ACh) is the cholinergic component and the other neurotransmitter is an atropine-resistant, nonadrenergic, noncholinergic (NANC) neuronal component (Hoyle and Burnstock, 1993). This NANC component has been identified in a number of studies as adenosine triphosphate (ATP) (Andersson, 1993; Hoyle, 1994; Burnstock, 2001). Thus, the contraction of the mammalian bladder is mediated by the two neurotransmitters, acetylcholine (ACh) and adenosine triphosphate (ATP).

In vitro contraction of isolated detrusor muscle was obtained by perfusing bladder muscle strips, suspended in an organ bath, with the cholinergic receptor agonist carbachol, which is an analogue of acetylcholine not hydrolyzed by anticholinesterases (Damaser et al., 2000) (Fig. 15). Contractile responses were also achieved by the brief exogenous application of the co-neurotransmitter α,ß methylene ATP, an agonist to purinergic receptors (Wu et al., 1999) (Fig. 15). Exposure to α, ß methylene ATP was limited to short bursts to prevent desensitization (Brading and Williams, 1990; Yoshida et al., 2001; Noda et al., 2002).

A receptor is a protein present on the cell membrane or within the cytoplasm or cell nucleus that binds to a specific molecule (a ligand), such as a neurotransmitter, and initiates the cellular response to the ligand. On binding to the receptor proteins, the ligands induce structural and behaviour changes in the receptor proteins that constitute the biological actions of the ligands. Once the neurotransmitters are released from the nerve terminal as the result of an action potential, they bind to specific receptors on the surface of the postsynaptic cell. The receptors are also present on the presynaptic neurons acting mainly to inhibit further release of neurotransmitters. One additional characteristic of neurotransmitter receptors is that

they are subject to ligand-induced desensitization, which means that they can become unresponsive upon prolonged exposure to their neurotransmitter.

5.2.2 Agonists and antagonists

An 'agonist' is a chemical compound which binds to its receptor to initiate the process of contraction. On administration of a range of concentrations of an agonist, the concentration-response curve takes a sigmoid shape ranging from the lowest concentration to high, where it reaches the maximum contractile response and plateaus. A 'full agonist' will produce a sigmoid curve whereas a 'partial agonist' will have a maximum response which is less than the maximum response of a full agonist. An 'antagonist' is a drug that does not provoke a response itself, but blocks agonist-mediated responses. An antagonist can occupy, although not always, the same receptor as the agonist and therefore denies access to that receptor by the agonist. An example of this is atropine which produces no direct response in muscle tissue. However, when the muscle tissue is stimulated with acetylcholine in the presence of atropine no contractile response is obtained. Acetylcholine at a much higher concentration is required to obtain a contractile response. Where the antagonism is overcome by increasing the concentration of agonist, the antagonism is known as competitive, whereas if the antagonism is not overcome, it is known as non-competitive.

Black and Leff (1983) in their operational model of agonist action have shown in spite of the complexity of the linkage between agonist binding and response, dose-response curves have shapes identical to receptor binding curves. The simplest explanation was that the link between receptor binding and response was direct, so response was found to be proportional to receptor binding. They have explained that though in a response, several messengers might be involved, if each messenger binds to a single

binding site according to the law of mass action, the dose-response curve will follow the same sigmoid shape as a receptor binding curve.

5.2.3 L-Type voltage gated Ca^{2+} channels

In smooth muscle cells, Ca^{2+} transport through voltage-operated Ca^{2+} channels is the key event in depolarization-mediated changes in intracellular calcium concentration (Ganitkevich and Isenberg, 1992). This depolarization-induced influx of Ca^{2+} through L-type Ca^{2+} channels induces a release of Ca^{2+} from intracellular stores, which constitutes a major portion of the phasic intracellular rise in calcium concentration (Ganitkevich and Isenberg, 1992).

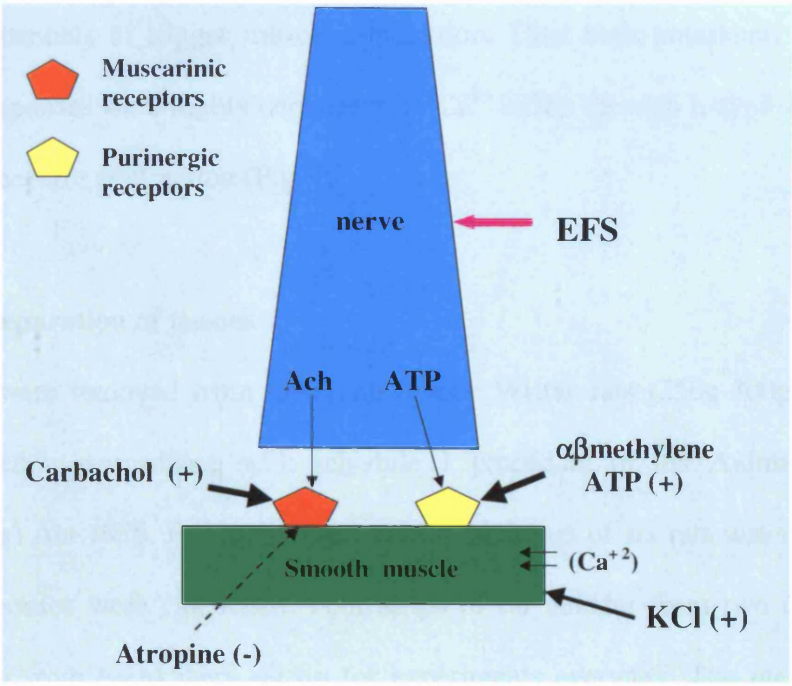


Fig. 15 *Schematic diagram showing mechanism of action of different stimuli for bladder muscle contraction: purinergic, cholinergic & Ca^{2+} channels; agonists (+) & antagonists (-). EFS: electrical field stimulation; ACh: acetylcholine; ATP: adenosine triphosphate*

The mechanisms mentioned above were used to build the framework of our investigations (Fig. 15). A contractile response to EFS was obtained via activation of

both cholinergic and purinergic pathways. Atropine was used to block the cholinergic component of the EFS-induced bladder muscle contraction to allow evaluation of the purinergic contribution. Alpha, β methylene ATP and carbachol were used to stimulate directly post synaptic purinergic and muscarinic receptors respectively. Potassium was used to induce contraction via depolarisation to investigate calcium influx through L-type voltage-gated Ca^{2+} channels, which is independent of receptor activation. High concentration of potassium depolarizes the cell membrane thereby opening the voltage-gated Ca^{2+} channels (Maggi et al., 1989a; Nakayama & Brading, 1995). Known amounts of calcium were then added to enter the already opened calcium channels to trigger muscle contraction. Thus both potassium and calcium evoked responses were highly dependent on Ca^{2+} influx through L-type voltage-gated Ca^{2+} channels for contraction (Fig. 15).

5.3 Preparation of tissues

Bladders were removed from male and female Wistar rats (250g-300g) which had been culled in accordance with schedule 1 procedure of the Animal (Scientific Procedures) Act 1986. For each experiment a minimum of six rats was used, and the mean responses were calculated. Four strips of rat bladder from two different rats (two strips from each) were set up for experiments everyday. The mean values of contractile force were calculated for each pair of bladder strips from the same rat, giving two mean values for each experiment day. The experiments were repeated for three days, giving a total of six mean values (n=6, strips=12). The bladders were removed and placed into cold Kreb's solution which was made up fresh and consisted of: NaCl 119 mM, KCl 4.4 mM, $NaHCO_3$ 20 mM, NaH_2PO_4 1.2 mM, $MgCl_2$ 1.2 mM, $CaCl_2$ 2.5 mM and Glucose 11 mM made up in distilled water (pH 7.2). Bladders

were then dissected free of any adhering fat or serosa and cut into longitudinal muscle strips (4 x 0.5 x 1 mm). The dissection was always performed exactly the same way, by the same person. The rat bladders were cut transversely at the top and bottom end and then were unfolded to open into a rectangular piece. Four strips of similar size were cut (Fig 16 A&B) and two strips of the bladder from the same rat were used to obtain the mean value of contractile force to minimize error.

A B

Fig. 16 *(A). Bladder was cut transversely at the dome and the base and then longitudinally at the middle to give a rectangular tissue sample. (B). The rectangular tissue was then cut into 4 equal longitudinal strips.*

These were suspended in a perspex organ bath chamber of 0.2ml volume (Brading and Sibley, 1983). The bladder strips were constantly perfused with Kreb's solution at the rate of 1 ml per minute, aerated with 95% oxygen and 5% carbon dioxide and the temperature was maintained at 37° C throughout the experiments. The bladder muscle strips were suspended using a fine silk suture attached to the base of the muscle strip and secured at the bottom of the chamber and another suture was attached to the apex of the muscle strip and tied to an isometric force transducer connected to a four-channel Harvard Universal Oscillograph. The strips were allowed to equilibrate for 1 hour under tension of 1 g before experimentation (Fig 17).

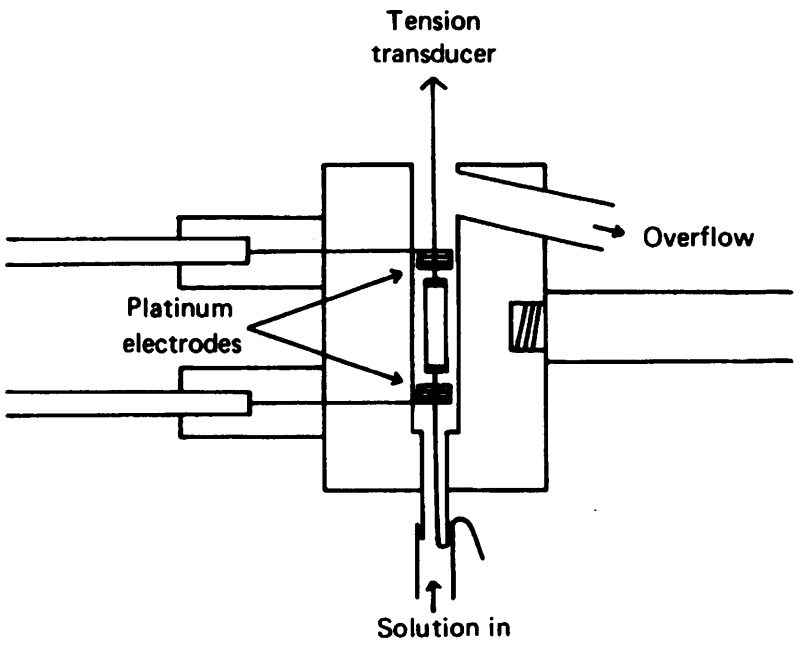


Fig. 17 Diagram of organ bath used for studying isolated detrusor muscle contraction. (Brading AF and Sibley GNA, 1983)

The transducer was connected to a Harvard Universal Oscillograph which converted isometric tension into an electrical signal. Contraction of the muscle strips shortened its length and the tension generated was transmitted to the oscillograph being represented by a deflection of the pen recorder. The oscillograph was calibrated at the beginning of the experimentation by suspending a known weight of 1g from the transducer and counting the number of squares covered by the deflection of the pen. A relationship between the deflection of the pen and tension generated is therefore known at the beginning of each experiment and was used to calculate the amount of tension generated during the experiments. For quality assurance, throughout the study it was ensured that the tension measured in each of the four organ baths did not vary.

5.4 Preparation of different concentrations of soft drink additives

Carbonated soft drinks are complex mixtures containing a variety of substances such as colouring compounds, flavouring agents, acidifiers, sweeteners, preservatives, and caffeine (Kuntz, 1993). Some of these compounds (including aspartame and ascorbic acid) have previously been investigated in animals to study toxicity and carcinogenic effects. We chose to investigate primarily the artificial sweeteners (aspartame, acesulfame K, sodium saccharine and phenylalanine), flavouring agents (citric acid), preservatives (sodium benzoate, potassium sorbate and sodium citrate) and antioxidants (ascorbic acid). We did not include caffeine, though it is an important constituent in cola drinks (as a stimulant). Caffeine has been investigated extensively for its effect on urinary system; its diuretic and stimulant effect on detrusor muscle is well established (Gray, 2001; Bryant et al., 2002).

Different concentrations (10^{-8} M to 10^{-2} M) of the carbonated soft drink components were freshly prepared before the experiments by dilution in Kreb's universal solution. 10^{-1} M concentration of any individual component was prepared by weighing out a known amount, calculated from the molecular weight (Table 2), and dissolving it in 20 mL of Kreb's solution to obtain the desired concentration. For the EFS 10 Hz experiments, subsequent ten fold dilutions were carried out from the 10^{-1} M concentration down to 10^{-8} M. For example, a concentration of 10^{-4} M was prepared by transferring 200μL of the soft drink component into 200 mL of Kreb's solution. Subsequent required concentrations were diluted as required.

Table 2 ***Molecular weights of different carbonated soft drink components***

Chemical	Molecular weight
Sodium saccharine	205
Acesulfame K	201
Aspartame	294
Phenylalanine	65
Ascorbic acid	176
Citric acid	294
Potassium sorbate	150
Sodium citrate	294

5.5 Concentration of carbonated soft drink

Different dilutions of a popular carbonated soft drink were prepared from 1: 100 to 1: 500 by a dilution method. 1 mL of the drink was mixed with 99 mL of Kreb's solution to obtain a concentration of 1: 100. This was followed by further dilutions of 1: 100 to obtain the lowest concentration, i.e. 1: 500. The effect of electrical field stimulation (EFS) on these different concentrations was investigated to obtain the concentration, which produced significant muscle contraction.

5.6 10 Hz Electrical field stimulation (EFS) - a screening test

The concentration of soft drink components in body fluids after the consumption of a can of drink was not known. The evaluation of the effects of any such drink additive *in vivo* is complicated by many factors, such as individual variations in absorption, distribution, metabolism and alteration of measured target organ action by excretion, homeostatic and compensatory mechanisms of different systems. Therefore preliminary investigations were carried out using a wide range of concentrations, starting from the lowest 10^{-8} M to the highest 10^{-2} M. This was a screening test to determine which component of carbonated soft drink, and at what concentration, significantly modulated bladder muscle contraction. The lower concentration (a concentration between 10^{-8} M and 10^{-4} M) producing a significant enhancement of muscle contraction was chosen for further investigations. The pH of these solutions was found to be within range of 7.30-7.34. Different concentrations (10^{-8} M to 10^{-2} M) of the carbonated soft drink additives were freshly prepared before the experiments by dilution in Kreb's universal solution. Thus by doing the screening test, the specific soft drink ingredient was identified along with the concentration to use, for detailed investigation.

Bladder strips were equilibrated and then stimulated with electrical field stimulation (EFS) 10 Hz to obtain control contractile responses. This frequency was chosen because both acetylcholine and ATP are released from the nerve terminals (Brading and William, 1990). At lower frequencies of EFS, muscle contraction is mainly due to the release of ATP from the nerves with an increasing component due to the release of ACh as the frequency of stimulation increases (Brading & Williams., 1990).

Individual soft drink components at the lowest concentration (10^{-8} M) were perfused through the organ bath for 20 minutes followed by three stimulations with EFS 10 Hz with an interval of 3 minutes between each to allow restoration of resting muscle tone in the bladder strips. Subsequent concentrations, up to 10^{-2} M, were added in the same manner followed by three stimulations with EFS 10 Hz. The average of the three contractile responses was determined for each concentration. Those low concentrations (10^{-4} M – 10^{-8} M) found to significantly modify the bladder contractile response to 10 Hz EFS were subsequently used for further experiments. The term 'previously determined concentration' is used later in this section to refer to these concentrations that have a significant effect on the contractile response to 10 Hz EFS.

5.7 Spontaneous contractions

Detrusor smooth muscle exhibits spontaneous contractile activity during the filling phase in normal guinea-pig bladder (Drake et al., 2003b; Gillespie, 2004a) or overactive human bladders (Brading 2006). Isolated rat detrusor strips also generate spontaneous phasic contractions. The effect of soft drink components at 'previously determined concentrations' on spontaneous rat detrusor contractions was determined by comparing the frequency and amplitude of contractions before and after the addition of each soft drink component for 5 minutes.

5.8 Time course experiments

Three frequency response curves (detailed below) were obtained at 21 minutes, 63 to 84 minutes and 210 to 231 minutes. This was to determine if the contractile response of the detrusor muscle to stimulation with EFS altered significantly over the time period of the experiment.

5.9. Response curves

5.9.1. Frequency response curves

Electrical field stimulation (EFS) was delivered by platinum electrodes recessed within the organ bath chamber and connected to a Harvard Dual Impedance Research Stimulator capable of delivering electrical impulses at different frequencies, voltage and pulse width. A frequency response curve was constructed by stimulating the bladder muscle strips with increasing frequencies of 0.5, 1.5, 10, 20, 40 and 60 Hz at 50 volts with a pulse width of 0.05 ms in 10 trains at 3 minutes interval. The response curve to EFS was plotted using a linear scale and is known as the **frequency response curve.**

Electrical field stimulation (EFS) not only generates a contractile response due to release of endogenous neurotransmitters from nerve terminals but also due to direct muscle stimulation. The magnitude of direct muscle stimulation is proportional to the setting of the pulse width; more direct muscle stimulation is obtained at higher setting. A pulse width of 0.05 ms was chosen to ensure stimulation of the nerves and not direct stimulation of the muscle. It was demonstrated previously that in rat bladder samples, tetrodotoxin 1.6 x 10^{-6} M, a sodium channel blocker, abolishes the response to EFS at this pulse width but not to pulse widths higher than this setting (Hudman et al., 2000). Thus muscle contractile response obtained on delivering electrical field stimulation with the chosen setting of 0.05 ms was due to the effect of the released neurotransmitters, acetylcholine and ATP.

5.9.1.1 EFS at different frequencies

After the equilibrium period of 60 minutes, neurally evoked isotonic contractions were induced using trains of electrical field stimulations at frequencies of 0.5, 1, 5, 10, 20, 40 and 60 Hz (at 50 V, 0.05-ms pulse width and 10-s trains) delivered at 3-min

intervals to obtain a control frequency response curve. The tissue samples were then perfused with Kreb's solution containing a soft drink component, at the previously determined concentration, for 20 minutes. The experiment was then repeated in the presence of a different soft drink component.

5.9.1.2 EFS in the absence and presence of atropine 10^{-6} M

Atropine, a cholinergic receptor antagonist, was used to block the cholinergic component of EFS (Yono et al., 2000). Atropine 10^{-6} M was used in our experiment to block the cholinergic pathway (Downie and Dean, 1977).

The bladder tissue preparation was perfused with Kreb's universal solution and control frequency response curve was obtained. Following this, the bladder tissue preparation was perfused with Kreb's solution containing atropine 10^{-6} M for 20 minutes, and another frequency response curve was obtained. The third frequency response curve was obtained in the presence of atropine 10^{-6} M plus individual soft drink additive at predetermined concentration to establish its effect on atropine resistant component of EFS (purinergic component).

5.9.2 Concentration response curves

This is a graph produced to show the relationship between the exposure concentration of a drug (or other chemical) and the degree of response it produces. Thus a concentration response curve was constructed by plotting the contractile response of rat bladder on the 'y axis,' expressed as force in grams, against the concentration of an agonist, such as carbachol, using logarithmic scale, on 'x axis'. The graph produced was a sigmoidal curve (Motulsky and Christopoulos, 2003).

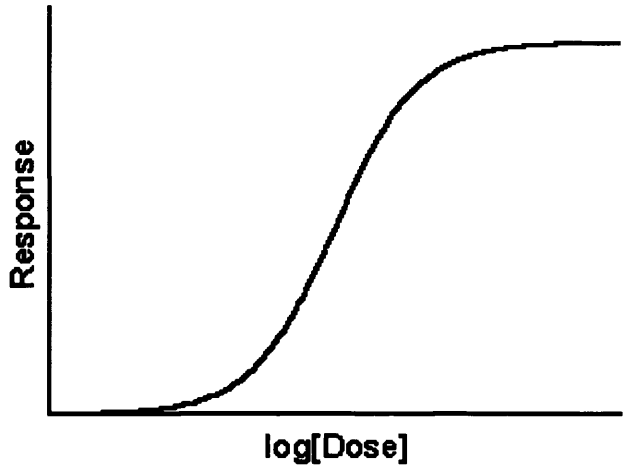


Fig. 18 A standard concentration response curve. X axis: concentrations of agonist and Y axis: Contractile response (Motulsky and Christopoulos, 2003)

A standard concentration-response curve is defined by four parameters: the baseline response (bottom), the maximum response (top), the slope, and the drug concentration that provokes a response halfway between baseline and maximum (EC_{50}).

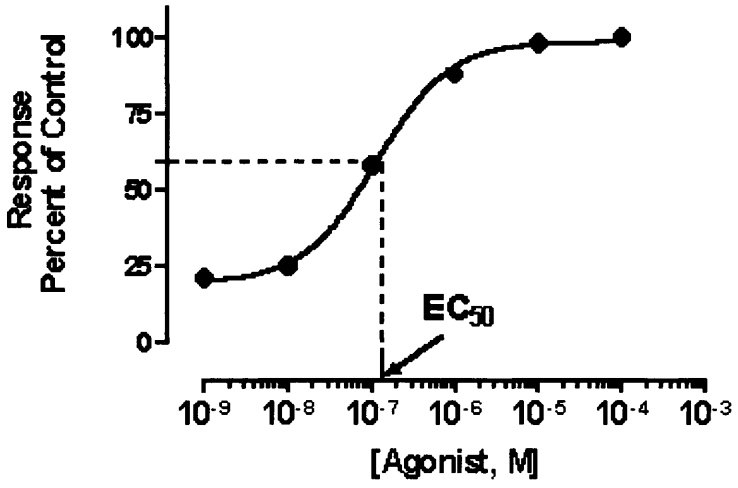


Fig. 19 EC_{50} of a standard concentration response curve (Motulsky and Christopoulos, 2003)

Experiments were performed to establish the effect of individual soft drink components on the concentration response curves to agonists such as carbachol (cholinergic) and α, β methylene ATP (purinergic), and chemicals such as KCl and calcium to investigate the role of L-type voltage-gated Ca^{2+} channels.

5.9.2.1 Concentration response curves to carbachol

Carbachol directly activates cholinoceptors in bladder smooth muscle (Damaser et al., 2000). Stock solution of carbachol, 10^{-2} M, in Kreb's solution was prepared and diluted to obtain concentrations from 10^{-8} M to 10^{-2} M immediately before use. Concentration response curves were obtained in the presence of carbachol by perfusing the muscle strips with increasing concentrations 10^{-8} M - 10^{-2} M for 30 seconds. The concentration response curve was repeated after the addition of individual soft drink component for 20 minutes, at previously specified concentrations.

5.9.2.2 Concentration response curves to α, β methylene ATP

Alpha, β methylene ATP directly activates purinergic receptors which depolarizes the cell membrane opening L-type Ca^{2+} channels, resulting in Ca^{2+} influx and muscle contraction (Wu et al., 1999).

Alpha, β methylene ATP was made up as stock in Kreb's solution 10^{-3} M and then immediately diluted further to make concentrations 10^{-7} M - 10^{-3} M before use. Concentration response curves were obtained after perfusing the organ bath with increasing concentrations of α, β methylene ATP from 10^{-7} M to 10^{-3} M for 45 seconds. The tissue samples were allowed to relax to base line between exposures. The strips were then perfused with Kreb's solution for 15 minutes to allow the tension to return to base line and to ensure that purinoceptors were not desensitised by prolonged exposure to α, β methylene ATP (Brading & Williams, 1990; Yoshida et

al., 2001; Noda et al., 2002). The α, β methylene ATP concentration response curve was repeated following 20 minutes of perfusion with Kreb's solution containing the individual carbonated soft drink component at previously determined concentrations, and compared to the control response.

5.9.2.3 Concentration response curves to potassium chloride

High concentration of extracellular potassium depolarizes the cell membrane and triggers opening up the L-type voltage-gated Ca^{2+} channels resulting in Ca^{2+} entry into the cell thereby initiating contractile response (Maggi et al., 1989a). The cholinergic and purinergic receptors are not activated by potassium. Thus the contractile response obtained in the presence of potassium is solely due to the influx of Ca^{2+} through L-type Ca^{2+} channels (Visser and Van Mastrigt, 2000a).

KCl was dissolved in potassium free Kreb's solution to produce concentrations of 10 mM - 80 mM immediately before use. Concentration response curves to KCl were obtained by perfusing the organ bath for 60 seconds with each concentration of KCl. Samples were allowed to relax to base line between exposures. Another concentration response curve was obtained in the presence of individual carbonated soft drink additive at previously specified concentrations.

5.9.2.4 Concentration response curves to calcium chloride

The detrusor muscle strips were depolarised by placing them into calcium-free, potassium-rich Kreb's solution containing 127 mM of KCl. This resulted in an initial contraction of the detrusor muscle followed by relaxation. Known amounts of calcium were then added to enter the already opened calcium channels to trigger muscle contraction.

Calcium-free, high potassium Kreb's solution (127 mM KCl) was prepared first and 500 mL was removed before adding EGTA (1.2mM), which chelates Ca^{2+}. Calcium

chloride was dissolved in the calcium free Kreb's solution without EGTA to prepare different concentrations of Ca^{2+} 0.25 to 2.5 mM (0.25, 0.5, 1.0, 1.5, 2.0, 2.5 mM). Previously determined concentrations of individual soft drink component were prepared by dissolving them in Ca^{2+} free high potassium Kreb's solution. After an equilibration period of 60 minutes in Kreb's universal solution, the bladder strips were perfused with calcium free high potassium Kreb's solution for about 20 minutes, when a contractile response was obtained (as mentioned above due to membrane depolarization by high potassium). Once the contractile response returned to baseline, each concentration of calcium (0.25, 0.5, 1.0, 1.5, 2.0, 2.5 mM) was perfused through the organ bath chamber for 60 seconds to produce a concentration response curve to calcium. Samples were allowed to relax in between exposures. The bladder muscle strips were then washed with calcium free Kreb's solution for 20 minutes and the procedure was then repeated in the presence of individual carbonated soft drink additive at previously specified concentrations.

To summarize, the sequence in which the following experiments were performed was:

1. 10 Hz EFS- screening test
2. Spontaneous contractions
3. EFS: time course experiment
4. EFS: control and carbonated soft drink component
5. EFS: control, atropine , atropine plus carbonated soft drink component
6. Carbachol response
7. Alpha, beta methylene ATP response
8. Potassium response
9. Calcium response

5.10 Statistical analysis

Sample size calculations (SPlus 6.2) indicated that muscle strips from six animals were required to construct each contractile response curve. Data are expressed as means ± standard error of the mean (SEM) and analysed using paired t tests when comparing two groups such as the maximum contractile response only. Repeated Measures One Way Analysis of Variance and One Way Analysis of Variance followed by Tukey's or Dunnett's multiple comparisons where appropriate. A p value of < 0.05 was regarded as significant. Log EC_{50} was determined by using non-linear regression analysis on GraphPad Prism.

CHAPTER 6

10 Hz

ELECTRICAL FIELD STIMULATION

SCREENING TEST

RESULTS

6.1 Introduction

This chapter presents the results of screening the carbonated soft drink ingredients by determining their effect on the contractile response of rat bladder muscle to stimulation with EFS 10 Hz. The results identified those ingredients which required further investigation and also identified the concentrations of respective soft drink ingredients, which significantly enhanced the contractile response to EFS compared to control. The concentrations of soft drink ingredients used for further investigations were no higher than 10^{-4} M.

6.2 Artificial sweeteners

The contractile response of rat detrusor muscle to 10 Hz EFS was significantly enhanced compared to control in the presence of acesulfame K 10^{-8} M - 10^{-2} M ($p<0.01$) (Fig 20A), sodium saccharin 10^{-8} M - 10^{-3} M ($p<0.01$) (Fig 20B) and aspartame 10^{-7} M – 10^{-2} M ($p<0.01$) (Fig. 20C). The following concentrations of sweeteners were used for subsequent experiments: acesulfame K 10^{-6} M, sodium saccharin 10^{-7} M and aspartame 10^{-7} M. Acesulfame K 10^{-6} M and 10^{-7} M enhanced the contractile response to EFS 10 Hz by 8% (± 1, $p<0.01$) and by 6% (± 2, $p<0.01$) compared to control respectively (10^{-6} M was chosen due to the slight enhancing effect). Sodium saccharin 10^{-7} M enhanced the contraction by 12% (± 4, $p<0.01$) and aspartame 10^{-7} M increased it by 14% (± 2, $p<0.01$) compared to control. These low concentrations were chosen as they produced significant enhancement of muscle contraction compared to the control.

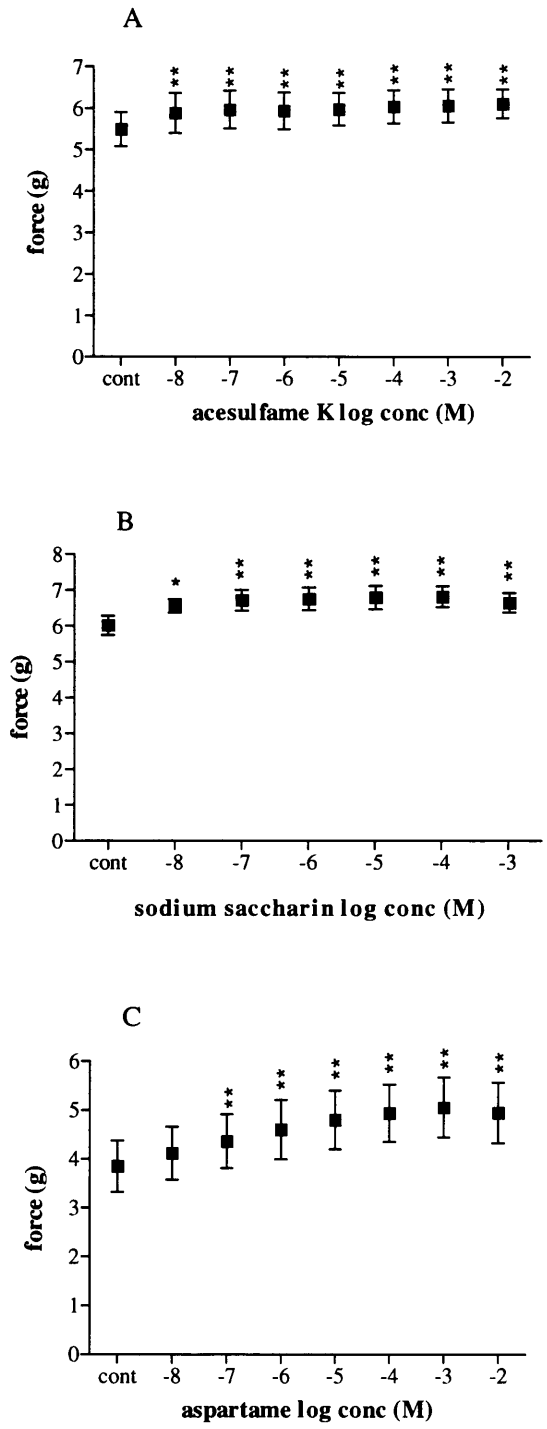


Fig. 20 *The effect of acesulfame K 10^{-8} M-10^{-2} M (A), sodium saccharin 10^{-8} M-10^{-3} M (B) and aspartame 10^{-8} M-10^{-2} M (C) on rat detrusor muscle contractile response to 10 Hz EFS. (*p<0.05, **p<0.01). n=6*

6.2 Ascorbic acid and citric acid

The contractile response of the bladder muscle to 10 Hz EFS was enhanced significantly compared to control in the presence of ascorbic acid 10^{-6} M - 10^{-3} M ($p< 0.01$) (Fig 21A) and citric acid 10^{-6} M - 10^{-3} M ($p< 0.05$) (Fig 21B). Ascorbic acid 10^{-4} M enhanced the bladder muscle contraction by 22% (±7) compared to control and citric acid 10^{-5} M enhanced contractile response by 13% (± 4) compared to control. Amongst all the concentrations used, the maximum enhancement of the contractile response was found to be at 10^{-4} M. As citric acid also enhanced contractile response to EFS 10 Hz at concentrations of 10^{-6} M upwards, there being very little difference between these effects, 10^{-5} M was chosen for further investigation as being roughly in the middle of those concentrations which effectively enhanced bladder contraction. Further investigations were performed using these concentrations of ascorbic acid 10^{-4} M and citric acid 10^{-5} M.

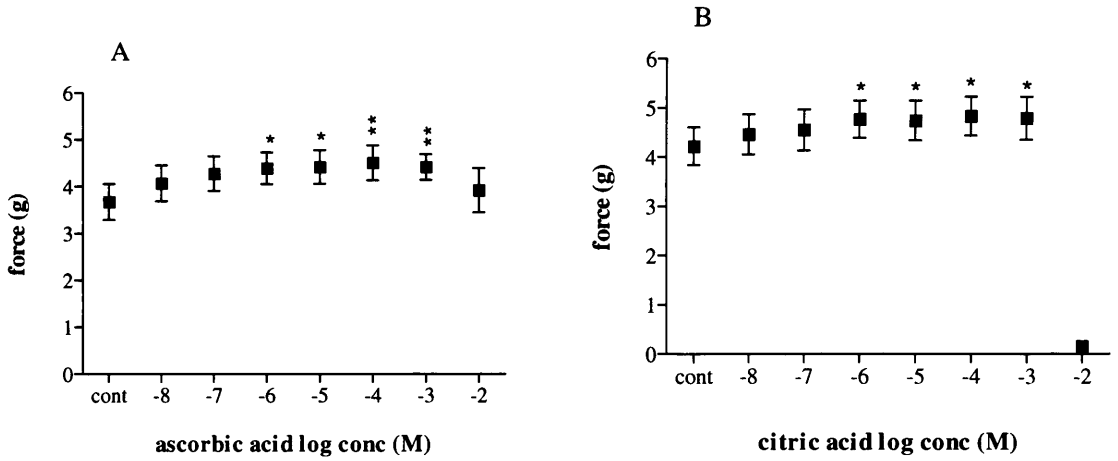


*Fig. 21 The effect of ascorbic acid 10^{-8} M-10^{-2} M (A) and citric acid 10^{-8} M-10^{-3} M (B) on rat detrusor muscle contractile response to 10 Hz EFS. (*p<0.05). n=6*

6.3 Other ingredients:

Although phenylalanine (Fig. 22A) and potassium sorbate (Fig. 22B) produced some significantly enhanced contractile responses, these were sporadic and not consistent over a range of concentrations. However, sodium citrate (Fig. 22C) and sodium benzoate (Fig. 22D) did not have any significant effect on contractile response. No further investigations were carried out using these four soft drink components.

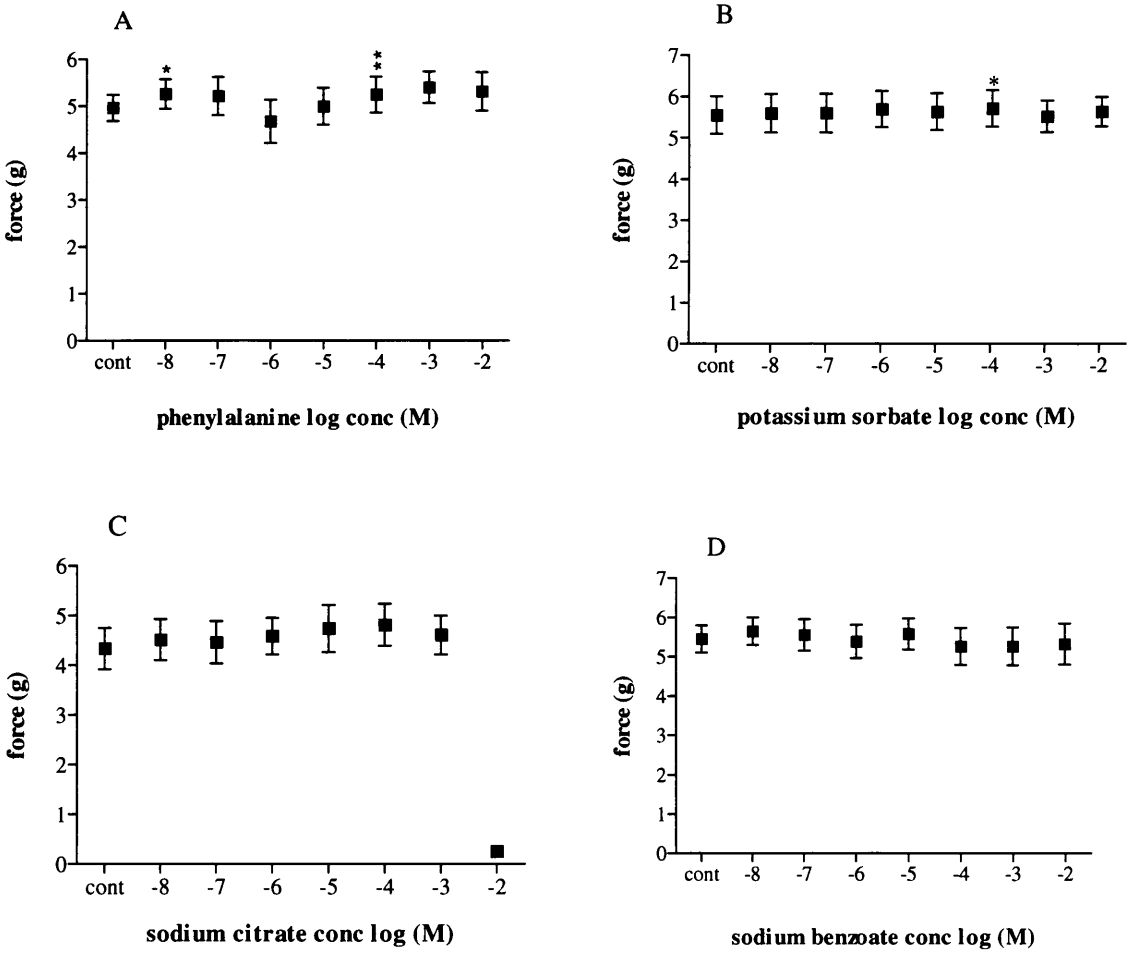


Fig. 22 The effect of phenylalanine 10^{-8} M-10^{-2} M (A), potassium sorbate 10^{-8} M-10^{-2} M (B), sodium citrate 10^{-8} M-10^{-2} M (C) and sodium benzoate 10^{-8} M-10^{-2} M (D) on rat detrusor muscle contractile response to 10 Hz EFS. n=6

It is difficult to explain why sodium citrate did not have any significant effect on the contractile response whereas citric acid was found to be stimulating the muscle contraction.

Table 3 *List of concentrations of individual soft drink components used for detailed investigations*

Soft drink component	concentration
Acesulfame K	10^{-6} M
Aspartame	10^{-7} M
Sodium saccharin	10^{-7} M
Ascorbic acid	10^{-4} M
Citric acid	10^{-5} M

Key points:

- Artificial sweeteners (acesulfame K, sodium saccharin and aspartame), ascorbic acid and citric acid were found to enhance the contractile response to 10 Hz EFS.
- Other constituents of carbonated soft drinks which were investigated similarly, such as, phenylalanine, potassium benzoate, sodium citrate and sodium sorbate, did not modulate the contractile response to 10 Hz significantly and consistently.

CHAPTER 7

ARTIFICIAL SWEETENERS

RESULTS & DISCUSSION

7.1 Introduction

Artificial sweeteners are added to a wide variety of food, drinks, drugs and hygiene products. The most commonly used artificial sweeteners in soft drinks are aspartame, acesulfame K and sodium saccharine (Frazier et al., 2000).

The screening tests which were performed in this study using 10 Hz EFS, suggested that all the three artificial sweeteners caused significant enhancement of muscle contraction. Further investigations were carried out on all three artificial sweeteners using the concentrations previously determined, i.e., acesulfame K 10^{-6} M, sodium saccharin 10^{-7} M and aspartame 10^{-7} M.

7.2 Results

7.2.1 Spontaneous contractions

At the moment when the perfused solution of acesulfame 10^{-6} M reached the bladder smooth strips in the organ bath, the frequency of the spontaneous contractions was reduced for about 60 seconds but the amplitude of the contractions was increased. Following this immediate effect of acesulfame on spontaneous contractions, the mean amplitude of the contractions was increased by a maximum of 39% compared to control and the frequency of spontaneous contractions was enhanced by 25% compared to control (from 6 bladders) (Fig. 23 A).

A similar effect was seen after the addition of sodium saccharin 10^{-7} M to the organ bath. The amplitude and frequency of spontaneous contractions were increased by 65% and 18% respectively, compared to control (Fig. 23 B). However, the effect of aspartame 10^{-7} M on spontaneous contractions was much less than the other sweeteners with the frequency and

amplitude being increased by only 14 and 16% respectively compared to control (from 6 bladders) (Fig. 23 C).

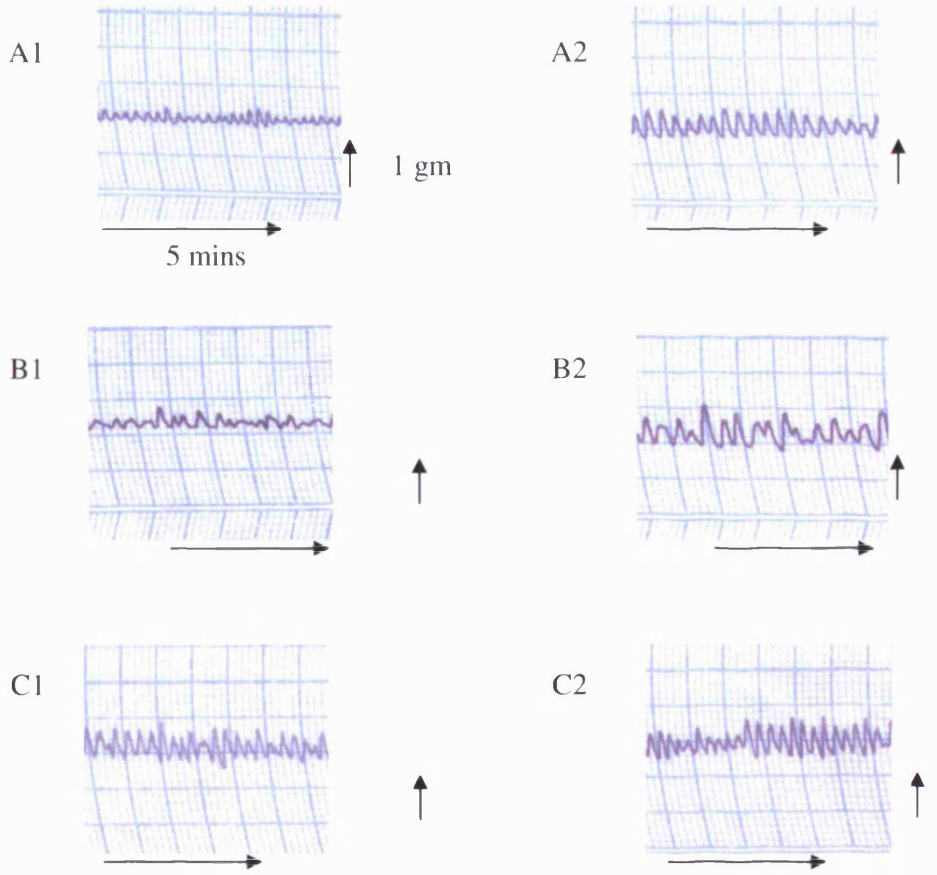


Fig. 23 *Spontaneous contractions of bladder muscle in presence of (A) acesulfame K 10^{-6} M, (B) sodium saccharin 10^{-7} M and (C) aspartame 10^{-7} M. A1, B1 & C1 are controls and A2, B2 & C2 are contractions in presence of respective sweeteners. The horizontal arrows correspond to time (5 big square$_=$ 5 mins) and the longitudinal arrows correspond to the amplitude of contraction (each 6 squares$_=$ tension of 1gm). Time of observation: 5 minutes.*

7.2.2 Frequency response curves

Time course experiments:

Results of the time course experiments showed no significant difference in the frequency–response curve obtained at 0 - 21 min, 63 - 84 min or 210 - 231 min compared to the control response (Fig 24).

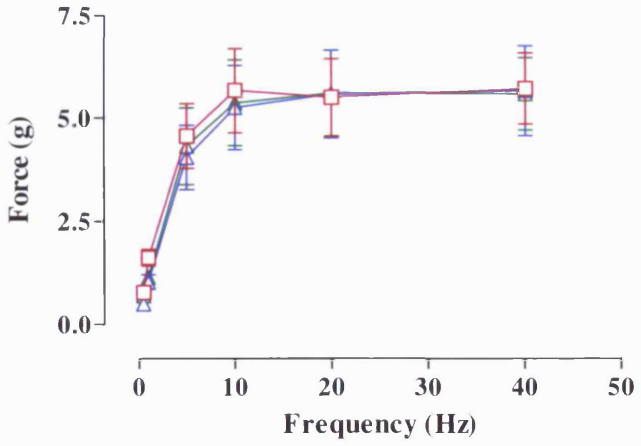


Fig. 24 *Frequency response curves at different time intervals (Time Course Experiments). △ 0-21 minutes, △ 63-84 minutes and □ 210-231 minutes. n=6.*

Acesulfame K 10^{-6} M increased the maximum contractile response of bladder muscle to 20 Hz EFS by 9% (± 4, $p<0.05$) (Fig. 25A). The maximum contractile response to 20 Hz EFS was increased by 12% (± 4, $p<0.05$) in presence of sodium saccharin 10^{-7} M (Fig. 25B) and by 10% (± 2, $p<0.05$) in presence of aspartame 10^{-7} M compared to control (Fig. 25C).

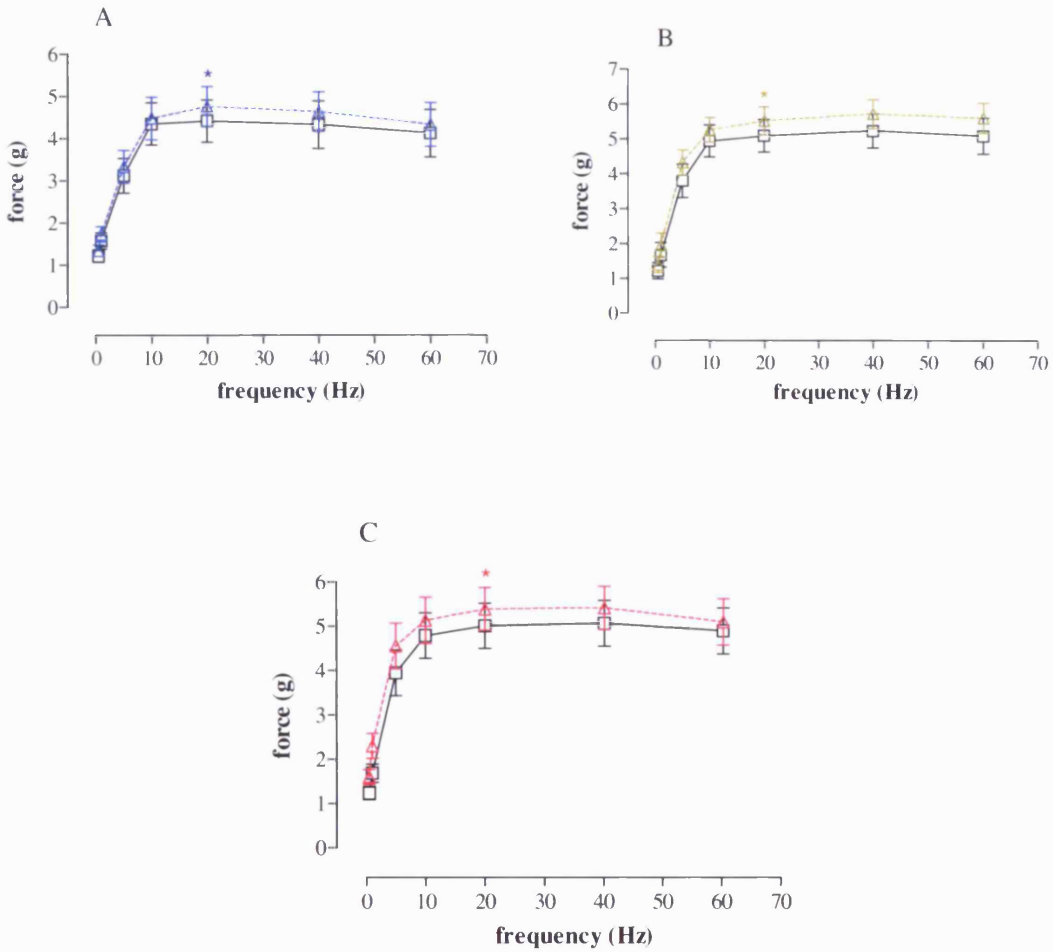


Fig. 25 *The effect of acesulfame K 10^{-6} M (A), sodium saccharin 10^{-7} M (B) and aspartame 10^{-7} M (C) on rat detrusor muscle contractile response to EFS. □ Control frequency-response curves. Frequency response curves in presence of △ acesulfame K 10^{-6} M (A), △ sodium saccharin 10^{-7} M (B) and △ aspartame 10^{-7} M (C), compared to control. (* $p<0.05$). n=6.*

7.2.3 EFS in the absence and presence of atropine 10^{-6} M plus sweetener

Atropine blocks the cholinergic component of the nerve mediated response with the remaining atropine-resistant component being due to the release of the neurotransmitter, ATP (Brading and Williams, 1990). Acesulfame K 10^{-6} M increased the maximum contraction of atropine resistance response to 20 Hz EFS by 9% (± 2, $p<0.05$) (Fig. 26A) compared to the atropine resistance response with atropine only. Sodium saccharin 10^{-7} M and aspartame 10^{-7} M also increased the maximum contraction of atropine resistant component to 20 Hz EFS by 8% (± 3, $p<0.05$) (Fig. 26B) and by 10% (± 2), respectively compared to the contraction with atropine alone (Fig. 26C).

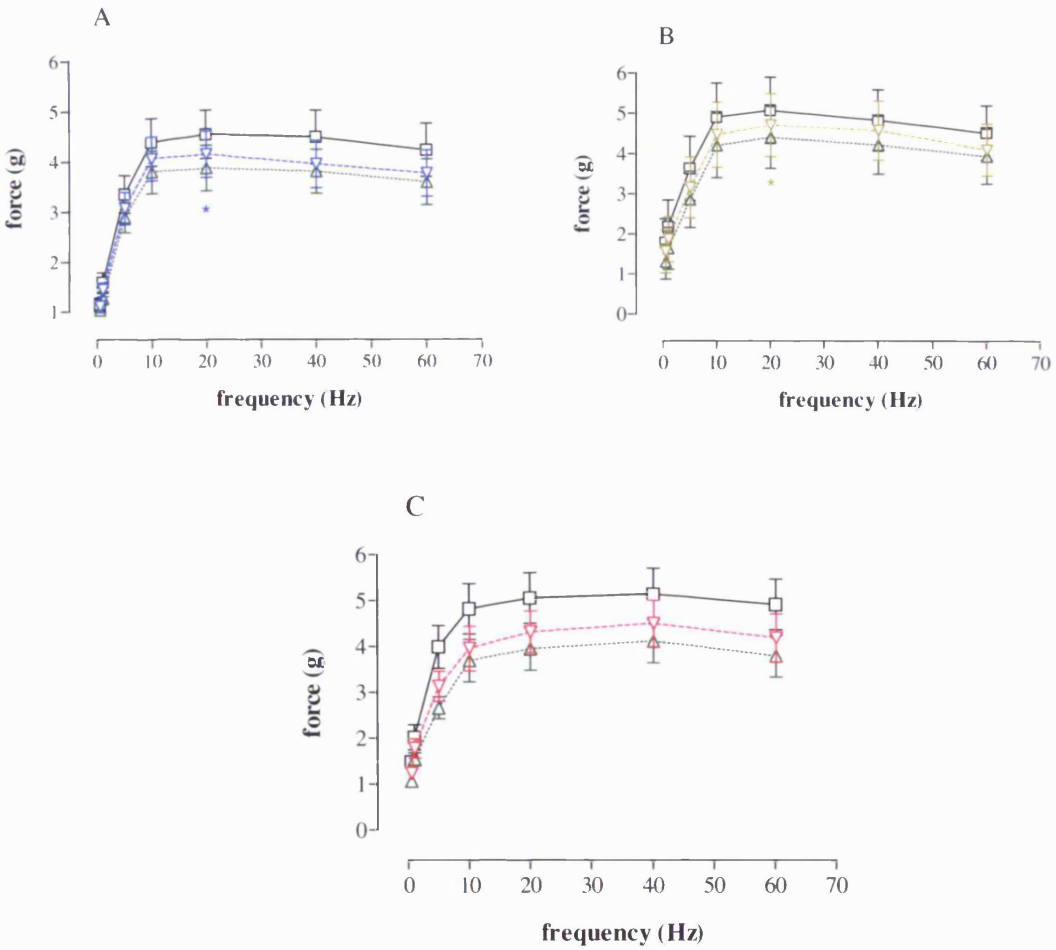


Fig. 26 *The effect of acesulfame K 10^{-6} M (A), sodium saccharin 10^{-7} M (B), and aspartame 10^{-7} M (C) on rat detrusor muscle contractile response to EFS in the presence of atropine 10^{-6} M. □ Control frequency-response curves. △ Frequency-response curves in presence of atropine without sweetener. ▽ Frequency-response curves in presence of atropine and acesulfame K 10^{-6} M (A), ▽ sodium saccharin 10^{-7} M (B), and ▽ aspartame 10^{-7} M (C). (* $p<0.05$, when compared between the two frequency-response curves, one with atropine and sweetener and the other with atropine alone). n=6.*

7.2.4 Concentration-response curves to carbachol

Carbachol directly activates cholinergic receptors in bladder smooth muscle. The sweeteners had not significant effect on the maximum contractile response to carbachol 10^{-3} M. Acesulfame K 10^{-6} M (Fig. 27A) and sodium saccharin 10^{-7} M (Fig. 27B) slightly enhanced the maximum response to carbachol by 5% (± 1) and by 6% (± 1) respectively. Aspartame 10^{-7} M had no effect on the concentration–response curve to carbachol (Fig. 27C).

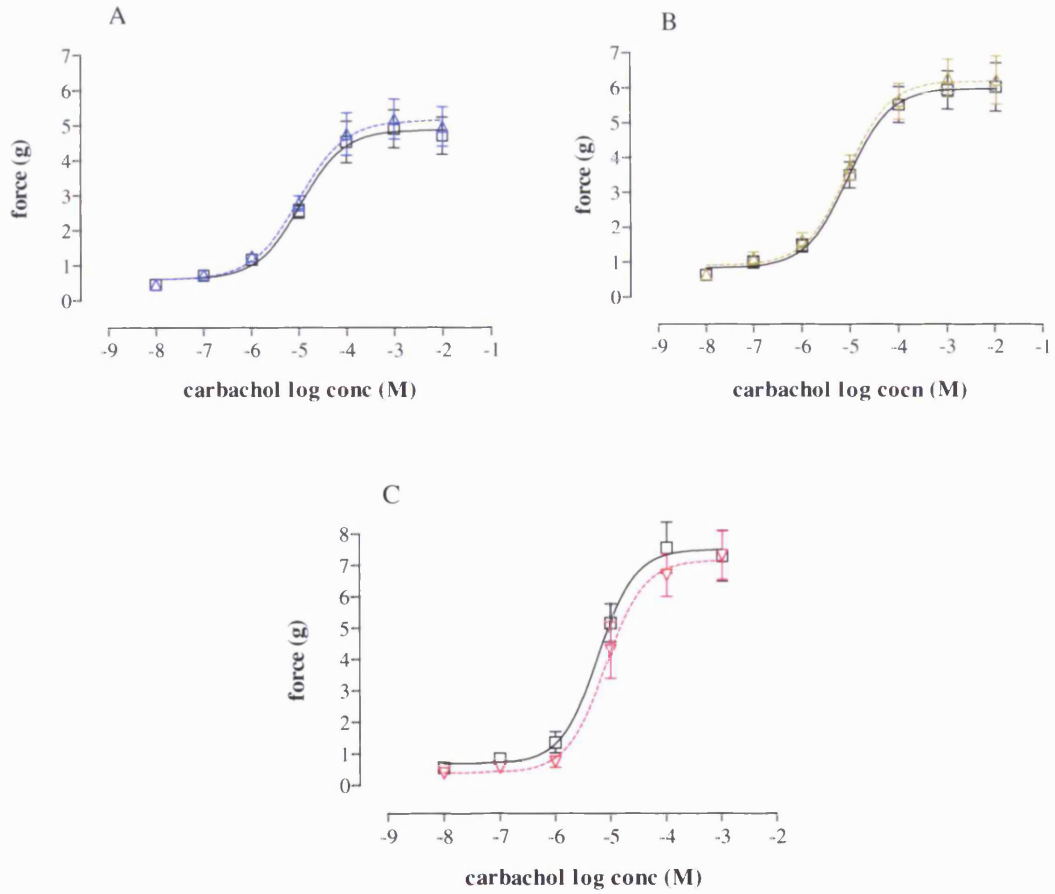


Fig. 27 *The effect of acesulfame K 10^{-6} M (A), sodium saccharin 10^{-7} M (B) and aspartame 10^{-7} M (C) on rat detrusor muscle contractile response to carbachol. □ Control concentration-response curves to carbachol. △, Concentration-response curves to carbachol in presence of acesulfame K 10^{-6} M (A), △ sodium saccharin 10^{-7} M (B) and △ aspartame 10^{-7} M (C). n=6.*

7.2.5 Concentration-response curves to α, β Methylene ATP (ABMA)

Alpha, β Methylene ATP 10^{-4} M directly activates purinergic receptors which depolarizes the cell membrane opening L-type Ca^{2+} channels, resulting in Ca^{2+} influx and muscle contraction (Wu et al., 1999). Acesulfame K 10^{-6} M significantly enhanced the maximum contractile response to α, β methylene ATP 10^{-3} M by 35% (± 9, $p<0.01$) compared to control (Fig. 28A). Sodium saccharin 10^{-7} M increased the maximum contractile responses to α, β methylene ATP 10^{-3} M by 16% (± 4, $p<0.05$) (Fig. 28B). Aspartame 10^{-7} M enhanced the maximum contractile response evoked by α, β methylene ATP 10^{-3} M by 15% (± 8, $p<0.05$), when compared to control (Fig. 28C).

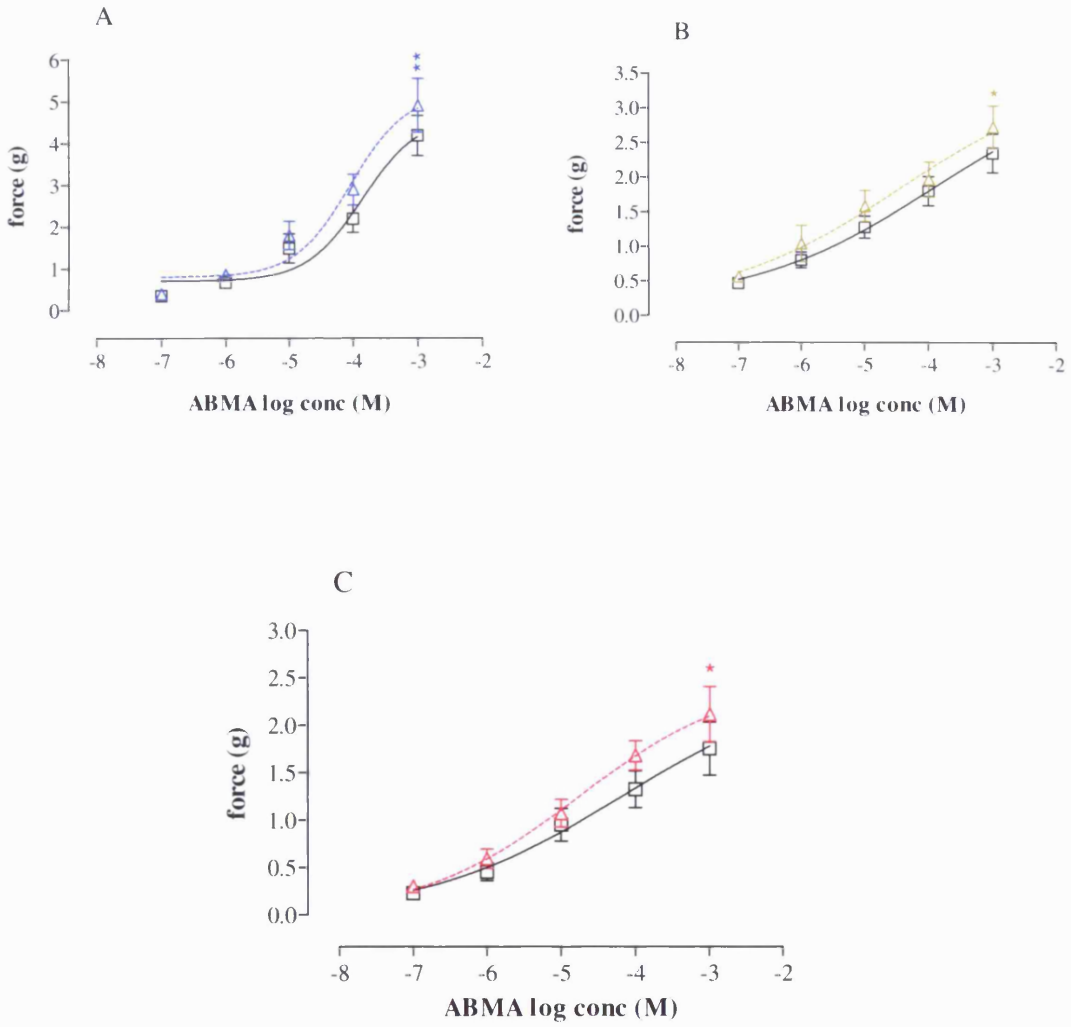


Fig. 28 *The effect of acesulfame K 10^{-6} M (A), sodium saccharin 10^{-7} M (B) and aspartame 10^{-7} M (C) on rat detrusor muscle contractile response to α, β methylene ATP. □ Control concentration-response curves to α, β methylene ATP. △ Concentration-response curves to α, β methylene ATP in the presence of acesulfame K 10^{-6} M (A), △ sodium saccharin 10^{-7} M (B) and △ aspartame 10^{-7} M (C). (*p<0.05, **p<0.01). n=6.*

7.2.6 Concentration-response curves to KCl

Potassium induces detrusor muscle contraction solely by opening L-type Ca^{2+} channels in the sarcolemma (Maggi et al., 1989a). It was therefore determined if the sweeteners, acesulfame K, aspartame and sodium saccharin directly affected Ca^{2+} influx by investigating their effect on contractile responses to KCl. Acesulfame K 10^{-6} M significantly enhanced the contractile response to KCl 60 mM and 70 mM by 21% (± 3, $p<0.001$) and 12% (± 3, $p<0.01$) respectively (Fig. 29A). The log EC_{50} −1.33 (± 0.0109) of the control concentration–response curve to KCl was significantly increased to −1.39 (± 0.013, $p<0.01$), shifting the concentration–response curve to the left. The contractile responses to KCl (60 mM, 70 mM and 80 mM) were enhanced in the presence of sodium saccharin 10^{-7} M by 29% (± 16), 29% (± 16) and 37% (± 15, $p<0.05$), respectively, compared to control (Fig. 29B). The control log EC_{50} −1.21 (± 0.01) was significantly increased to −1.31 (± 0.01, $p<0.001$) after the addition of sodium saccharin 10^{-7} M. Aspartame 10^{-7} M had no significant effect on the contractile response to KCl (Fig. 29C).

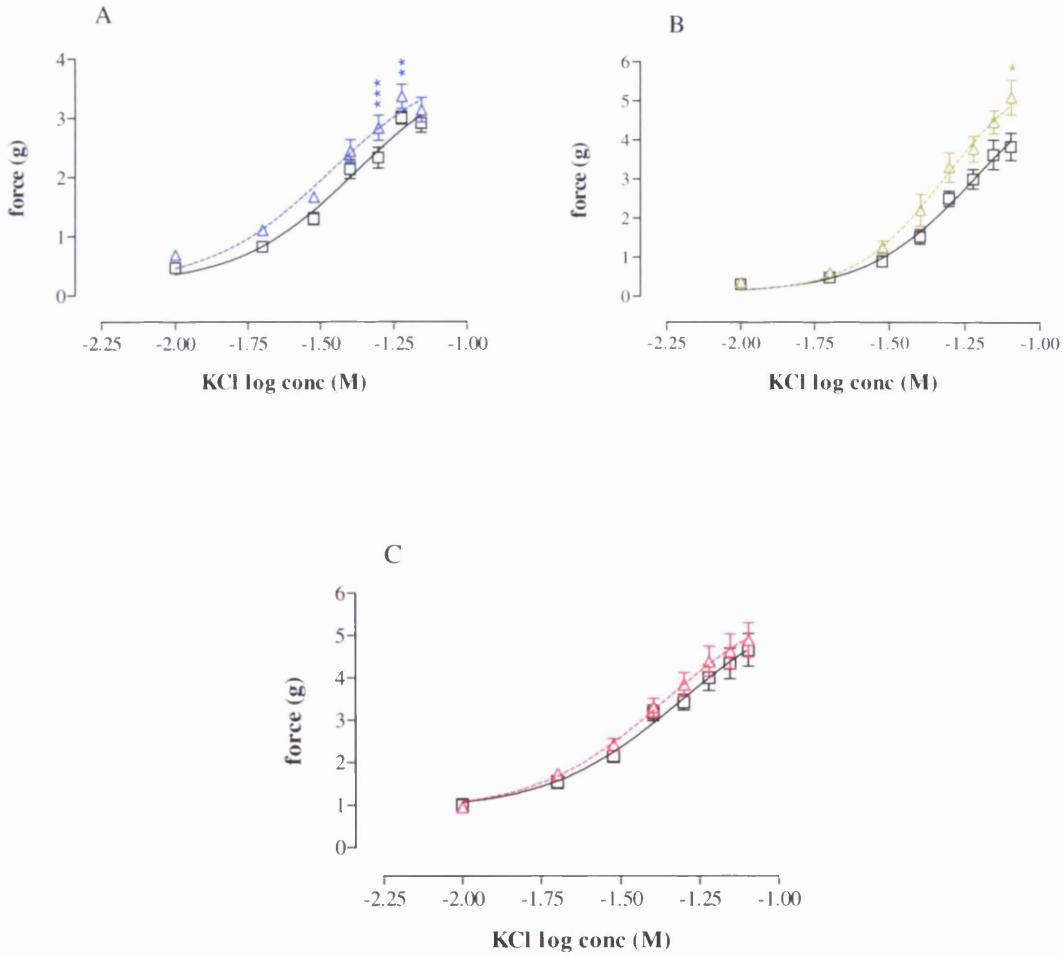


Fig. 29 *The effect of acesulfame K 10^{-6} M (A), sodium saccharin 10^{-7} M (B) and aspartame 10^{-7} M (C) on rat detrusor muscle contractile response to KCl. □ Control-concentration response curve. △ Concentration-response curve to KCl in presence of acesulfame K 10^{-6} M (A), △ sodium saccharin 10^{-7} M (B) and △ aspartame 10^{-7} M (C). (*p<0.05, **p<0.01).n=6.*

7.2.7 Concentration–response curves to calcium

In depolarized bladder muscle strips, acesulfame K 10^{-6} M enhanced the contractile response to calcium, 1.0, 2.0 and 2.5 mM by 64% (± 16, $p<0.05$), 45% (± 19, $p<0.05$) and 39% (± 13, $p<0.05$), respectively (Fig. 30A). The concentration–response curve to calcium was significantly shifted to the left after the addition of acesulfame K 10^{-6} M. Sodium saccharin 10^{-7} M significantly increased the maximum contractile response to calcium by 69% (± 17, $p<0.01$) (Fig. 30B). Aspartame 10^{-7} M enhanced the contractile response to 1.0 mM calcium by 35% (± 4, $p<0.001$) (Fig. 30C).

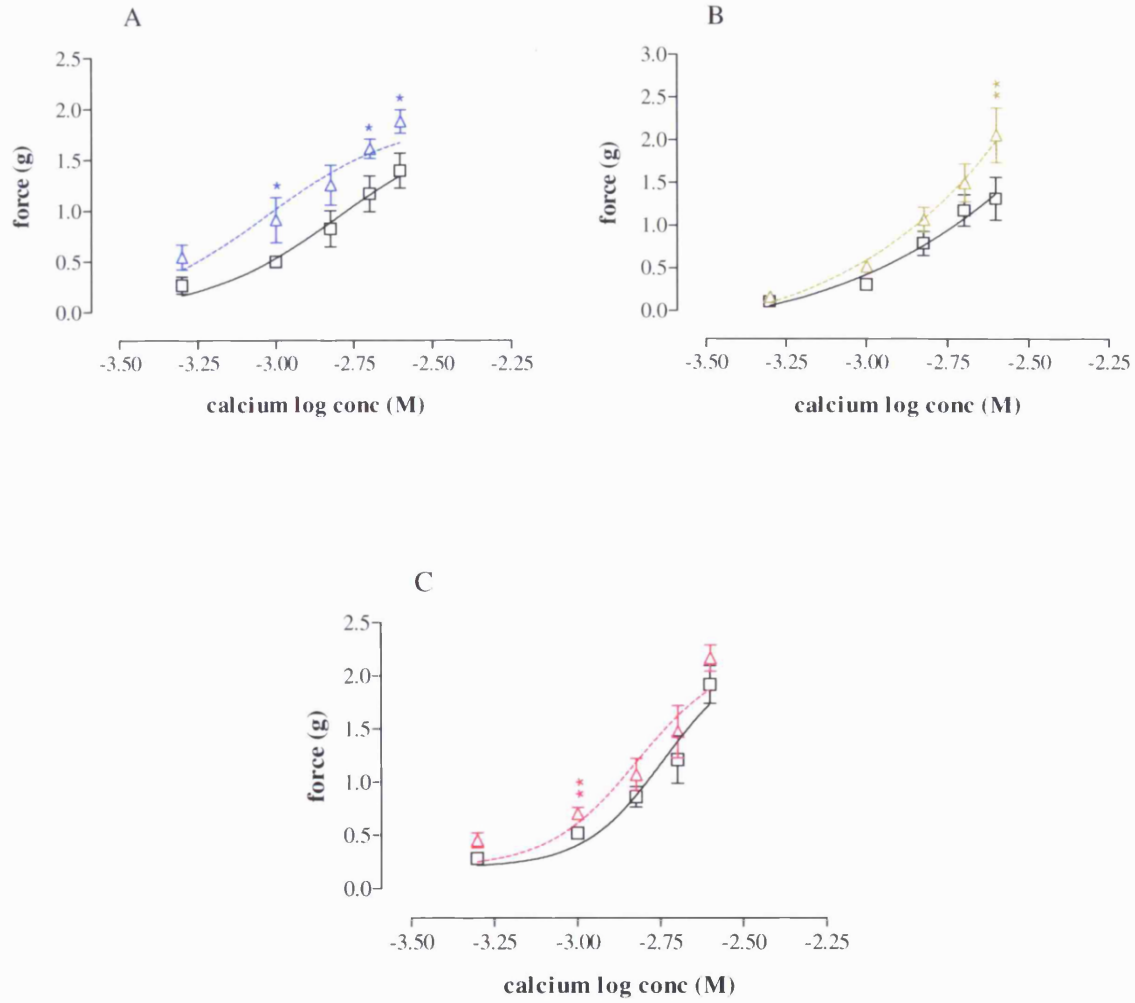


Fig. 30 *The effect of acesulfame K 10^{-6} M (A), sodium saccharin 10^{-7} M (B) and aspartame 10^{-7} (C) on concentration response curve to calcium. □ Control calcium concentration-response curve △ Calcium concentration-response curve in presence of acesulfame K 10^{-6} M (A) △ sodium saccharin 10^{-7} M (B) and △ aspartame 10^{-7} (C).(* $p<0.05$, ** $p<0.01$). $n=6$.*

Table 4 *Summary of the effects of artificial sweeteners on the muscle contraction in response to different stimuli.*

Component	Atropine resistant response to EFS	Response to ATP	Response to KCl	Response to calcium	Response to carbachol
Acesulfame	↑	↑↑	↑↑	↑↑	NS
Sodium saccharin	Marginally ↑	Marginally ↑	↑	↑	NS
Aspartame	↑	Marginally ↑	NS	↑	NS

(NS: not significant; marginally↑: enhanced contraction, but not statistically significant; ↑: statistically significant enhancement, $p<0.05$; ↑↑ enhanced contraction, $p<0.01$)

7.3 Discussion

When the cholinergic component of the muscle contraction was blocked by atropine, during EFS stimulation with increasing frequencies, the magnitude of the remaining contractile response was slightly enhanced in the presence of the sweeteners, suggesting that a possible mode of action was through the atropine resistant component, mediated by ATP. Although the contractile response was found to be significantly enhanced at 10Hz EFS in the screening tests, this effect was not observed at 10 Hz in the EFS experiments when a range of frequencies were used (pp 173). This may be due the desensitization of the receptors.

This hypothesis is supported by the finding that the maximum contractile response to α,β, methylene ATP was also enhanced by some of these sweeteners, indicating their direct action on purinoceptor-associated mechanisms. Activation of purinoceptors P2X, which are ligand-gated ion channels, by α β methylene ATP, a non- hydrolysable stable analogue of ATP, causes depolarisation and release of Ca^{2+} from intracellular stores, followed by opening of L-type Ca^{2+}

channels increasing Ca^{2+} influx and thereby initiating contractile response (Foresta et al., 1995; Wu et al., 1999). Alpha, beta-methylene ATP is not only a potent agonist of the P2X purinoceptor, but it causes desensitisation of purinoceptors after prolonged exposure (Brading and Williams, 1990, Yoshida et al., 2001, Noda et al., 2002). In the present study, the bladder strips were exposed to α β methylene ATP for 45 seconds after which they were washed out with Kreb's solution. This would be considered an inadequate length of time to cause desensitisation of purinoceptors (Bo and Burnstock, 1989).

This study investigated the effect of sweeteners on the contractile response of detrusor muscle strips to potassium (KCl) to determine the possibility that the sweeteners affect calcium influx through L-type calcium channels. Nifedipine, an L-type Ca^{2+} channel antagonist, completely suppresses the phasic and tonic contractile response to KCl. Exposure to calcium free medium also rapidly abolishes the response to KCl (Maggi et al., 1989a). This suggests that the contractile response to KCl is solely dependent on the influx of calcium through L-Type Ca^{2+} channels. As acesulfame 10^{-6} M and sodium saccharin 10^{-7} M enhanced the maximal contractile response to potassium and significantly shifted the concentration response curves to the left, this suggests that their mode of action involves augmentation of extracellular Ca^{2+} influx through L-type Ca^{2+} channels, which is independent of muscarinic or purinergic receptor activity (Maggi et al., 1989a; Visser and Van Mastrigt, 2000). Further confirmation of this mechanism was obtained by investigating the effect of acesulfame 10^{-6} M, aspartame 10^{-7} M and sodium saccharin 10^{-7} M on the contractile response of rat bladder muscle to calcium $0.5 - 2.5 \times 10^{-3}$ M. When the rat bladder strips were placed in Ca^{2+} free, high potassium medium, a contractile response was observed initially, due to the depolarizing effect of potassium. In the absence of extracellular calcium in the Krebs solution, the intracellular calcium was not replenished and the tone was not maintained. The

high potassium concentration depolarised the smooth muscle cells and opened L-type Ca^{2+} channels. As the medium did not contain Ca^{2+}, contraction of the bladder muscle strips was obtained by the addition of increasing concentrations of Ca^{2+} to produce a concentration response curve. Acesulfame and sodium saccharin significantly shifted the calcium concentration response curve to the left, thereby suggesting the mechanism of action of these sweeteners on calcium entry during excitation contraction coupling in bladder muscle.

The chemical structure of acesulfame K resembles that of saccharin in that they both contain the SO_2 and N-C=O functionalities in a rigid framework (Venanzi and Venanzi, 1988). These two sweeteners have had very similar effects on rat bladder contraction whereas aspartame, having a very different chemical structure, has had little effect on rat bladder contractile response. It is therefore likely that acesulfame K and saccharin are interacting with the same structure within the bladder muscle but it is unlikely that this would be 1, 4 dihydropyridine calcium channels as the activators of this receptor have a very different chemical structure to acesulfame K and saccharin.

Isolated bladder smooth muscle strips exhibit spontaneous phasic contractions which depend on Ca^{2+} entry through dihydropyridine-sensitive voltage-dependent Ca^{2+} channels (VDCC) (Mostwin, 1986). It would also appear that combined small-conductance Ca^{2+} - activated K^+ (SK) channels, ryanodine receptors (RyRs) and large-conductance Ca^{2+} - activated K^+ (BK) channels function as negative – feedback elements through differential modulation of contractile amplitude, duration and frequency (Herrera et al., 2000). Both acesulfame and sodium saccharin increased the amplitude and frequency of the rat bladder smooth muscle spontaneous contractions, which may involve modulation of these K^+ channels and activation of RyRs. Further investigation into the effect of these sweeteners on spontaneous contraction of bladder smooth muscle would be required to differentiate the mechanisms involved.

The results showed that aspartame only modified rat detrusor muscle contractile response to EFS 10Hz and to low concentrations of calcium. This sweetener is different from acesulfame K and sodium saccharin in its effect on bladder contraction. It is possible that it has much weaker effects on calcium influx or it may be affecting a completely different pathway to those described so far. The other possibility is that we have observed non-specific effects. *In vivo* aspartame is not the active compound, because after ingestion it is metabolised to aspartic acid, methanol and phenylalanine (Karikas et al., 1998). Methanol is further metabolized to formaldehyde in both humans and rats (Ranney et al., 1976). Phenylalanine seems to play a role in the transport of precursors of monoamine neurotransmitters into the brain (Pin and Duvoisin, 1995), and aspartic acid (aspartame) has excitatory neurotransmitter functions in the gut mediated by inotropic receptors (ligand gated ion channels) and metabotropic receptors, coupled to G-proteins (Slobodan et al., 1999). Preliminary experiments were carried out to investigate the effect of aspartic acid and phenylalanine on rat bladder contraction to 10 Hz EFS which showed no consistent significant effect on contractile response (data not shown). It is therefore possible that aspartame itself had non-specific effects on bladder contraction due to direct actions on the muscle rather than through cellular pathways.

The results did not suggest that the sweeteners were acting via the cholinergic pathway, as evident from the carbachol concentration response curve. The purinergic receptors were therefore not desensitized by prolonged exposure to α, β-Methylene ATP (Inoue and Brading, 1990) to evaluate the effect of sweeteners on cholinergic component in isolation.

In conclusion, the stimulatory effect of artificial sweeteners on bladder muscle contraction, although small, is clearly evident from the results of the experiments and is greater than any difference found over the time course of the experiments. The concentration response curves to α

β, methylene ATP, potassium and calcium suggest that the possible mechanism of action is by increasing the intracellular Ca^{2+} concentration via extra cellular Ca^{+2} influx through L-type voltage gated Ca^{2+} channels, either via purinergic receptor activation or by some unknown mechanism, such as some other receptor that specifically bind the sweeteners. Specific sweet taste receptors are present in the tongue to detect sweet taste and interestingly these receptors were also found in other tissues in human body, such as lung, liver, brain and testes (Max et al., 2001; Kitagawa et al., 2001). The results with artificial sweeteners thus led the hypothesis that there may be sweet taste receptors present in urinary bladder that act via the same mechanism of Ca^{2+} influx. This hypothesis was examined and has been detailed in chapter 10.

Key points:

- Acesulfame and sodium saccharin were found to enhance the bladder muscle contraction possibly by increasing the intracellular Ca^{2+} concentration via extra cellular Ca^{2+} influx through L-type voltage gated Ca^{2+} channels, which may involve purinergic receptor activation.
- The results did not suggest that the sweeteners were acting via the cholinergic pathway.
- The effects of aspartame on the muscle contractile response were not strong compared to that of acesulfame and sodium saccharin.

CHAPTER 8

ASCORBIC ACID AND CITRIC ACID

RESULTS & DISCUSSION

8.1 Introduction

The role of preservatives, acidulants and antioxidants in relation to the antimicrobial action, buffering action to maintain pH and delaying oxidation to stabilise the drink, has already been discussed in detail in chapter 4 (pp 135-136). Amongst the preservatives tested in this study such as sodium benzoate, sodium citrate, potassium sorbate, ascorbic acid and citric acid, only ascorbic and citric acids had significant effects on contractile response. The aim of the work described in this section was to investigate the effect of ascorbic acid and citric acid on the contractile response of isolated rat detrusor muscle and to elucidate the mechanism of action of the effect observed.

8.2 Results

8.2.1 Spontaneous contractions

When the perfused solution containing ascorbic acid 10^{-4} M reached the bladder smooth strips in the organ bath, the frequency and amplitude of the spontaneous contractions were increased by 14% and 21% compared to the control (Fig. 31A). A similar effect was seen after the addition of citric acid 10^{-5} M to the organ bath. The frequency and amplitude of spontaneous contractions were increased by 21% and 11% respectively compared to the control (Fig. 31B).

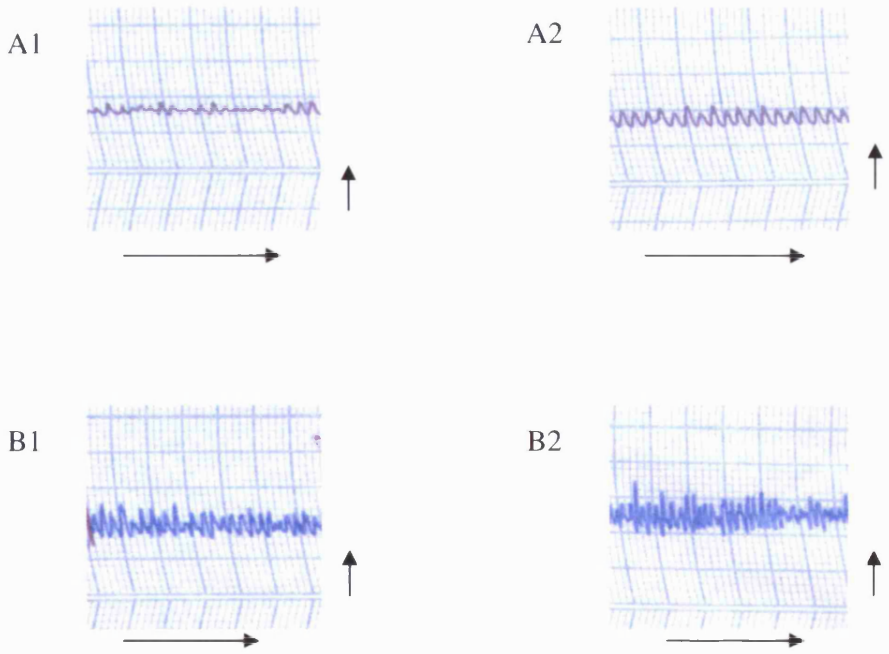


Fig. 31 *Spontaneous contractions of rat bladder muscle before and after addition of ascorbic acid 10^{-4} M (A) and citric acid 10^{-5} M (B); A1 & B1 correspond to controls (without ascorbic or citric acid) and A2 & B2 correspond to contractions after addition of ascorbic or citric acid respectively. Horizontal arrows indicate time: 5 big squares= 5 minutes (time of observation), longitudinal arrows indicate amplitude of contraction, 6 small squares= tension of 1g.*

8.2.2 Frequency response curves

Ascorbic acid 10^{-4} M and citric acid 10^{-5} M increased the contractile response of bladder muscle to all frequencies of EFS from 1 Hz to 60 Hz respectively. The enhancement of maximum contractile response of bladder muscle strips to EFS 20 Hz was by 10% (± 3.0, $p<0.01$) in the presence of ascorbic acid 10^{-4} M (Fig. 32 A). Citric acid 10^{-5} M also enhanced the maximum contractile response to 40 Hz by 11% (± 4.0, $p<0.01$) (Fig. 32 B).

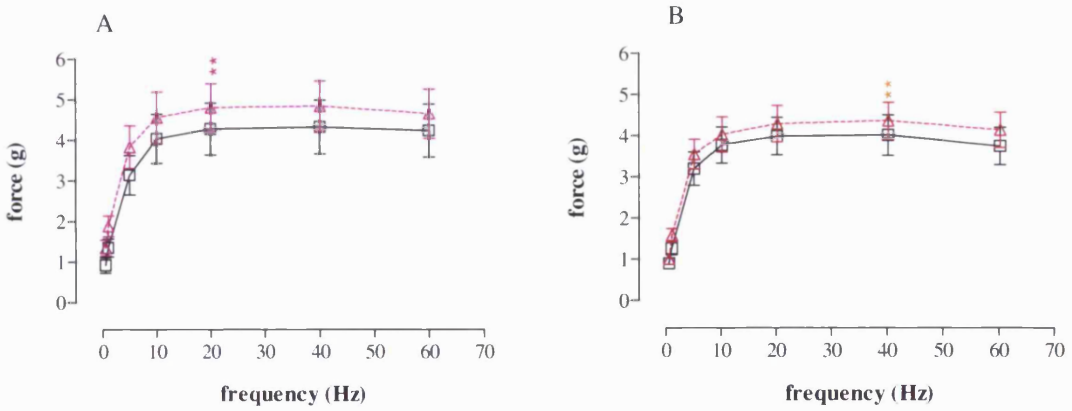


Fig. 32 *The effect of ascorbic acid 10^{-4} M (A) and citric acid 10^{-5} M (B) on rat detrusor muscle contractile response to EFS. □ Control frequency-response curves. △ Frequency-response curves in presence of ascorbic acid 10^{-4} M (A) and △ in presence of citric acid 10^{-5} M (B). (** $p<0.01$). n=6.*

8.2.3 EFS in the absence and presence of atropine 10^{-6} M plus ascorbic acid /citric acid

Ascorbic acid 10^{-4} M increased the atropine resistant response to EFS at all the frequencies used from 0.5 Hz to 60 Hz. The atropine resistant contractile response to electrical field stimulation at 1 Hz, 5 Hz, 10 Hz, 20 Hz, 40 Hz and 60 Hz, was increased by 59% (± 8), 43% (± 6, $p<0.01$), 32% (± 7, $p<0.01$), 26% (± 7, $p<0.01$), 24% (± 7) and 22% (± 7) respectively in presence of ascorbic acid 10^{-4} M plus atropine 10^{-6} M, compared to the contractile response in the presence of atropine alone (Fig. 33 A).

Citric acid 10^{-5} M also enhanced the atropine resistant component of EFS at all frequencies from 0.5 Hz to 60 Hz, although the enhancement was not as much as that produced by ascorbic acid. The maximum contractile response to EFS was obtained at 20 Hz and the presence of citric acid 10^{-5} M enhanced the atropine resistant component of the maximum contractile response by 9% (± 2.0, $p<0.05$) compared to the contraction in the presence of atropine alone (Fig. 33 B).

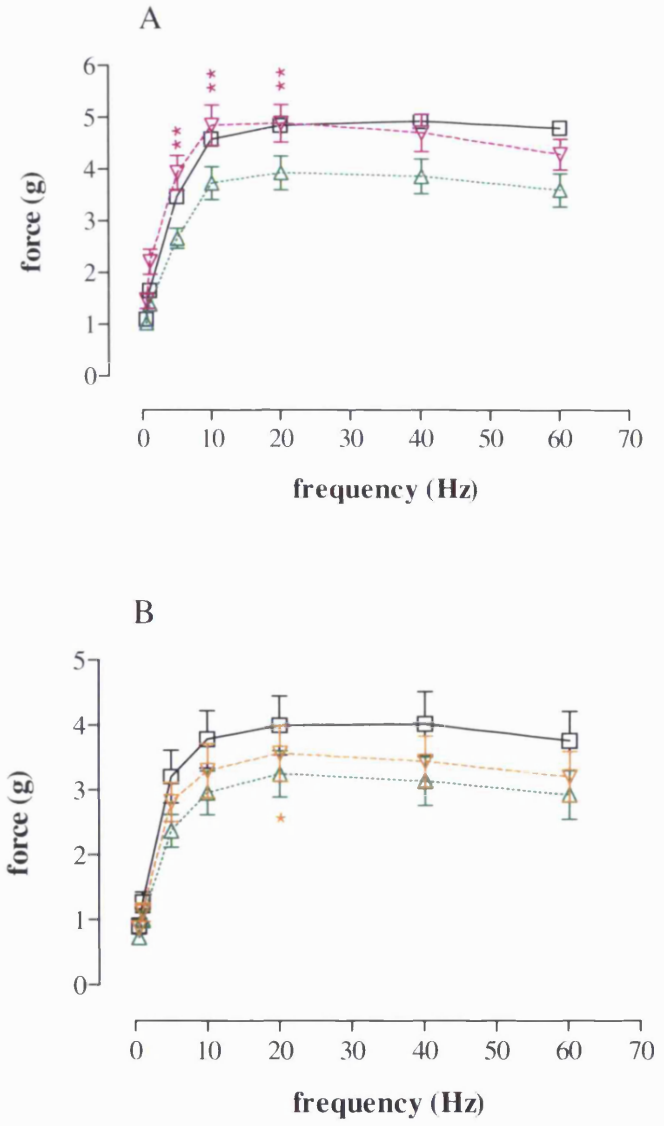


Fig. 33 *The effect of ascorbic acid 10^{-4} M (A) and citric acid 10^{-5} M (B) on rat detrusor muscle contractile response to EFS in the presence of atropine 10^{-6} M. □ Control frequency-response curves. △ Frequency-response curves in presence of atropine without ascorbic acid or citric acid. ▽ Frequency-response curves in presence of atropine 10^{-6} M and ascorbic acid 10^{-4} M (A), and ▽ Frequency-response curves in presence of atropine and citric acid 10^{-5} M (B). (* $p<0.05$, ** $p<0.01$, when compared between the two responses, with atropine plus ascorbic or citric acid and atropine alone). n=6.*

8.2.4 Concentration–response curves to carbachol

Ascorbic acid 10^{-4} M inhibited the carbachol evoked contractile response in all concentrations from 10^{-8} M to10^{-2} M. The maximum contractile response to carbachol 10^{-3} M was found to be inhibited by 24% (± 4, $p<0.05$) compared to control (Fig. 34A). However, citric acid 10^{-5} M did not show any affect on the contractile response to carbachol (Fig. 34B).

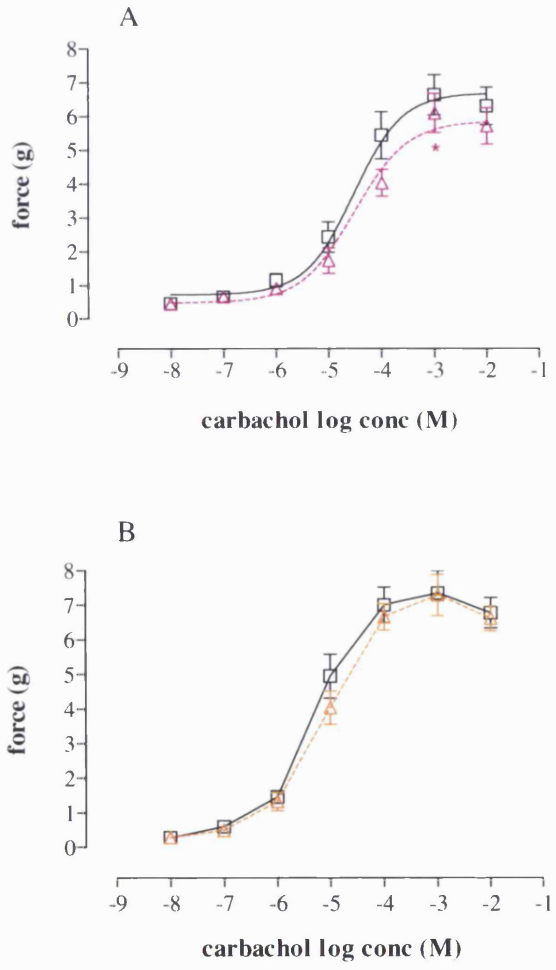


Fig. 34 *The effect of ascorbic acid 10^{-4} M (A) and citric acid 10^{-5} M (B) on rat detrusor muscle contractile response to carbachol. □ Control concentration-response curves to carbahol. △ Concentration-response curves to carbachol in the presence of ascorbic acid 10^{-4} M (A) and △ citric acid 10^{-5} M (B). (*p<0.05). n=6.*

8.2.5 Concentration–response curves to α, β methylene ATP (ABMA)

Ascorbic acid 10^{-4} M increased rat bladder muscle contractile response to stimulation with the purinergic receptor agonist, α, β methylene ATP, at concentrations of 10^{-5} M to 10^{-3} M. The enhancement of the maximum contractile response to α, β methylene ATP 10^{-3} M, in the presence of ascorbic acid 10^{-4} M, was found to be 20% (± 5.0, $p<0.01$) compared to the control (Fig. 35A).

Citric acid 10^{-5} M was also found to enhance the maximum contractile response to α, β methylene ATP 10^{-3} M by 16% (± 2.0, $p<0.001$) (Fig.35B).

The log EC_{50} of the concentration response curves to ATP in the presence of either ascorbic acid or citric acid did no show any significant difference from the log EC_{50} of the control concentration response curve.

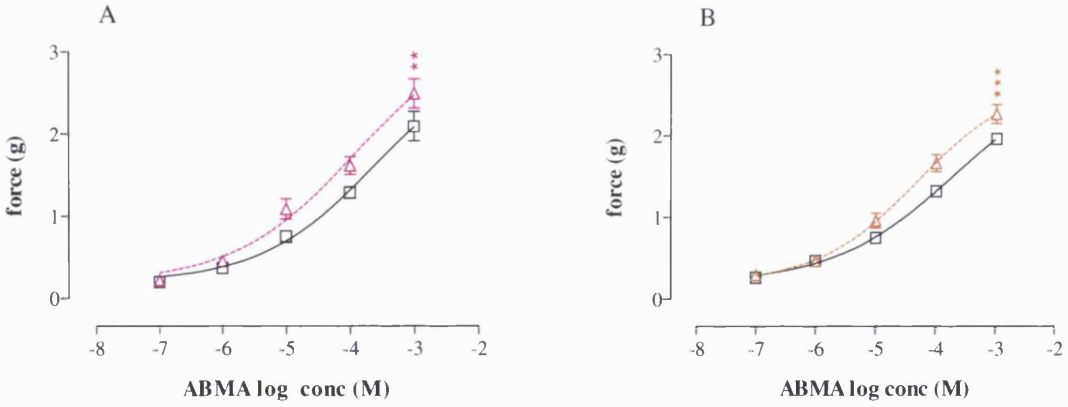


Fig. 35 *The effect of ascorbic acid 10^{-4} M (A) and citric acid 10^{-5} M (B) on rat detrusor muscle contractile response to α, β methylene ATP. □ Control concentration-response curves to α, β methylene ATP. △ Concentration response curves to α, β methylene ATP in the presence of ascorbic acid 10^{-4} M (A) and △ citric acid 10^{-5} M (B).(** $p<0.01$, *** $p<0.001$). n=6.*

8.2.6 Concentration–response curves to KCl

Ascorbic acid 10^{-4} M enhanced the contractile response of the bladder muscle strips to KCl throughout the range of concentration 20mM to 80mM. The contractile responses to 60, 70 and 80 mM KCl were found to be increased by 14 % (± 3, $p<0.05$), 11 % (± 2, $p<0.05$) and 17 % (± 3, $p<0.01$) respectively in the presence of ascorbic acid 10^{-4} M (Fig. 36A). The contractile response to 70 mM KCl was found to be increased by 21 % (± 5, $p<0.01$) in the presence of citric acid 10^{-5} M (Fig. 36B).

The log EC_{50} of the concentration response curves to KCl in the presence of either ascorbic acid or citric acid did no show any significant difference from the log EC_{50} of the control concentration response curve.

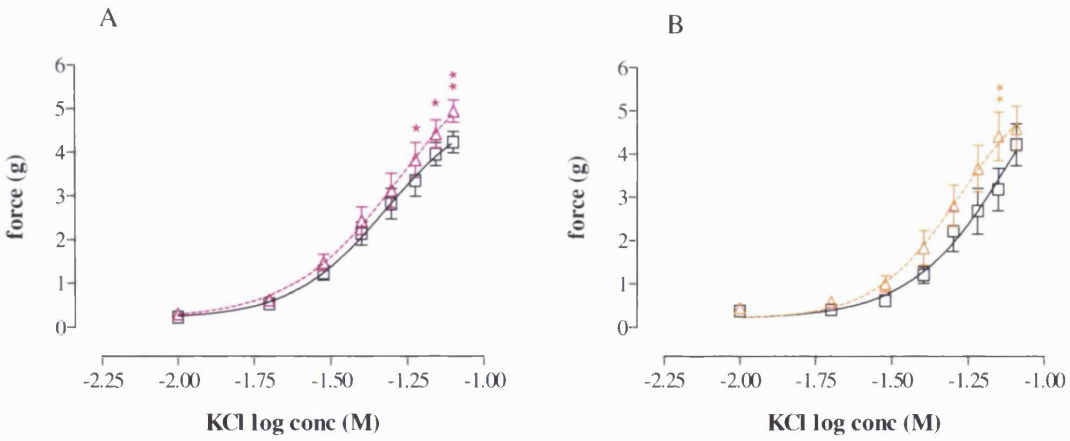


Fig. 36 *The effect of ascorbic acid 10^{-4} M (A) and citric acid 10^{-5} M (B) on rat detrusor muscle contractile response to KCl. □ Control concentration-response curve. △ Concentration response curve to KCl in presence of ascorbic acid 10^{-4} M (A) and △ citric acid 10^{-5} M. (* $p<0.05$, ** $p<0.01$). n=6.*

8.2.7 Concentration–response curves to calcium

In depolarized bladder muscle strips, ascorbic acid 10^{-4} M enhanced the contractile response to calcium, 1.5, 2.0 and 2.5 mM by 71% (± 8.0, $p<0.01$), 40% (± 5.0, $p<0.01$) and 30% (± 4.0, $p<0.01$) respectively, compared to the control response (Fig. 37A).

Citric acid 10^{-5} M also enhanced bladder muscle contraction to calcium at concentrations of 1.5, 2 and 2.5 mM by 44% (± 10), 62% (± 6.0, $p<0.001$) and 49% (± 3.0, $p<0.001$) respectively compared to the control (Fig.37B).

The log EC_{50} of the concentration response curves to calcium in the presence of either ascorbic acid or citric acid did not show any significant difference from the log EC_{50} of the control concentration response curve.

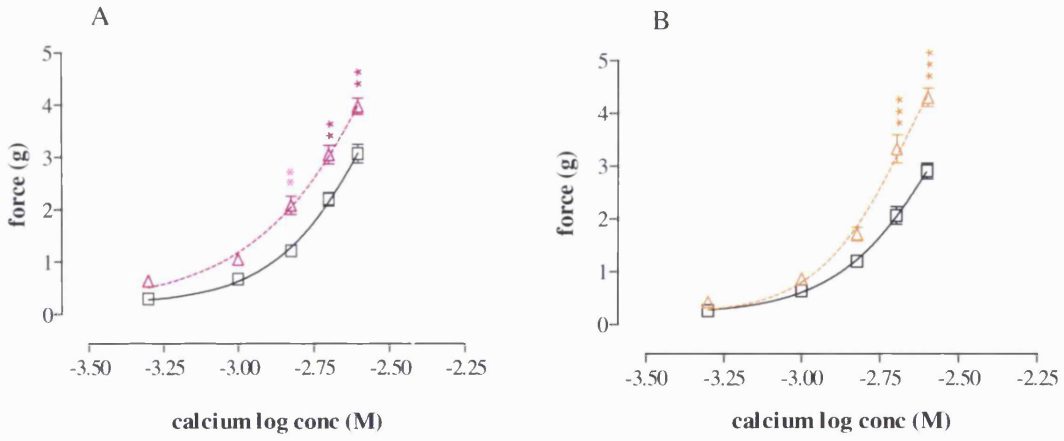


Fig. 37 *The effect of ascorbic acid 10^{-4} M (A) and citric acid 10^{-5} M (B) on concentration response curve to calcium. □ Control calcium concentration-response curve. △ Calcium concentration-response curve in the presence of ascorbic acid 10^{-4} M (A) and △ citric acid 10^{-5} M (B). (** $p<0.01$, *** $p<0.001$). n=6.*

Table 5 *Summary of the effects of ascorbic acid and citric acid on the muscle contraction in response to different stimuli.*

Component	Atropine resistant response to EFS	Response to ATP	Response to KCl	Response to calcium	Response to carbachol
Ascorbic acid	↑↑	↑↑	↑↑	↑↑	↓
Citric acid	↑	↑↑	↑↑	↑↑	NS

(NS: not significant; ↑: statistically significant enhancement, $p<0.05$; ↑↑: enhanced contraction, $p<0.01$, ↓: inhibition of contraction, $p<0.05$)

8.3 Discussion

The bladder muscle contraction in response to EFS involves activation of both purinergic and cholinergic pathways. Both ascorbic acid and citric acid increased the muscle contraction due to EFS throughout the range of frequencies, indicating that the enhancement of muscle contraction may be as a result of activation of both the pathways (as at higher frequencies both the neurotransmitters are released). When the muscarinic receptors were blocked by atropine, the effect of ascorbic and citric acid on the purinergic component of the muscle contraction was evaluated.

When the cholinergic component of the muscle contraction to nerve stimulation was blocked by atropine, during EFS, the magnitude of the remaining contractile response, i.e. the atropine resistant component, was markedly enhanced in the presence of ascorbic acid, indicating that a possible mode of action is through the atropine-resistant component,

mediated by ATP. Although all the soft drink components under investigation showed enhancement of the atropine resistant response of the muscle contraction to EFS, ascorbic acid produced the maximum enhancement compared to the enhancement of contractile response by the other soft drink ingredients. It has been mentioned earlier that (pp 158), atropine resistance is higher at low frequencies of stimulation (Brading and Williams, 1990). Hammarstrom and Sjostrand (1984) showed that the neurogenic contractile response to low frequency stimulation of control bladders was not only completely resistant to atropine or scopolamine *in vitro*; but the response was enhanced by atropine or scopolamine, suggesting increased release of ATP was responsible for the detrusor contraction. Thus the enhancement of the atropine resistant muscle contraction to EFS in presence of ascorbic acid suggests either an increased release of ATP or enhanced postsynaptic receptor activation or a combination of both.

Further investigation showed that although ascorbic acid enhanced muscle contraction due to purinergic receptor stimulation by exogenous applied ATP, the enhancement was less than the enhancement of the atropine resistant component of the contractile response to EFS. It is therefore possible that the presynaptic mechanism, i.e. increased neurotransmitter release (ATP), is playing a more prominent role than the post synaptic receptor activation.

In contrast, enhancement of the atropine resistance of muscle contraction due to EFS was found to be far less by citric acid compared to that of ascorbic acid. However, the increase in muscle contraction due to purinergic receptor activation was very similar in both ascorbic and citric acid. As, the enhancement of muscle contraction by both presynaptic (by increased neurotransmitter release) and postsynaptic (by receptor activation) mechanisms in the presence of citric acid were found to be similar, it would be prudent to suggest that perhaps

the stimulatory effect of citric acid on muscle contraction was not due to increased ATP release from the nerves but by some other common mechanism, such as calcium influx.

As mentioned earlier, purinoceptor activation is coupled to L-type Ca^2 channels, so it was aimed to determine the effect of ascorbic acid and citric acid on Ca^{2+} influx through L-type Ca^{2+} channels by determining their effect on potassium (KCl) evoked contractile responses. The enhancement of maximum contractile response to potassium in presence of ascorbic acid 10^{-4} M and citric acid 10^{-5} M enhanced suggested an increased Ca^{2+} influx through L-type voltage gated Ca^{2+} channels to be a possible mechanism of action for the enhancement of bladder contraction, which is independent of muscarinic or purinergic receptor activation (Maggi et al., 1989a; Visser and van Mastrigt, 2000a). This mechanism was further confirmed by investigating the effect of ascorbic acid 10^{-4} M and citric acid 10^{-5} M on the contractile response of rat bladder muscle to calcium 0.5–2.5 mM. Ascorbic and citric acid enhanced the maximum contractile response to calcium similar to the effect on the contractile response to potassium, where the extracellular Ca^{2+} influx plays the key role in the contractile machinery.

The bladder contractile response to muscarinic receptor stimulation by cholinergic agonist carbachol was found to be significantly inhibited in the presence of ascorbic acid. This suggested that the stimulatory effect of ascorbic acid on muscle contraction is perhaps not via cholinergic pathway. Although it was unclear why this inhibitory effect on cholinergic stimulation was only demonstrated by ascorbic acid, it certainly warrants further investigation. The main difference between the contractile response produced by muscarinic receptor activation and the other agonists used in this study to evoke contraction is the involvement of IP_3 induced intracellular Ca^{2+} release. It may well be worth investigating the

effect of ascorbic acid on this pathway. Citric acid did not show any significant modulation on muscle contraction due to carbachol.

Direct myogenic activity of ascorbic acid was highlighted by Joiner (1973) and Terada et al. (1980). A possible explanation of the contractile effect of ascorbate was suggested by Joiner (1973) that it interacted with Ca^{2+} in the extracellular space, decreasing the level of free Ca^{2+} near the cell surface. Calcium then dissociates from the cell membrane and elicits an alteration in membrane permeability, which results in influx of Ca^{2+} into the smooth muscle cell cytoplasm. Terada et al. (1980) have shown ascorbic acid enhanced ileal smooth muscle contraction via increased acetylcholine release. Basu and Biswas (1940) also showed that ascorbic acid (0.1 mM - 1mM) potentiated electrically induced contractions of guinea-pig intestinal muscle. Results in this study seem to be in agreement with these findings. Although the effects of ascorbic acid on the cholinergic component of the muscle contraction to EFS was not isolated, results of the experiments looking into the purinergic pathway in isolation suggested that ascorbic acid may be influencing the release of ATP from the intrinsic nerves during stimulation. As acetylcholine and ATP are co-transmitters in mammalian bladder smooth muscle (MacKenzie, 1982) it seems highly probable that ascorbic acid would also influence acetylcholine release in rat bladder muscle nerve terminals. Also, ascorbic acid enhanced the response to nerve stimulation with EFS but inhibited post synaptic muscarinic receptor activation with carbachol, suggesting an effect on pre synaptic mechanisms such as neurotransmitter release.

Grossmann et al., (2001) described the relaxation effect of ascorbic acid on vascular smooth muscles by activation of cGMP-dependent potassium channels that hyperpolarize the smooth muscle cell membrane, inducing vasodilatation. This suggests that there may be different

pathways through which ascorbic acid and the carbonated soft drink are acting. Tsao (1984) have suggested that *in vivo*, chelation of extracellular Ca^{2+} should decrease Ca^{2+} influx into vascular smooth muscle cells, resulting in vasodilatation. Ascorbic acid causes hyperpolarization in various cells (Asamoto et al., 1992; Bergsten et al., 1994) which can originate from activation of potassium channels or of membrane Na^{+}-K^{+} ATPase (Ferrer et al., 1999). Quinidine, a nonselective potassium channel inhibitor, completely blocked the ascorbic acid–induced venodilation, suggesting possible involvement of potassium channels (Grossmann et al, 2001). Heller et al. (1999) suggested a different mechanism of action of ascorbic acid where they have shown that intracellular ascorbic acid enhances NO synthesis in endothelial cells which may explain, in part, the beneficial vascular effects of ascorbic acid. Thus it is interesting to find different studies have suggested different possible mechanism of action of ascorbic acid on smooth muscle and endothelium.

Kitto and Bohr (1953) showed that some agents like cysteine, cystine, tyrosine and ascorbic acid can potentiate the positive inotropic response of the papillary muscle to epinephrine. They suggested that possible sites of action where such substances might act to alter the response of the papillary muscle to epinephrine are: a) the tissue enzymes involved in the destruction of epinephrine; b) the autoxidation of epinephrine; c) the sensitivity of the muscle to epinephrine; d) the general responsiveness of the muscle.

They also showed that the potentiating agents like ascorbic acid act by removing the trace amounts of heavy metals which catalyze the autoxidation of epinephrine. It is thus possible for ascorbic acid to act by potentiation of adrenergic system (α_1 mediated contraction). However, this pathway was not investigated this study.

To summarise, although the experiments in this study suggested that the stimulatory effect of ascorbic and citric acid on bladder smooth muscle was possibly by enhancing Ca^{2+} influx, it was difficult to specify the pathway of Ca^{2+} influx. This may be via activation of purinergic pathway, or by any other mechanism independent of purinergic receptor activation. Ascorbic acid enhanced the presynaptic neurotransmitter (ATP) release, which may contribute to its stimulatory effect. However, ascorbic acid slightly inhibited the response to muscarinic receptor activation with carbachol, indicating a possible different intracellular action.

Key points:

- Ascorbic acid and citric acid enhanced the muscle contraction possibly by enhancing Ca^{2+} influx.
- The presynaptic neurotransmitter release was found to be enhanced by ascorbic acid, which was a unique observation amongst all the components that were investigated.
- Ascorbic acid inhibited the response to muscarinic activation by carbachol.

CHAPTER 9

CARBONATED SOFT DRINK

RESULTS & DISCUSSION

9.1 Introduction

The results of experiments conducted so far suggested that certain soft drink components can individually modulate detrusor muscle contraction. The next logical step was to find out how the carbonated soft drink as a whole modulated detrusor muscle contraction. It is important to remember that the Leicester MRC group epidemiological survey found an association between overactive bladder and carbonated soft drink as a whole, rather than any individual components. Moreover, no epidemiological study so far has reported any association between individual soft drink components and overactive bladder, apart from caffeine (Holroyd-Ledue and Straus, 2004).

9.2 Results

9.2.1 Spontaneous contractions

When the perfused solution of carbonated soft drink (1:200) reached the bladder smooth strips in the organ bath, the frequency and amplitude of the spontaneous contractions were increased by 21% and 25% respectively, compared to that of control spontaneous contractions (Fig. 38).

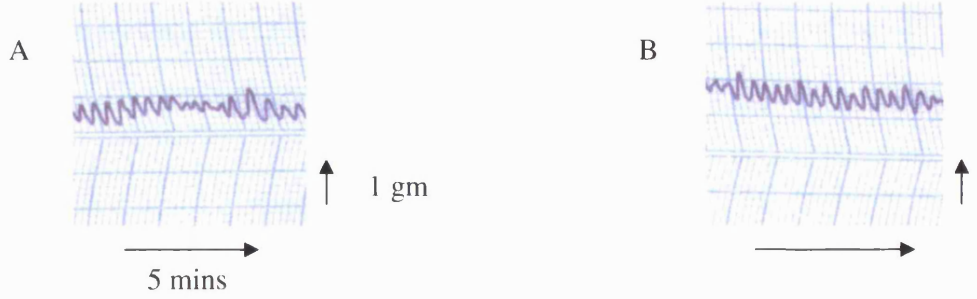


Fig. 38 *Spontaneous contractions of rat bladder muscle before (A) and after (B) addition of the carbonated soft drink. Horizontal arrows indicate time: 5 big squares= 5 mins, longitudinal arrows indicate amplitude of contraction, 6 small squares= tension 1g*

9.2.2 Frequency Response Curve

The contractions of the bladder muscle in response to EFS in the presence of differing concentrations of the whole carbonated soft drink, ranging from 1:500 to 1:100, were compared to identify the concentration causing maximum enhancement of muscle contraction.

Although the rat detrusor muscle contraction to EFS was enhanced by all the concentrations of the carbonated soft drink from 1:500 to 1:100 dilutions, the enhancement was more profound in the presence of 1:300, 1:200 and 1:100 dilution of the soft drink. The maximum contractile response was enhanced by 9% (± 4, $p<0.05$) in the presence of soft drink 1:300 at 40 Hz EFS (Fig. 39A), by 15% (± 4, $p<0.01$) in the presence of soft drink 1:200 at 20 Hz (Fig. 39B) and by 12% (± 4, $p<0.05$) in the presence of 1:100 dilution of the soft drink at 40 Hz (Fig. 39C). As the maximum enhancement of muscle contraction was obtained with carbonated soft drink 1:200, this concentration was chosen for further investigation. The enhancement of the contractile response was not significant when 1:400 and 1:500 dilutions were used; the frequency response graphs for those two concentrations of the soft drink are not shown here.

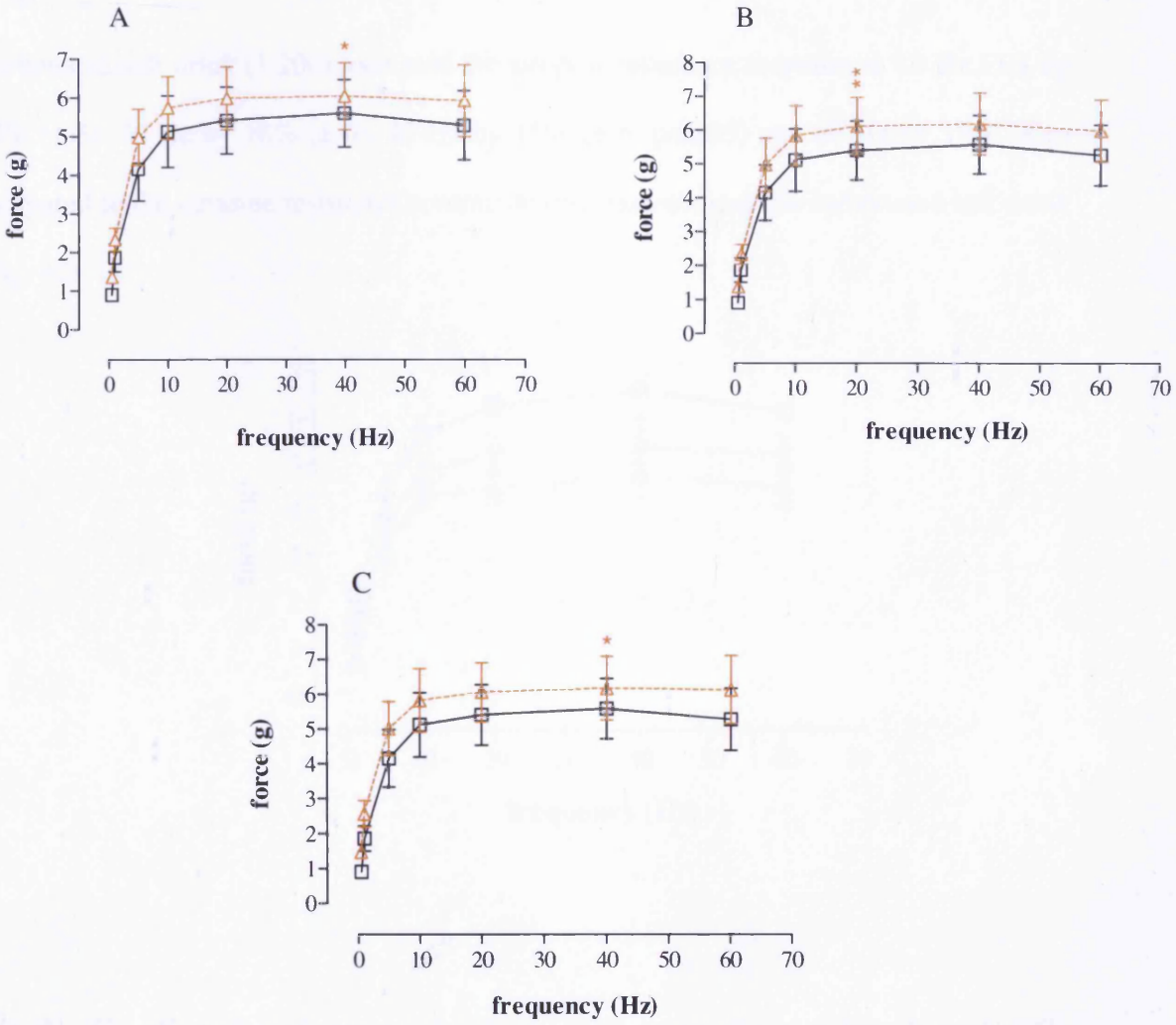


Fig. 39 *The effect of the carbonated soft drink, dilution 1:300 (A), dilution 1:200 (B) and dilution 1:100 (C) on rat detrusor muscle contractile response to EFS. □ Control frequency-response curves. △ Frequency-response curves in presence of the carbonated soft drink. (*p<0.05, **p<0.01). n=6.*

9.2.3 EFS in the absence and presence of atropine 10^{-6} M plus carbonated soft drink

Carbonated soft drink (1:200) increased the atropine resistance response to 10 Hz EFS by 12% (± 5), 20 Hz by 18% (± 6), 40 Hz by 15% (± 6; $p<0.05$) and 60 Hz by 18% (± 6) compared to the atropine resistance contractile response without the carbonated soft drink (Fig. 40).

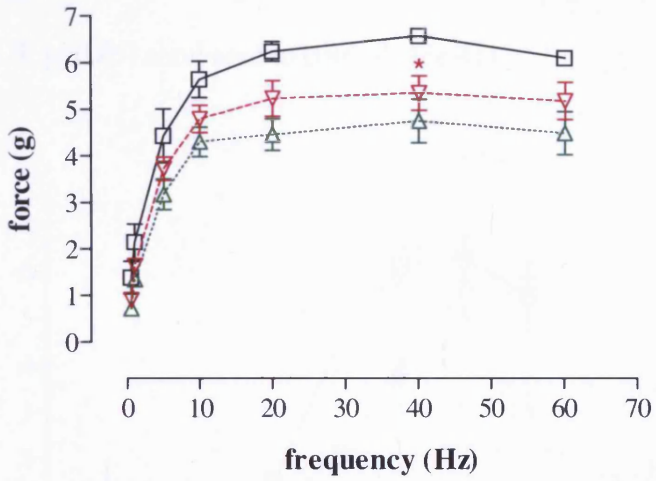


Fig 40 *The effect of carbonated soft drink (1:200) on rat detrusor muscle contractile response to EFS in the presence of atropine 10^{-6} M. □ Control frequency-response curves. △ Frequency-response curves in presence of atropine without carbonated soft drink. ▽ Frequency-response curves in presence of atropine and carbonated soft drink. (* $p<0.05$, when compared between the response curves, with atropine alone △, and atropine plus carbonated soft drink ▽). n=6.*

9.2.4 Concentration response curves to carbachol

Carbonated soft drink inhibited the carbachol evoked contractile response in concentrations from 10^{-7} M to10^{-3} M. The contractile response to carbachol 10^{-5} M was found to be significantly inhibited by 50% (± 8, $p<0.01$) compared to the control in the presence of carbonated soft drink at concentration of 1:200. The carbachol (10^{-4} M) evoked response was also found to be significantly inhibited in the presence of carbonated soft drink, by 36% (± 3, $p<0.05$) compared to control (Fig.41).

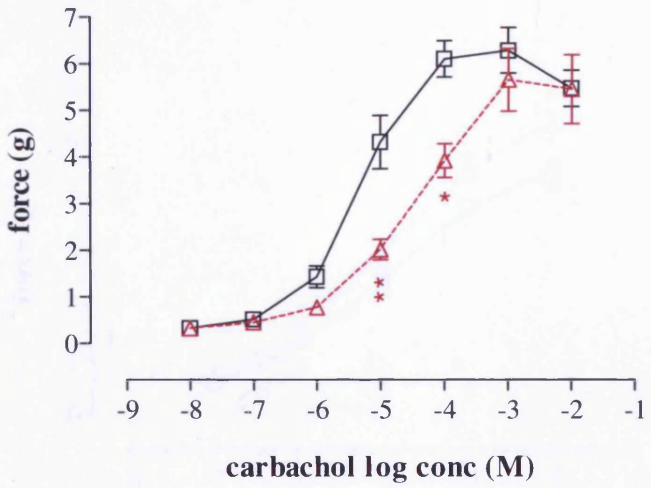


Fig 41 *The effect of carbonated soft drink (1:200) on rat detrusor muscle contractile response to carbachol. □ Control concentration-response curve to carbachol. △ Concentration-response curve to carbachol in the presence of carbonated soft drink. (* p<0.05, ** p<0.01). n=6.*

9.2.5 Concentration response curves to α, β Methylene ATP (ABMA)

Carbonated soft drink (1:200) enhanced the contractile response to α, β methylene ATP (10^{-7} M – 10^{3} M). The maximum contractile response to α, β methylene ATP 10^{-3} M was enhanced by 21% (± 6, $p<0.01$) (Fig. 42). There was no significant difference between the control log EC_{50} -4.95 (± 0.21) and the log EC_{50} after the addition of carbonated soft drink -4.91 (± 0.26).

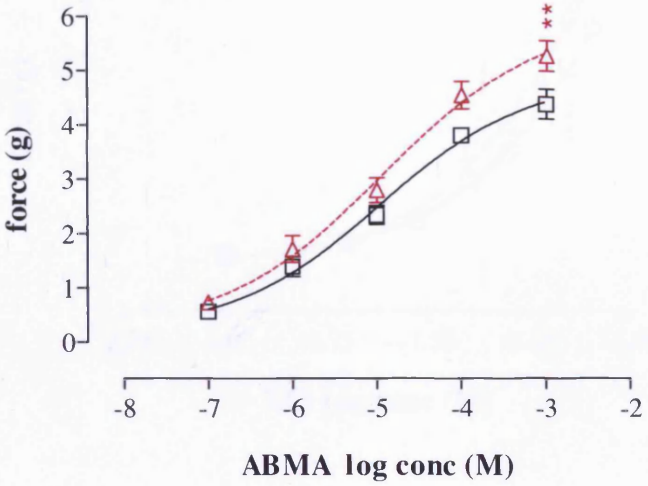


Fig 42 *The effect of carbonated soft drink (1:200) on rat detrusor muscle contractile response to α, β methylene ATP. □ Control concentration-response curves to α, β methylene ATP. △ Concentration-response curves to α, β methylene ATP in the presence of carbonated soft drink. (** $p<0.01$). n=6.*

9.2.6 Concentration response curves to KCl

Carbonated soft drink significantly enhanced the contractile response to KCl 70 mM and 80 mM by 28% (± 8, $p<0.01$) and 19% (± 3, $p<0.05$) respectively (Fig. 43). There was no significant difference between the control log EC_{50} and the log EC_{50} after the addition of carbonated soft drink.

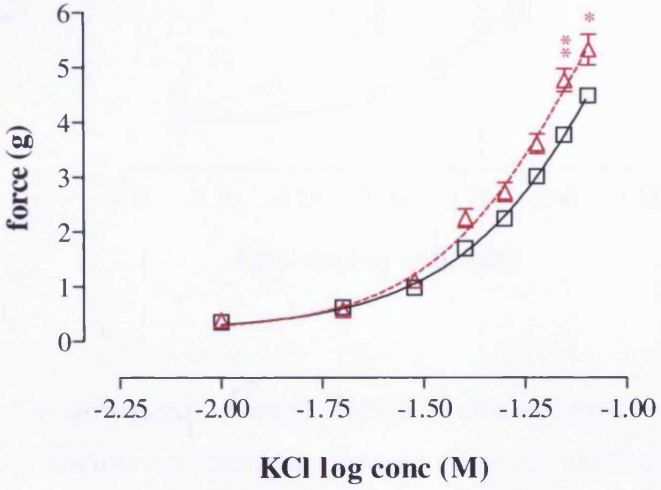


Fig. 43 *The effect of carbonated soft drink (1:200) on rat detrusor muscle contractile response to KCl. □ Control concentration-response curve. △ Concentration-response curve to KCl in presence of carbonated soft drink. (* $p<0.05$, ** $p<0.01$). n=6.*

9.2.7 Concentration response curves to calcium

Carbonated soft drink enhanced the contractile response to calcium 1.5mM to 2.5mM significantly; 1.5mM increased the contraction by 92% (± 13, $p<0.01$), 2mM by 95% (± 12, $p<0.001$), 2.5mM by 95 % (± 7, $p<0.001$) compared to the control response (Fig. 44).

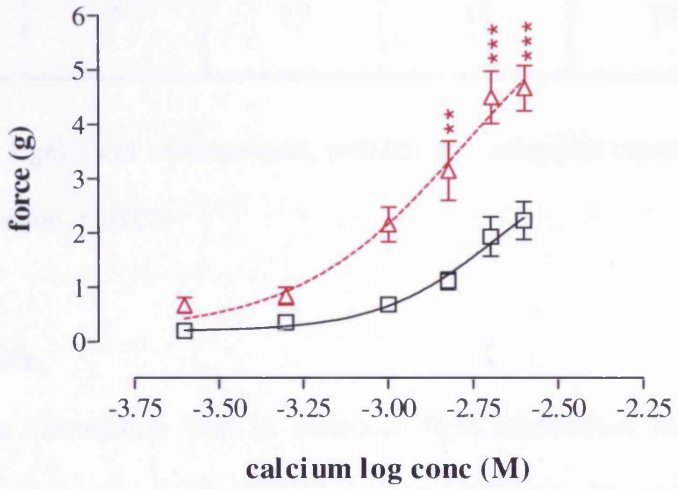


Fig 44 *The effect of carbonated soft drink (1:200) on concentration response curve to calcium. ☐ Control calcium concentration-response curve. △, Concentration- response curve to calcium in the presence of carbonated soft drink. (** $p<0.01$, *** $p<0.001$). n=6.*

Table 6 *Summary of the effects of carbonated soft drink (1:200) on the muscle contraction in response to different stimuli.*

	Atropine resistant response to EFS	Response to ATP	Response to KCl	Response to Calcium	Response to carbachol
Carbonated soft drink	↑	↑↑	↑↑	↑↑	↓↓

(↑: statistically significant enhancement, $p<0.05$; ↑↑: enhanced contraction, $p<0.01$; ↓↓: inhibited contraction, $p<0.01$)

9.3. Discussion

Smooth muscle contraction due to electrical field stimulation involves presynaptic neurotransmitter release from the nerve terminals followed by the post synaptic receptor activation by the released neurotransmitter; the enhancement of muscle contraction due to EFS in the presence of carbonated soft drink does not particularly suggest whether this is due to presynaptic or post synaptic mechanisms. Carbonated soft drink enhanced the atropine resistant component of the contractile response to EFS and also the response to post synaptic purinoceptor activation by exogenously applied ATP. The results of these experiments suggest that the enhancement of muscle contraction in response to exogenously applied ATP, in presence of carbonated soft drink, was greater than the enhancement of the atropine resistant response due to nerve stimulation. This indicates

that probably the enhancement of muscle contraction to electrical stimulation in the presence of carbonated soft drink was mainly the result of its effects on the post synaptic receptor mechanisms, and not by increasing neurotransmitter release.

It has been mentioned earlier in the methods section, that both acetylcholine and ATP are released from the nerve terminals when a higher frequency of EFS is delivered to the smooth muscle (such as 40 Hz). In order to isolate selectively the purinergic component of the muscle contraction, the bladder muscle was exposed to muscarinic receptor blocker, atropine. The way ATP mediates detrusor muscle contraction is possibly by acting on a ligand-gated cation channel (P2X receptor) thus leading to Ca^{2+}influx into cell cytoplasm (Burnstock and Williams, 2000). The bladder contractile response to ATP (in rabbit) is generally biphasic, but the response becomes monophasic after desensitization with α, β-Methylene ATP. This suggests that possibly two different types of receptors are involved in the mechanism of action (Cheng and Brading, 1991). It has been well established that ATP is the transmitter responsible for the NANC component of detrusor contraction (Burnstock, 2001), which acts mainly on purinoceptor $P2X_1$ subtype in different species (Lee et al., 2000; Vial and Evans, 2000). Hashitani et al. (2000) demonstrated that the activation of purinoceptors by neurally released ATP induces Na^+ influx that causes a rapid plasma membrane depolarization (Foresta et al., 1995), leading to increased intracellular Ca^{2+} by both Ca^{2+} entry into cell cytoplasm and Ca^{2+}-induced Ca^{2+} release (CICR) from intracellular stores to initiate bladder contraction. The CICR is possibly responsible for generation of the second component of excitatory junctional potentials (EJPs) (Ganitkevich & Isenberg, 1992). Thus modulation of post synaptic purinergic

receptor activation resulting in Ca^{2+} influx may be a probable mechanism of action for carbonated soft drink to enhance bladder contraction.

The muscle contraction to carbachol was found to be inhibited in the presence of carbonated soft drink whereas the response to nerve stimulation was enhanced. Interestingly this inhibitory effect of whole carbonated soft drink on muscle contraction to carbachol was very similar to the effect of ascorbic acid on this response (pp 198). Studies with cat detrusor muscle showed that acetylcholine-induced bladder contraction was mediated via M_3 receptor-dependent activation IP_3, resulting in Ca^{2+} release from the sarcoplasmic reticulum (An et al., 2002). However, it has been highlighted that relatively high concentrations of muscarinic receptor agonists have been used in most studies that suggested a role of IP_3 in the muscarinic receptor-mediated detrusor contractions (Mostwin, 1985; Iacovou et al., 1993). There is a possibility that the concentration of acetylcholine released by nerve-evoked response may not be high enough to stimulate IP_3 production. Hashitani et al. (2000) suggested that lower concentration of muscarinic receptor agonist may actually stimulate M_2 receptors, which do not involve production of IP_3. The authors also suggested an alternative explanation that there may be a difference in muscarinic receptor type depending on their localization in bladder smooth muscle. The junctional receptors are likely to be of M_2 receptors, while extrajunctional receptors are M_3 muscarinic receptors. M_2 muscarinic receptors inhibit the release of neurotransmitters from autonomic nerves (Coulson et al., 2004). Carbonated soft drink might inhibit the carbachol-induced muscle contraction by modulating any of the steps leading to Ca^{2+} release from the sarcoplasmic reticulum, involving the $G_{q/11}$, PLC-β_2 and IP_3 pathway.

However the effects of carbonated soft drink on nerve mediated responses and neurotransmitter release must be due to enhancing effects on presynaptic mechanisms.

The contractile response evoked by potassium was significantly enhanced in the presence of carbonated soft drink compared to control. Potassium depolarizes the smooth muscle cell membrane and opens voltage gated calcium channels, resulting in an influx of extracellular Ca^{2+} and an activation of contractile machinery (Karaki et al., 1984). Ganitkevich and Isenberg (1992) demonstrated that depolarization of plasma membrane results in extracellular Ca^{2+} influx into cell cytoplasm via voltage-gated Ca^{2+} channels which in turn stimulates Ca^{2+} release from the intracellular stores generating the phasic contractile response. As the two processes overlap, it is difficult to analyze the individual components separately. The replenishment of Ca^{2+} in the intracellular stores occurs via more Ca^{2+} influx which is regulated by a feedback mechanism involving Ca^{2+}-activated K^+ channels. A fall in cytoplasmic Ca^{2+} concentration inhibits activity of Ca^{2+}-activated K^+ channels resulting in increased K^+ leading to depolarization of plasma membrane and an ultimately enhancing the conductance of voltage-gated Ca^{2+} channels (Wu et al., 2002).

Further confirmation of the effect of carbonated soft drinks on bladder muscle contraction via Ca^{2+} influx was obtained by demonstrating its enhancing effect on the contractile response of rat bladder muscle to calcium, as described in previous chapters.

In conclusion, these experiments suggested that the carbonated soft drink as a whole enhanced the detrusor muscle contraction by enhancing calcium influx by a yet unknown mechanism. Carbonated soft drink inhibited the response to carbachol evoked muscle contractions. This action may be due to modulation of the pathway involved in Ca^{2+} release from sarcoplasmic reticulum.

Key points:

- ❖ Carbonated soft drink as a whole enhanced the muscle contractile response by enhancing calcium influx into cell cytoplasm.
- ❖ Carbonated soft drink also found to inhibit the carbachol induced muscle contraction (muscarinic effect).

CHAPTER 10

SWEET TASTE RECEPTORS

10.1 Introduction

The enhancing effect of artificial sweeteners on rat detrusor muscle contraction led to the hypothesis that these sweeteners might be acting through specific high affinity membrane receptors similar to the sweet taste receptors found in the tongue. This conclusion was reached because of the following: i) this study has shown that Ca^{2+} influx through L-type Ca^{2+} channels is involved in the mechanism of enhancement of muscle contraction by artificial sweeteners and ii) sweet taste receptors act on Ca^{2+} influx via second messenger pathway (Margoskee, 2002). Previous studies have demonstrated by Northern blot analysis (Max et al., 2001) and reverse transcriptase polymerase chain reaction (RT-PCR) (Kitagawa et al., 2001) that transcripts for the taste receptor proteins are expressed in a wide variety of tissues such as brain, testis, liver and lung. However, the possibility of the presence of these receptors in bladder has not been explored so far. Therefore, it seemed prudent to carry out a preliminary investigation to determine whether a sweet taste receptor protein, similar to the sweet taste receptor in rat tongue, is present in rat bladder.

10.2 Review of Literature

10.2.1 Taste perception

Humans experience five distinctive types of taste: sweet, sour, bitter, salty, and umami, a savoury flavour chiefly due to the amino acid glutamate (Roper, 1989; Lindemann, 1996, Chaudhari and Roper, 1998; Herness and Gilbertson, 1999; Lindemann, 2000). The different taste sensations are mediated by divergent taste receptors present on the surfaces

of taste receptor cells within the taste buds of the tongue. In animals this ability to differentiate various tastes is crucial for survival as it enables recognition of nutritionally rich food sources by sweet and umami receptors, while bitter taste receptors elicit aversive responses to noxious and toxic taste stimuli.

10.2.2 Taste receptors

Taste receptors have been classified into several groups: the T1R family which senses sweetness and umami-taste, the T2R family involved in detecting bitter compounds, and ion channels which are responsible for detecting acidic and salty tastes (Chandrashekar et al., 2000; Dulac, 2000; Lindermann, 2001).

Sweet and amino acid tastes (umami) are mediated by three members of the T1R family of G-protein-coupled receptor proteins (T1R1, T1R2 and T1R3) that combine to generate at least two heteromeric receptors: T1R1/T1R3 and T1R2/T1R3. T1R1/T1R3 forms a universal L-amino acid sensor (Nelson et al., 2002; Li et al., 2002), while T1R2/T1R3 functions as a broadly tuned sweet receptor (Hoon et al., 1999; Nelson et al., 2001; Li et al., 2002; Damak et al., 2003; Xu et al., 2004). The T1R2/T1R3 sweet taste receptor binds with variety of sweet-tasting compounds, including natural sugars and the artificial sweeteners saccharin and acesulfame K (Zhao et al., 2003). T1R2 is the protein responsible for the binding of artificial sweeteners in the tongue (Zhao et al., 2003). T1R3 is a seven-transmembrane, G protein-coupled receptor protein (GPCR) that has been identified as a component of a taste receptor essential for the perception of several types of sweeteners (Li et al., 2002; Montmayeur et al., 2001; Sainz et al., 2001).

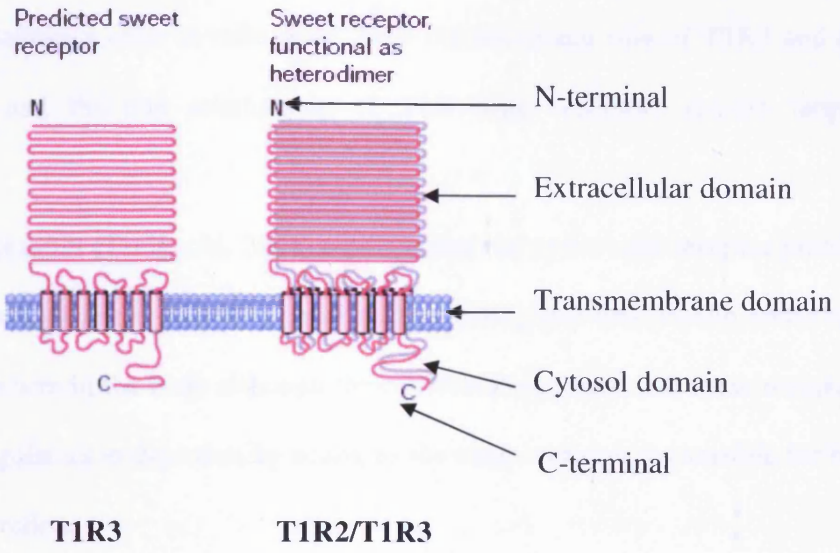


Fig. 45 *T1R3 monomer and T1R2/T1R3 heterodimer sweet taste receptor, showing N-terminal, extracellular domain, transmembrane domain and intracellular (cytosol) domain with C-terminal. (Reproduced from Linderman, 2001)*

Although the T1R3 molecule (Fig. 45) is one of the monomers in the heterodimeric structure of sweet- and umami-tasting receptors, there is no evidence that T1R3 monomer alone can function in recognizing similar ligands. Ariyasu et al. (2003) demonstrated for the first time that T1R3 alone, perhaps as a homodimer, functions as a molecule essential for the recognition and response to the disaccharide trehalose. Xu et al. (2004) suggested that there is a possibility that T1R3 is not a functional component of sweet and umami taste receptors, but merely a chaperone protein, which facilitates the proper folding or intracellular translocation of T1R1 and T1R2. The distinct ligand specificities of T1R1/T1R3 and T1R2/T1R3 receptors suggest that T1R1 and T1R2 play more important roles in ligand binding in sweet and umami taste receptors than T1R3. Moreover, Damak et al. (2003) demonstrated that T1R3-independent sweet- and umami-responsive

receptors and/or pathways exist in taste cells. Thus the functional role of T1R3 and the overall structure and function relationship of T1R taste receptors remain largely unknown.

Taniguchi and colleagues (Taniguchi, 2004) reported that the sweet taste receptor protein, T1R3, is also expressed in the liver and pancreas suggesting that this protein could also be expressed elsewhere in the body although these authors suggested that these receptors function as key regulators in digestion by acting as the chemosensors responsible for bile and pancreatic secretion.

Studies have suggested that the detection of both bitter and sweet taste by taste receptor cells is likely to involve G-protein-coupled receptors (Spielman et al., 1992; Lindemann, 1996; Kinnamon and Margolskee, 1996). Although two putative G-protein-coupled bitter/sweet taste receptors have been identified (Hoon et al., 1999), the chemical diversity of bitter and sweet compounds leads one to expect that there is a larger number of different receptors (Spielman et al., 1992; Lindemann, 1999). The signal transduction mechanism related to sour and salty tastes are likely to act via depolarization of the taste receptors by directly interacting with ion channels (Herness and Gilbertson, 1999). Sweet, bitter and umami-tasting substances, on the other hand, function by activating G-protein-coupled receptors (GPCRs) (Li et al., 2002).

The T1R taste receptors are most closely related to glutamate receptors (mGluRs), Ca^{2+}-sensing receptors (CaSRs), and some pheromone receptors (Bachmanov et al., 2001; Montmayeur et al., 2001; Kitagawa et al., 2001). All of these receptors belong to the GPCR family, and possess a large clam shell-shaped extracellular domain composed of an N-terminal (amino terminal domain) often referred to as the 'Venus fly trap module'

(VFTM) (Pin et al., 2003). The amino terminal domain is connected to a heptahelical transmembrane domain (HTD) via a cysteine-rich domain (CRD) and terminated by a variable C-terminal (carboxyl-terminal) intracellular tail containing several binding sites for intracellular proteins (Jiang et al., 2005a) (Fig. 45). The VFTM was found to be responsible for agonist recognition (Takahashi et al., 1993; Tones et al., 1995; Parmentier et al., 1998). Over the past few decades, multiple models of the sweet receptor's hypothetical ligand binding site have been generated based on the structures of existing sweeteners but without direct knowledge of the nature of the sweet receptor itself (Jiang et al., 2005b).

Xu et al. (2004) commented on an intriguing observation about the T1R2/T1R3 receptor combination and the structural diversity of its ligands. This receptor was able to recognize every sweetener tested, including carbohydrate, amino acids and derivatives, proteins, and synthetic sweeteners (Li et al., 2002). On the other hand, the receptor exhibits stereo-selectivity for certain molecules, e.g. it responds to D- tryptophan but not L- tryptophan (Li et al., 2002). It is still a puzzle as to how this single receptor can recognize such a large collection of diverse chemical structures. There are differences in human and rodent sweet taste in terms of the ligand specificity, G protein-coupling efficiency, and sensitivity to inhibitors (Xu et al., 2004).

Xu et al. (2004) have also confirmed in their study with human taste receptors that both T1R2 and T1R3 are required in a functional sweet taste receptor, that aspartame and neotame require the N-terminal extracellular domain of T1R2, that G protein coupling requires the C-terminal half of T1R2, and that cyclamate and lactisole require the transmembrane domain of T1R3. These findings demonstrated the different functional

roles of T1R subunits in a heteromeric complex and the presence of multiple sweetener interaction sites on the sweet taste receptor. They have also speculated that natural carbohydrate sweeteners bind to the N-terminal domain of T1R2, similar to aspartame and neotame, and there may be other ligand-binding sites on the sweet taste receptor, for example, the transmembrane domain of T1R2.

When a sweet-tasting molecule binds to the GPCR, it creates a conformational change in the receptor, causing activation of a G protein. Activation of G protein allows replacement of G protein bound GDP with GTP, which in turn stimulates adenylate cyclase enzyme activity resulting in production of cAMP (Nelson and Cox, 2005) (Fig. 44). The elevation of cAMP activates the taste receptor cells either by opening a cyclic nucleotide-gated channel (Misaka et al., 1997) or by activating protein kinase A (PKA), to phosphorylate K^+ channels in the plasma membrane, causing them to close. Reduced efflux of K^+ depolarizes the cell to generate an action potential (Cummings et al., 1996; Margolskee, 2002). Phospholipase C (PLC), a major signalling effecter of GPCRs, and TRPM5 (Taste Receptor Protein), are co-expressed with T1Rs and T2Rs and are essential for transduction of major tastes such as sweet, bitter and umami (Zhang et al., 2003). The activation of PLC, either directly or indirectly results in gating of TRPM5 leading to development of a depolarizing receptor potential (Minke, 2001; Runnels et al., 2002).

This complex nature of functioning was also highlighted in other studies. These studies suggested that although natural and artificial sweeteners activated the same receptor T1R2/T1R3 (Nelson et al., 2001; Li et al., 2002), they probably stimulated different signalling pathways resulting in the production of multiple second messengers (Bernhardt et al., 1996; Cummings et al., 1996). Based on biochemical and electrophysiological

studies of taste cells, Margolskee (2002) suggested activation of two pathways of sweet taste signal transduction, first is a GPCR-G_s-cAMP pathway, activated by sucrose and other sugars and the second is by GPCR-G_q/G$\beta\gamma$-IP_3 pathway, activated by artificial sweeteners (Gilbertson et al., 2000; Smith and Margolskee, 2001; Margolskee, 2002) (Fig. 46).

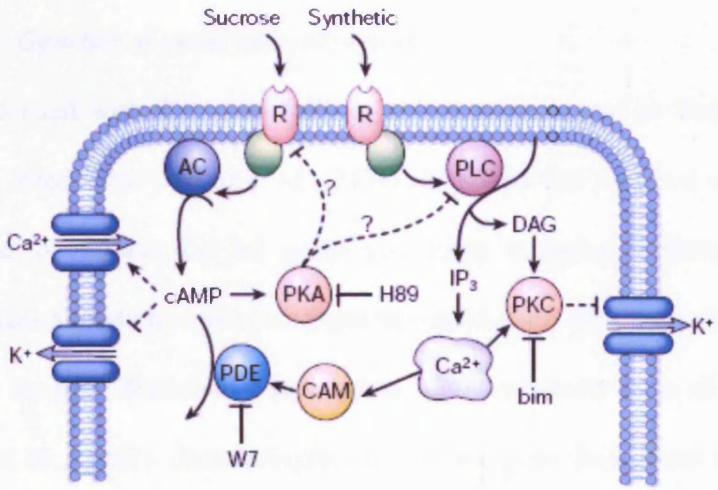


Fig. 46 *Signal transduction of sweet taste. Two separate sweet taste receptors shown. R: receptors; AC: adenyl cyclase; cAMP: cyclic adenosine monophosphate; PDE: phosphodiesterase & inhibitor W7; CAM: calmodulin; PKA: phosphokinase A and inhibitor H89; PLC: phospholipase C; DAG: diacylglycerol; IP_3: Inositol tris phosphate; PKC: protein kinase C and inhibitor bim (bisindolylmaleimide). (Reproduced from Linderman, 2001)*

Both the transduction pathways ultimately lead to a rise in Ca^{2+} concentration in cytoplasm followed by neurotransmitter release. Artificial sweeteners activate GPCRs (T1R heterodimers) and the subsequent mechanism probably involve phospholipase C (PLC), production of inositol trisphosphate (IP_3) and release of Ca^{2+} from intracellular stores. On the other hand sugars, after activating GPCRs, likely to act via increasing

cAMP production by activating adenyl cyclase pathway (AC). Increase in cAMP possibly inhibits basolateral K^+ channels through phosphorylation by cAMP-activated protein kinase A (PKA) resulting in membrane depolarization (Avenet et al., 1988). These two pathways (Fig. 46) can coexist in the same taste receptor cells (Bernhardt et al., 1996). It has also been suggested that both artificial sweeteners and sucrose depolarize taste receptor cell membrane possibly via inhibiting K^+ conductance (Cummings et al., 1996).

10.2.3 Genetics of sweet taste receptors

The genes associated with the sweet taste receptors were named as *Tas1r2* and *Tas1r3* (McDaniel and Reed, 2004). Reed et al. (2004) suggested that minimal alterations in the DNA sequence of mouse *Tas1r3* gene can have a major impact on sweetener consumption. This alteration in sweetener preference in mice with different *Tas1r3* alleles was explained by their diminished perception of the concentration of the sweeteners (Bachmanov et al., 1997). Interestingly, in *Tas1r3* gene knock-out mice, the taste perception for glucose and maltose was almost similar to mice with a normal *Tas1r3* gene and that for other sugars and sweeteners the taste perception was diminished, but not completely absent (Damak et al., 2003). This indicated the presence of other receptors or mechanisms to sense the sweet taste in mice. It may well be that the unaffected monomer T1R2 acted as a taste receptor by itself in these *Tas1r3* knockout mice (Zhao et al., 2003). If alterations in DNA sequence have a significant impact on the consumption of sweet substances in mice, then this may also be applicable to humans. The three sweet receptor genes present in humans were designated as *TAS1R1, TAS1R2,* and *TAS1R3* (Liao and Schultz, 2003) having a considerable sequence similarity to that in mice.

10.3 Methodology

To examine the presence of artificial sweet taste receptor in rat bladder, immunoblotting was used. **Immunoblotting** is a technique in molecular biology or immunogenetics that can be used to detect protein in a given sample of tissue homogenate or extract. It normally utilises polyacrylamide gel electrophoresis to separate native proteins by shape and size (Towbin et al., 1979). Electrophoresis is an important technique for the separation of proteins, based on the migration of charged molecules in an electric field. The proteins are then transferred out of the gel and onto a membrane (typically nitrocellulose), where they are "probed" using antibodies specific to the protein of interest (Renart et al, 1979; Burnette, 1981). As a result, size and amount of different proteins in a given sample can be detected and compared simultaneously. This method is especially useful as an analytical method because proteins can be visualised as well as separated, permitting the number of different proteins in a mixture, and approximate molecular weight to be determined quickly.

Electrophoresis of proteins is generally carried out in gels made up of cross-linked polyacrylamide polymer, which acts as molecular sieve, slowing the migration of native proteins approximately in proportion to their charge-to-mass ratio. Migration may also be affected by protein shape (Towbin et al., 1979) and thus to overcome this technical difficulty, SDS is added to the gel, so that proteins are separated by size only. Therefore, SDS-PAGE was used in the following studies.

10.3.1 Tissue preparation

Samples of rat tongue and bladder were dissected from culled animals and after removing all excess fat and visceral components minced in a small amount of homogenisation

buffer (~1ml/g of tissue) with an Ultra-Turrax T8 (Ika Labortechnik) and transferred as aliquots to microfuge tubes. The samples were then centrifuged at 14,000 x g for 10 minutes at 4°C. The supernatants were aliquoted (50-100 µl) and stored at -80°C.

10.3.2 Preparation of reagents

Homogenisation buffer (Stock and working solution)

To prepare the homogenisation buffer solution, we added 4 ml of 1 M Tri-HCl (pH 7.4), 4 ml of 0.1 M EDTA (pH 7.4), 4 ml of 0.1 M EGTA (pH 7.6) and 80 µl of β-mercapto-ethanol in a beaker and made the solution up to 200 ml with distilled water. The solution was then mixed thoroughly and stored at 4°C. Immediately before use, 10 ml of the above solution was taken and 1 mini-complete protease inhibitor tablet (Roche Diagnostics) was added to the homogenisation buffer and dissolved.

Ammonium persulphate (10%)

To prepare 10% ammonium persulphate, 0.1 g of ammonium persulphate (APS) was weighed in a 1.5 ml Eppendorff tube and then dissolved in 1 ml distilled H_2O.

Layering solution

To prepare the layering solution, the following chemicals were mixed in a 50 ml measuring cylinder: 12.5 ml of Tris (pH 6.8), 100 µl of 0.5 M EDTA (pH 8.0), 500 µl of 20% Sodium dodecyl sulphate (SDS) and 36.9 ml of distilled H_2O.

PAGE running buffer

PAGE running buffer was prepared by adding 28.8 g of glycine, 6 g of Tris base, 2 g of sodium dodecyl sulphate (SDS) and 1.25 litre distilled H_2O in a 2 litre measuring cylinder and the solution was dissolved thoroughly. The volume was increased to 2 litres.

SDS-PAGE gel loading buffer

SDS-PAGE gel loading buffer solution was prepared by adding: 1.2 ml of 0.5 M Tris-HCl (pH 6.8), 5 ml of 50% glycerol, 2 ml of 0.1% sodium dodecyl sulphate (SDS), 0.5 ml of 2-(β) mercaptoethanol, 1 ml of 1% bromophenol blue and 0.3 ml of distilled water in a 15 ml Sterilin tube. The mixture was then stored at 4°c.

SDS-PAGE gel transfer buffer

This buffer solution was prepared by dissolving: 2.9 g of Tris base, 14.4 g of glycine and 400 ml of methanol in 2 litres of distilled water in a Duran bottle.

Blocking buffer 4% (Blotto)

This buffer solution was prepared by dissolving: 2 g of non-fat milk powder (Carnation® or Coffee-mate® or Marvel®) in 50 ml of Tris-buffered saline (R15) in a 50 ml Sterilin bottle.

Tris-buffered saline (R15; pH 7.5)

10 ml of 1M Tris-HCl (pH 7.5) was mixed with 37.5 ml of 4 M NaCl and the solution was made up to 800- 1000 ml (deionised water) at pH 7.5.

Antibodies

Two primary antibodies were used: V-20 and T-20 (goat anti-human taste receptor, Santa Cruz). V-20 is specific for the N-terminal (extracellular) domain of the receptors and T-20 is specific for the C-terminal (membrane/ intracellular) domain of the receptor. It should be noted that antibodies were generated against peptides based on predicated amino acid sequences of the protein molecule.

Donkey anti-goat-HRP conjugated antibody (Santa Cruz) was used as a secondary antibody.

10.3.3 Gel electrophoresis

10.3.3.1 Preparation of Western Blot

Gel forming apparatus was set up and 8% separating and 4.7% stacking gels containing 14% glycerol and 0.1% SDS were poured into the respective compartments of the apparatus and allowed to polymerise and the equipment set up. After filling the chambers with gel running buffer, a maximum of 20 μg of total protein was loaded carefully using a micropipette into the appropriate wells along with Rainbow molecular size markers (GE Healthcare). Electrophoresis was carried out for 1 hr and 30 min at 125 Volts, which allowed the ion front to reach 5 mm from the bottom of the plate.

10.3.3.2 Western Transfer

After SDS-PAGE, the proteins were transferred to nitrocellulose by carefully placing the polyacrylamide gel within a Novex transfer apparatus on top of two porous pads and a piece of 3 MM filter paper that had been soaked in transfer buffer. After removing air bubbles from the nitrocellulose membrane, another piece of 3 MM filter paper was placed on top followed by two more soaked pads. The apparatus was assembled, the inner chamber filled with transfer buffer, the outer chamber with cold water and the proteins transferred at 125 mA for 1 hr and 30 min.

The nitrocellulose membrane was then briefly rinsed with water and allowed to dry completely for 5 min on filter paper.

10.3.3.3 Blocking

The membrane was placed in 4% blocking buffer (blotto) for an hour to prevent non-specific binding of proteins to the membrane. The membrane has the property to bind to any kind of protein, and as both the antibodies and target are proteins, necessary steps

must be taken to prevent interactions between the membrane and the antibody used for detection of the target protein (Renart et al., 1979).

10.3.3.4 Detection

A modified antibody linked to a reporter enzyme has been used to detect the specific protein present on the membrane. The reporter enzyme enables the production of a colour by a colorimetric reaction with the antibody. Although traditionally this process of detection happens in two-steps, now one-step detection methods are also being used for certain experiments. The two step procedure was used here.

Step 1: The primary antibody was added at 1:200 dilution to 20 ml of 4% Blotto and the preparation was sealed and incubated for 18 hr (overnight) at 4°C on an orbital shaker.

Step 2: On the following morning, the membrane was washed three times for 5 minutes to remove any unbound primary antibody. The membrane (in 4% Blotto) was then exposed to the secondary antibody, at 1:50,000, at room temperature on an orbital shaker for 1 hr and 30 min. This step confers an advantage in that several secondary antibodies will bind to one primary antibody, providing an enhanced signal.

10.3.3.5 ECL chemiluminescent detection

The membrane was then washed three times for 5 min with water, once with 0.05% Tween 20 (Sigma) in TBS for 5 min, and again very briefly with a small amount of water. The membrane was blotted dry on filter paper and then exposed to West-femto ECL reagent (Pierce) for 5 min. The membrane was once again blotted dry with filter paper, sealed in a plastic bag and exposed to X-ray film (ECL film GE Healthcare) for 5 min. The film was developed to obtain the result. The dark regions on the film corresponded to the protein bands of interest.

10.4 Results and analysis

10.4.1 Western blot with V-20 antibody

A single protein band was identified in both tongue and bladder tissue, with an approximate molecular mass of 50-54 kDa. In both tissues, the protein was present in whole cellular extracts and membrane extracts, but absent in the cytoplasmic extracts samples (Fig. 47).

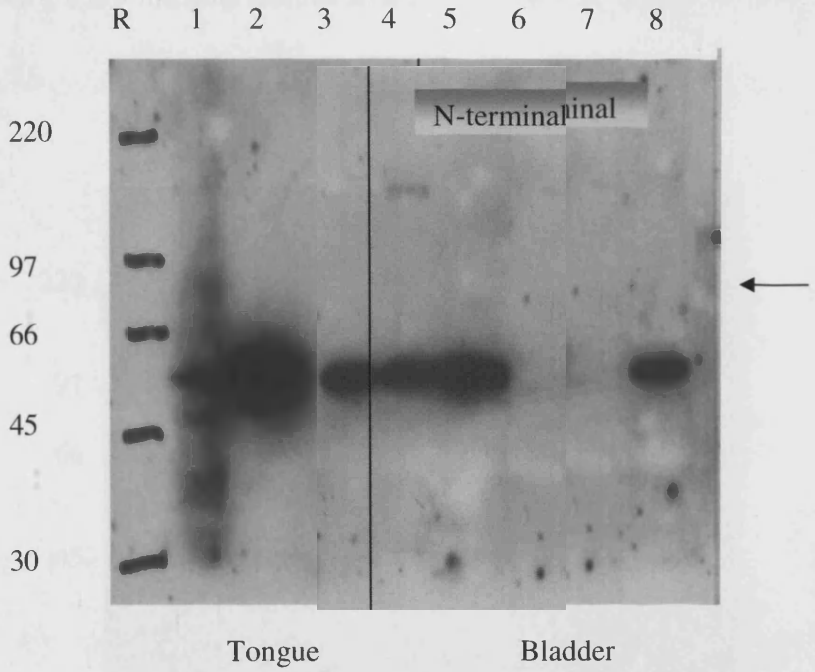


Fig. 47 *Developed film image from western blot using V-20 antibody. R: size markers (kDa size to left of image). Samples harvested on 22.08.06. Lanes 1& 5: whole cellular extract; lanes 2 & 6: membrane extracts; lines 3 & 7: cytoplasmic extracts. Lanes 4 & 8: whole cellular extract from samples harvested on 20.07.06.*
Arrow indicates predicted size of whole T1R2 receptor protein

10.4.2 Western blot with T-20 antibody

A single protein band was identified in the tongue, with an approximate molecular mass of 40- 43 kDa and was present in whole tissue and membrane extracts (Fig. 48, lanes 1-4). In the bladder, a single protein band was also identified. This band had an approximate molecular mass of 45 kDa and was present in whole cellular extract, and membrane fraction, but not in the cytoplasmic extracts (Fig 48, lanes 5-8).

In both tissues, a low intensity doublet around 180 kDa was seen in all tissue samples.

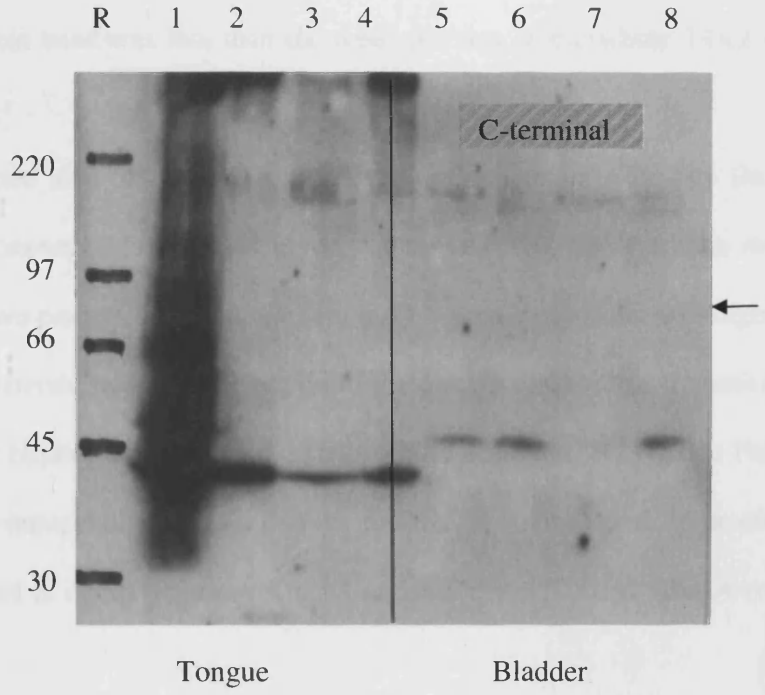


Fig. 48 *Developed film image from western blot using T-20 antibody. R: size markers (kDa size to left of image). Samples harvested on 22.08.06. Lanes 1& 5: whole cellular extract; lanes 2 & 6: membrane extracts; lines 3 & 7: cytoplasmic extracts. Lanes 4 & 8: whole cellular extract from samples harvested on 20.07.06*

Arrow indicates predicted size of whole T1R2 receptor protein

10.4.3 Interpretation of findings

The western blots demonstrated a single protein band for each antibody, without any apparent non-specific binding. The presence of a low intensity doublet at 180 kDa with the C terminal antibody (T-20) could be interpreted as indicating that incomplete dissolution of the T1R2/T1R3 couplet in the tissue extracts has occurred, although this is unlikely as the presence of β-mercaptoethanol and SDS in the sample buffers should have caused dissociation of receptor sub-units. For each antibody, the estimated size of the major protein band was less than the predicted size of the whole T1R2 receptor protein (URL 11).

The predicted size of the entire T1R2 precursor protein is 95,183 Da. The molecule consists of several domains, and glycosylation sites that may alter the structure and size of the mature protein. In addition, there are 17 putative disulphide bridges located on the predicted external hydrophobic region of the protein close to the transmembrane domain. The ligand binding domain is predicted to bind the Atrial Natriuretic Factor (ANF) and there is an intracellular domain that is predicted to interact with G-proteins, particularly those related to cGMP regulation, Ca^{2+} regulation via IP3 and GABA-receptor function (URL 12).

The combined size of the specific bands using the two T1R2-specific antibodies is between 90 – 97 kDa depending on tissue source (fig 47 and fig 48). These sizes are in keeping with the idea that the mature receptor protein lacking the first 19 amino acids (the signal peptide) would be 2150.65 Da smaller than the precursor molecule (i.e. 95,183-2151=93,032) producing a mature protein of ~93 kDa. However, although there

are some weak bands at ~93 kDa, the most intense bands are at much lower molecular mass. The 2 kDa signal peptide could explain the difference in size of the products using the T-20 antibody that is directed towards the C-terminal (intracellular domain) but this is unlikely.

There are three possible explanations for the receptor protein becoming fragmented: i) the tissue homogenisation process has cleaved the molecule at a junction close to the transmembrane domain; ii) there is a disulphide bond within the protein close to the membrane/extracellular domain junction of the protein that is destroyed using SDS-PAGE; iii) there was inadequate protease inhibition during the tissue preparation and a specific (weak) peptide bond close to the transmembrane domain was broken.

The slight size difference in the protein fragment observed with the C terminal antibody raises the possibility of post-translational structural modifications which are tissue specific and may be involved in specific post-receptor signalling pathways. Additionally, this region of the molecule putatively contains 7-specific glutamate metabotropic regions that are known to confer different signalling activities dependent upon co-activator and phosphorylation status (Tanabe et al., 1992). Indeed there are several possible glycosylation sites in this region of the molecule that could account for the 2 kDa apparent increase in molecular mass between tongue and bladder T1R2 protein.

10.5 Discussion

These results suggests the possibility of a single protein moiety in both tongue and bladder tissue. The primary antibody, V-20 is a goat polyclonal IgG specific antibody (200μg/ml) with epitope mapping near the N-terminal of T1R2, and T-20 is another goat

polyclonal IgG specific antibody (200μg/ml) with epitope mapping near the C-terminal of T1R2. Polyclonal antibodies are useful for detecting proteins which have lower expression levels allowing more antibodies to bind a single protein molecule, thus enhancing the detection signal. Although the size of protein detected with each antibody was smaller than the predicted size of the whole protein, the combined weights do approximate to 95 kDa, if one assumes that the whole protein has been cleaved during preparation of the samples. It is important to remember that the apparent difference in band sizes can still be attributed to non-specific binding of antibodies to different proteins in the gel, as antibody specificity was not established by using blocking peptide in the experiments. Peptide blocking to minimize the non-specific binding of antibodies needs to be included in future experiments to find a definitive answer.

The cleavage or fragmentation of the protein molecule was described above. This could be evaluated by repeating the experiment with fresh tissue and taking particular care during homogenisation to keep samples chilled and handled gently. This may be hard due to the elastic properties of bladder tissue which makes homogenisation difficult. It can also be suggested to repeat electrophoresis and Western blotting using a non-reducing gel without SDS, to minimise the disruption of disulphide bonds within the proteins. A single larger protein band of similar visualised using each antibody would be expected.

Thus the results suggest the possibility of presence of T1R2 protein in rat bladder similar to sweet taste receptors in rat tongue. It is worth mentioning that because T1R2 participates exclusively in sweet taste detection, while T1R3 is involved in both sweet and amino acid recognition, the antibody against T1R2 was chosen for Western blot analysis experiment.

There is only one study (Dyer et al., 2005) which has shown the presence of extra-oral T1R2 protein in mouse intestine. However, those authors did not include the size marker on their gel, so comparisons to the present data are difficult, because no data on the size of protein detected are available.

It has already been established that the artificial sweeteners enhanced the detrusor smooth muscle contraction possibly by the increase of Ca^{+2} influx from extracellular space via L-type voltage-gated Ca^{2+} channels. It has been explained earlier (Chapter 2, section 2.10 and 2.11), that activation of both receptor mediated (cholinergic and purinergic) pathway and the excitation-contraction pathway involves Ca^{2+} influx through voltage-gated Ca^{2+} channels.

Signal transduction of sweet taste has been studied in detail by several investigators. Striem et al. (1989) demonstrated that some sweeteners stimulated the formation of the second messenger cyclic AMP in a rat tongue membrane preparation. They suggested that the activation of the enzyme adenyl cyclase, responsible for formation of cAMP was mediated by specific protein receptors (Guanosine 5'-[γ-thio]triphosphate, GTP), coupled through a GTP binding protein (G-protein).

Uchida and Sato (1997) suggested two different pathways for sweet taste transduction for two different types of sweeteners such as sodium saccharin and D-tryptophan. The former was found to act by increasing intracellular cAMP levels and the later by increasing the intracellular IP_3 level. Margolskee (2002) and Lindemann (2001) both supported the view that that the molecular mechanism of sweet taste signal transduction was via two possible mechanisms and both the transduction pathways appeared to converge to a common point, which is a rise in intracellular Ca^{2+} followed by

neurotransmitter release. The first mechanism involved an increase in cyclic nucleotides (cGMP or cAMP) when sugars were the ligand and the second mechanism was via an increase in IP_3, when the sweet taste receptors were activated by artificial sweeteners. Membrane depolarization by inhibition of K^+ conductance may be a common feature of the two pathways (Lindemann, 2001; Nakashima and Ninomiya, 1999; Cummings et al., 1996) (pp 232-233). Membrane depolarization was originally thought to be due to inhibition of the K^+ conductance through activation of protein kinase A (PKA) (Cummings et al., 1996). More recently Lindemann (2001) suggested that perhaps PKA is not involved directly in the response to sugars, but may be involved in adaptation. This inference came from the observation that a PKA inhibitor was found not to inhibit the sugar-sweet response in the hamster anterior tongue (Varkevisser and Kinnamon, 2000). However, sweet-taste transduction is a complex phenomenon and our knowledge about it is far from complete (Lindemann, 2001).

Nevertheless, we have sufficient evidence to suggest that Ca^{2+} influx is a common factor in all the different pathways of sweet taste transduction. Our data found that Ca^{2+} influx plays an important role in the stimulating effect of artificial sweeteners on detrusor muscle contraction. This leads to the speculation that the protein molecules co migrating with sweet taste receptors detected in the rat bladder by our study may be implicated in the process of bladder muscle contraction in response to artificial sweeteners.

The identification of the sweet taste receptor(s) in the bladder will provide opportunities for a better understanding of molecular and cellular events involved in detrusor contraction in relation to sugar and artificial sweeteners. This may eventually help in the development of therapeutic compounds and dietary aids to modulate the function of the

receptor in the bladder. Such developments may be particularly useful in diabetic patients who as a group are bigger users of artificial sweeteners than the general population and may therefore be more vulnerable to overactive bladder symptoms. The sweet taste receptor mediated mechanism of bladder contraction may also be postulated as causing urgency and frequency in pregnancy, which may be related to the glycosuria due to lower renal threshold for excretion of sugars.

CHAPTER 11

DISCUSSION

Urinary incontinence is a common condition with significant physical, psychological and economic impact (Wagg et al., 2007), affecting quality of life to a great extent (Burgio and Ouslander, 1999). The prevalence of overactive bladder in the general adult population is approximately 17%. Incontinence events are reported in approximately one-third of these patients. Symptoms of overactive bladder have a negative impact on quality of life in every second patient and on sexuality in every fourth patient (Schumacher, 2006). In Britain alone, an estimated 5.15 million adults over the age of 40 have symptoms attributable to overactive bladder syndrome (OAB) (Abrams et al., 2002).

Although several risk factors have been associated with overactive bladder in different studies (pp 100), the pathophysiology of overactive bladder is still unclear. Consumption of carbonated soft drinks was recently reported to be associated with development of overactive bladder and urinary incontinence (Dallosso et al., 2003a). The aim of this study was to therefore determine if individual components of a popular carbonated soft drink modulate detrusor muscle contraction. Rat bladders were used in this study for two reasons- firstly, easy availability and secondly, rat bladders have similar innervation to abnormal human bladders, as detailed in the review of literature section (pp 43).

The effects of some of the individual components of soft drinks on rat detrusor muscle contraction were determined using organ bath methodology. The actual concentrations of the individual soft drink components in body fluids after consumption of one can was unknown, so a range of concentrations from the lowest (10^{-8} M) to the highest (10^{-2} M) was used in the initial experiment to detect the concentration that significantly modified bladder muscle contraction. It is worth mentioning that sophisticated technology has been developed to measure individual components such as artificial sweeteners in carbonated

beverages and also in human urine after consumption of individual components. Levels of individual components in a carbonated soft drink can be easily measured by the recently developed technique of a rapid capillary electrophoresis method (Frazier et al., 2000). Wilson et al. (1999) have developed a method to determine the levels of two sweeteners (sweeteners saccharin and acesulfame K) in human urine by using urinary biomarkers as a measure of intake of the sweeteners and high-performance liquid chromatography (HPLC). The two sweeteners, saccharin and acesulfame K were considered suitable for monitoring using biomarker approach as both these compounds are excreted rapidly almost unmetabolized (Renwick, 1996).

The results of a recent study which had estimated the concentration of four artificial sweeteners in food and beverages by ion chromatography (Zhu et al., 2005) were used to calculate possible levels of the above sweeteners in human body after consumption of one 300 ml can of the soft drink in question by drug kinetics, assuming equal distribution in a man weighing 70 kg. This data was then compared to the concentrations of these soft drink components used in the organ bath in this study. The average concentrations of sweeteners in the two cola drinks were: aspartame, 5030 mg/L, acesulfame, 1290 mg/L and sodium saccharin 381, mg/L. Out of the three sweeteners investigated in this study, acesulfame K and sodium saccharin were the two sweeteners that caused significant modulation of bladder contraction. Also aspartame is metabolised to phenylalanine and aspartic acid after consumption so it was not possible to make a presumptive calculation of levels in body fluids. After calculation, the concentrations of acesulfame K and sodium saccharin were found to be 6.04 mg/L and 23 mg/L respectively, assuming equal distribution in all body water. The concentrations of these sweeteners used in the organ

bath, excluding those used for the EFS 10Hz responses, were 20.1 ng/L for acesulfame K (10^{-7} M) and 20.5 ng/L for sodium saccharin (10^{-7} M) being much lower than the estimated blood levels of the sweeteners after soft drink consumption.

The concentration of acesulfame and sodium saccharin has also been determined in urine (Wilson et al., 1999). After an intake of up to 100 mg/L acesulfame and 70 mg/L sodium saccharin, the peak urine concentrations were 92 mg/L and 64 mg/L respectively. These levels were also far greater than the concentrations used in the organ bath (excluding EFS 10 Hz responses) and the concentration of these sweeteners in cans of cola have been determined to be even greater than the intake of sweeteners in the Wilson study. The concentrations of sweeteners used in the organ bath (excluding EFS 10 Hz responses) were therefore below the amount that would be found in body fluids after the consumption of a single can of carbonated soft drink.

In the stable human bladder, detrusor contraction is mediated by acetylcholine (ACh) release from parasympathetic nerve fibres, which is completely abolished by atropine (Sibley, 1984). Interestingly detrusor muscle contractions from dysfunctional bladders were found to have an atropine-resistant component, believed to be mediated by ATP (Ruggieri, 2006). In contrast, most non-primate bladder detrusor muscle contractions involve the muscarinic pathway, mainly via the M_3 receptor subtype. (Braverman et al., 1998) and purinergic pathway-mediated by ATP (Namasivayam et al., 1999).

Contraction of rat detrusor muscle in response to EFS has been shown to be via stimulation of the nerves, as demonstrated by the abolition of this response in the presence of tetrodotoxin (TTX), a sodium channel blocker (Brading & Williams, 1990; Hudman et al., 2000). Nerve stimulation releases the neurotransmitters ACh and ATP,

involving the cholinergic and purinergic pathways (Creed et al., 1983). At lower frequencies of EFS, muscle contraction is mainly due to the release of ATP from the nerves with an increasing component due to the release of ACh as the frequency of stimulation increases (Brading & Williams., 1990).

When the cholinergic component of the muscle contraction was blocked by atropine, during EFS stimulation, the slight enhancement in magnitude of the remaining contractile response in the presence of the sweeteners, suggested a possible mode of action through the atropine-resistant component mediated by ATP, which is coupled to L-type calcium channels. The activation of purinoceptors by neurally released ATP initiates excitatory junction potentials (EJP), which triggers action potentials by Na^+ influx causing a rapid plasma membrane depolarization (Foresta et al., 1995). The resultant influx of Ca^{2+} through L-type Ca^{2+} channels and the release of Ca^{2+} from intracellular stores produce contraction of the bladder smooth muscle. The activation of muscarinic receptors by neurally released ACh increases the frequency of action potentials, initiates oscillatory rises in the intracellular Ca^{2+} concentration causing oscillatory contractions. Both the influx of Ca^{2+} through L-type Ca^{2+} channels and the release of Ca^{2+} from intracellular stores appear to be involved in the generation of these oscillatory responses in guinea pig bladder smooth muscle (Hashitani et al., 2000).

The sweeteners enhanced muscle contraction through purinergic receptor activation by α, β methylene ATP. This enhancement was similar to the enhancement observed in the 'atropine resistant' component of muscle contraction due to EFS. Electrical stimulation triggers presynaptic nerve terminals to release neurotransmitters, which then activate postsynaptic receptors. Therefore it can be speculated that this enhancement of muscle

contraction by the sweeteners was possibly not by an increased release of ATP as the postsynaptic activation of purinergic receptors was equally affected. The sweeteners also had no significant effect on the contractile response to carbachol, indicating that the mechanism of action of sweeteners is not via direct muscarinic receptor activation.

The L-type channels present in the detrusor are important for mediating the upstroke of the actions potentials, as discussed earlier in the physiology chapter. The L-type channels in guinea pig detrusor display interesting behaviour in that they can switch into a long channel open mode in response to large depolarization (Nakayama & Brading, 1993; 1995). Potassium was used in our experiments to depolarize the cell membrane and investigate the effects of the individual components of the carbonated soft drink on Ca^{2+} influx through these L-type voltage gated Ca^{2+} channels. Ganitkevich and Isenberg (1992) demonstrated that depolarization-induced influx of Ca^{2+} through L-type Ca^{2+} channels induces the release of Ca^{2+} from intracellular stores, which causes the major part of the phasic contraction. Restoration of resting membrane potential after a phasic contraction is achieved by opening of two types of Ca^{2+}-activated K^+ (K_{Ca}) channels, iberiotoxin-sensitive, large-conductance K_{Ca} (BK) channels, and apamin-sensitive, small-conductance K_{Ca} (SK) channels (pp 65). Herrera et al. (2000) determined the roles of RyRs on sarcoplasmic reticulum, BK channels, and SK channels in the regulation of phasic contractions of bladder smooth muscle. They suggested that the amplitude of a phasic contraction was dependant on the increase in Ca^{2+} entry caused by membrane depolarization, as well as Ca^{2+} released from the sarcoplasmic reticulum (SR). The later mechanism of Ca^{2+} release from internal stores was due to activation of RyRs on SR. [Ca^{2+}-induced Ca^{2+} release (CICR)]. The results of their study (Herrera et al., 2000)

indicated that RyRs, BK channels, and SK channels act by a negative-feedback mechanism through differential modulation of contractile amplitude, duration, and frequency. In their later study, Hererra and Nelson (2002) suggested that although Ca^{2+} influx through VDCCs activated both BK and SK channels, Ca^{2+}-induced Ca^{2+} release from SR activated only BK channels.

The concentration response curves to α β, methylene ATP, potassium and calcium in presence of sweeteners suggested that the possible mechanism of action was by increasing the intracellular Ca^{+2} concentration via extra cellular Ca^{+2} influx through L-type voltage gated Ca^{+2} channels, either via purinergic receptor activation or by some unknown mechanism, such as some other receptor that specifically bind the sweeteners. The chemical structure of these artificial sweeteners are very different from calcium channel openers such as Bay K 8644, it is therefore unlikely that the soft drink components act via direct modulation of Ca^{2+} channels (Bo and Burnstock, 1990).

It has been discussed earlier (pp 60), that isolated detrusor strips often generate spontaneous phasic contractions, with frequencies that are species specific, but usually of the order of tens of contractions each minute (Sibley, 1984). The contractions are small compared to the contraction that can be evoked by stimuli; they rise and fall from a low resting tone, and do not normally show the type of tetanic fusion that is seen in other electrically excitable smooth muscles. It seems clear that ion channels do play an important role in determining the properties of the spontaneous contractile activity in the urethra and detrusor, and that altering their function can have profound effects on this activity (Brading, 2006). However, there are still many areas of uncertainty. Mostwin (1986) reported that the spontaneous phasic contractions depend on Ca^{2+} influx through

the voltage-dependent Ca^{2+} channels (VDCC). As mentioned earlier in the chapter, the small-conductance Ca^{2+} - activated K^+ (SK) channels, ryanodine receptors (RyRs) and large-conductance Ca^{2+} - activated K^+ (BK) channel contribute to the negative feedback system through differential modulation of contractile amplitude, duration and frequency (Herrera et al 2000). Both acesulfame K and sodium saccharin increased the amplitude and frequency of the rat bladder smooth muscle spontaneous contractions, which may involve modulation of these K^+ channels and activation of RyRs. Further investigation into the effect of these sweeteners on spontaneous contraction of bladder smooth muscle would be required to differentiate the mechanisms involved.

The structural resemblance between acesulfame K and saccharin (Venanzi & Venanzi, 1988) could explain their similar effects on rat bladder contraction whereas aspartame, having a very different chemical structure, has had little effect on rat bladder contractile response. It can therefore be speculated that acesulfame K and saccharin are interacting with the same structure within the bladder muscle but it is unlikely that this would be 1,4 dihydropyradine calcium channels as the activators of this receptor have a very different chemical structure to acesulfame K and saccharin. This raised the possibility of specific receptors being present on the cell membrane that bind to these sweeteners, similar to the sweet taste receptors in the tongue. It is also interesting to note a recent study demonstrated that activation of mammalian sweet taste receptors (T1R2/T1R3) is highly selective and aspartame was one of the sweeteners which did not produce a response in rodents (Nelson et al, 2001).

The results of Western blot experiments demonstrated the detection of a single protein species in both tongue and bladder tissue extracts using two separate monoclonal

antibodies against the T1R2 receptor protein. The size of protein detected with each antibody was smaller than the predicted size of the whole protein, but the combined weights approximated to 95 kDa. It has previously been discussed why it is reasonable to conclude that the protein species detected represent fragments of the intact sweet taste receptor molecule (pp 240). Fragmentation of the receptor adjacent to the extracellular membrane surface produced a larger fragment detected with the N-terminal specific antibody which was absent from the membrane bound fraction, and a smaller fragment detected with the C-terminal specific antibody which was present in the membrane fraction.

Further work is required to confirm that this is the case. Repeating the Western blot analysis whilst taking particular care during homogenisation, plus the addition of a non-reducing electrophoresis process would be required and should reveal a single large protein band of the same size with each antibody. It would then be necessary to further evaluate the presence and expression of the T1R2 protein using immunocytochemistry techniques to define the localisation of the expressed tissue within the bladder, and reverse transcriptase PCR to evaluate the presence of specific messenger RNA demonstrating transcription of the *Tas1r2* gene.

It is important to remember that humans and rodents exhibit some notable differences in their ability to detect certain artificial sweeteners and intensely sweet proteins. Nelson et al. (2001) demonstrated that the species-specific selectivity was due to the difference in sequencing of amino acids. High level of T1R receptor variability underlies the difference in sweet perception and this variability is true for other candidate chemoreceptors (Adler et al., 2000). This diversity can be expressed functionally in

different species via different transduction pathways. Once the presence of T1R2 is confirmed, the sequence of this bladder specific protein must be determined as a first step to elucidating the functional role of this protein in terms of signalling within the bladder.

The preservatives and antioxidant citric acid and ascorbic acid, both enhanced the atropine resistant component of muscle contraction due to EFS. This enhancement of muscle contraction was similar to the enhancement observed in the concentration response curve due to purinergic receptor agonist α, β methyl ATP, in the presence of citric acid. This indicates that the enhancement of muscle contraction due to EFS in presence of citric acid is probably not by an increased neurotransmitter release, as the postsynaptic activation of purinergic receptors is equally affected. In contrast, the enhancement of the 'atropine resistant' component of muscle contraction to EFS in the presence of ascorbic acid was greater than the enhancement of the contractile response to purinergic receptor activation, suggesting that modulation of presynaptic neurotransmitter release may be playing a role in the enhancement of rat bladder muscle contraction by ascorbic acid. It is possible that nerve stimulation and receptor activation could also be influenced by other mechanisms, such as activation of presynaptic receptors that regulate neurotransmitter release.

In healthy human detrusor muscle the neuromuscular transmission is predominantly cholinergic (Sibley, 1984), although a partially atropine-resistant response is occasionally found (Sjogren et al., 1982). Cholinergic responses in the rat urinary bladder are initiated via activation of muscarinic M_3 receptors (Giglio et al., 2001). The neurotransmitter acetylcholine released from nerve terminals, causes an increase in free cytoplasmic Ca^{2+} concentration, activating the contractile machinery of the detrusor smooth muscle cells.

The two possible pathways for this rise in free cytoplasmic Ca^{2+} involve the influx of Ca^{2+} from the extracellular matrix through voltage sensitive L-type Ca^{2+} channels, or release from intracellular stores (Fry and Wu, 1998). An increased intracellular Ca^{2+} concentration in turn can also influence the release of Ca^{2+} from the stores (CICR- Ca^{2+}-induced Ca^{2+} release) (Ganitkevich, and Isenberg, 1992). Carbachol directly stimulates the muscarinic receptors on cell membranes like acetylcholine, activating release of Ca^{+2} from sarcoplasmic reticulum through the second messenger inositol 1,4,5-trisphosphate (IP_3) (Oike et al., 1998). The carbachol concentration response curve was unaffected by citric acid, so it appears that the mechanism of action of citric acid was not via direct muscarinic receptor activation. In contrast, ascorbic acid inhibited the carbachol induced bladder muscle contraction. It is unclear why this inhibitory effect was not demonstrated by the other soft drink components such as artificial sweeteners or citric acid. The inhibitory effect of ascorbic acid was particularly puzzling as it enhanced all the other agonist-induced contraction of bladder smooth muscle. The only difference between carbachol induced contractile responses and those obtained via the other agonists used in this study, is the pathway involving IP_3 induced release of Ca^{2+} from the sarcoplasmic reticulum to raise intracellular Ca^{2+} levels. The activation of muscarinic receptors stimulates the second messenger pathway by activating G-protein. Activated G-protein in turn causes activation of PLC, which leads to increased production of IP_3; activated IP_3 subsequently triggers Ca^{2+} release from the sarcoplasmic reticulum (An et al., 2002). Any of the steps in this pathway may well be modulated by ascorbic acid to reduce contractile responses to carbachol. Further investigation of this hypothesis would be required.

Citric acid and ascorbic acid significantly enhanced the maximal contractile response to potassium, suggesting an increased Ca^{2+} influx through L-type voltage gated Ca^{2+} channels, which is independent of muscarinic or purinergic receptor activation (Maggi et al., 1989a; Visser and van Mastrigt, 2000). This mechanism was further confirmed by investigating the effect of ascorbic acid and citric acid on the contractile response of rat bladder muscle to calcium 0.5–2.5 mM. It appears that an increased Ca^{2+} influx through L-type voltage gated Ca^{2+} channels is a possible mechanism of action for the enhancement of bladder contraction by citric acid and ascorbic acid.

The results of the experiments with carbonated soft drink as a whole were found to be very similar to that of the findings with ascorbic acid. The enhancement of contractile response to the atropine resistant component of muscle contraction to EFS was found to be greater than the enhancement of the response to purinergic receptor activation. Similar to ascorbic acid, carbonated soft drink as a whole, was found to inhibit the carbachol induced muscle contraction, indicating possible modulation of any of the steps leading to calcium release from sarcoplasmic reticulum via IP_3. The stimulatory effects of whole carbonated soft drink on muscle contraction to potassium and calcium, suggested that increased Ca^{2+} influx was the probable mode of action.

All the individual components and the carbonated soft drinks as a whole slightly enhanced nerve mediated contraction in the absence and presence of atropine. The cholinergic component of nerve stimulation by EFS was not investigated in isolation by blocking the purinergic pathway with prolonged exposure to-α, β methylene ATP (Hoyle and Burnstock, 1985). It is therefore not possible to say whether the enhancement of the contraction to nerve stimulation by sweeteners, and citric acid involved modulation of

presynaptic acetylcholine release. However, acetylcholine and ATP are co-transmitters in small mammal bladders therefore any effects of these agents on nerve stimulation may affect both neurotransmitters.

After the completion of this study, the findings can be summarised in the following two statements:

a. ***Individual carbonated soft drink components and the carbonated soft drink as a whole enhanced rat bladder muscle contraction in response to α, β, methylene-ATP, KCl and calcium, indicating their action on bladder smooth muscle involves increased extracellular Ca^{2+} influx.***
b. ***Ascorbic acid, an important ingredient in most soft drinks, enhanced the atropine resistant component of the detrusor muscle contraction to EFS by a greater degree than it enhanced the contractile response to post synaptic purinoceptor activation, suggesting that increased neurotransmitter release (ATP) from intrinsic nerves may play a role in the action of ascorbic acid on bladder muscle contraction.***

Although the individual soft drink components and the soft drink itself as a whole enhanced bladder contraction by a small amount compared to the control response, the concentrations of soft drink components used in the organ bath to examine their effect on the atropine resistant response to EFS; the response to α, β, methylene ATP, KCl and the response to calcium, were also small. However the concentrations of the soft drink components used in the organ bath (excluding EFS 10 Hz responses) were below the amount that would be found in body fluids after the consumption of a single can of

carbonated soft drink. It is therefore possible that high consumption of carbonated soft drinks may have a greater effect on bladder smooth contractile properties.

Caution should be employed when extrapolating data from epidemiological studies into the laboratory, and vice versa, but nevertheless, the work described here is a necessary first step in the hypothesis testing which is required after epidemiology research has generated new hypotheses. These experiments describe the effects of the soft drink components on isolated rat detrusor muscle these can not necessarily be extrapolated to human muscle *in vitro*, or indeed to the detrusor muscle *in vivo*. Testing of these hypotheses requires further work, but this data is important to raise awareness of the potential for dietary factors to influence bladder and other organ function.

This study raises concerns about whether wide use of artificial sweeteners and preservatives in daily life is safe. This might be an easily modifiable risk factor indicating a public health issue. The study also suggests that confirmation of the presence and mechanism of action of sweet taste receptors in urinary bladder may provide an opportunity for a new pathway for pharmacological intervention in overactive bladder.

This study has highlighted several areas for further research, some of which have already been outlined above. Extensive laboratory investigations including immunohistochemistry and RT-PCR will be required to confirm the sweet taste receptor hypothesis and to explore the normal function of this receptor and how it may contribute to detrusor overactivity.

The present study demonstrated that the soft drink and individual components also affected the spontaneous contractions of rat bladder smooth muscle. This may involve modulation of the K^+ channels and activation of ryanodine receptors. Further

investigation into the effect of these sweeteners on spontaneous contraction of bladder smooth muscle would be required to differentiate the mechanisms involved.

The pathway of muscarinic receptor activation to generate bladder smooth muscle contraction via an increase in IP_3 warrants further investigation because ascorbic acid and the whole carbonated soft drink inhibited this response.

Bearing in mind the differences between rat detrusor and human detrusor and particularly the differences in taste receptor activation, it is essential to extend the laboratory work to confirm or refute the effects that have been demonstrated using human bladder samples. Confirmation of these changes will then naturally lead on to experimental studies to examine the *in vivo* effects of these compounds individually and also whole soft drinks upon urinary symptoms, urodynamic findings and the contractile response of human detrusor *in vitro*.

BIBLIOGRAPHY

Abbracchio M, Burnstock G. 1994. Purinoceptors: are there families of P2X and P2Y purinoceptors? *Pharm Therap* **64**: 445-75

Abrams P, Blaivas JG, Stanton SL, Andersen JT. 1988. Standardization of terminology of lower urinary tract function. *Neurourol Urodyn* **7**: 135-140

Abrams P, Kelleher CJ, Kerr LA, Rogers RG. 2000. Overactive bladder significantly affects quality of life. *Am J Manag Care* **6**: S580-S590

Abrams P, Wein AJ. 2000. Introduction: overactive bladder and its treatments. *Urology* **55 (Suppl 5A)**: 1-2

Abrams P. 2002a. Anatomy and Physiology. In *Urodynamics*, 2nd edn., pp 14

Abrams P, Cardozo L, Fall M, Griffiths D, Rosier P, Ulmsten U, van Kerrebroeck P, Victor A, Wein A. 2002b.The standardisation of terminology of lower urinary tract function: report from the Standardisation Sub-committee of the International Continence Society. *Am J Obstet Gynecol* **187(1)**: 116-26

Abrams P. 2003. Describing bladder storage function: overactive bladder syndrome and detrusor overactivity. *Urology* **62**: 28-37

Adler E, Hoon MA, Mueller KL, Chandrashekar J, Ryba NJP, Zuker CS. 2000. A novel family of mammalian taste receptors. *Cell* **100**: 693-702

Aggazzotti G. Pesce F. Grassi D. Fantuzzi G. Righi E. De Vita D. Santacroce S. Artibani W. 2000. Prevalence of urinary incontinence among institutionalized patients: a cross-sectional epidemiologic study in a midsized city in northern Italy. *Urology* **56(2)**: 245-9

Albertson AM, Tobelmann RC, Marquart L. 1997. Estimated dietary calcium intake and food sources of adolescent females: 1980–92. *J. Adolesc. Health* **20**: 20-26

al-Hiyasat AS, Saunders WP, Sharkey SW, Smith GM. 1998. The effect of a carbonated beverage on the wear of human enamel and dental ceramics. *J Prosthodontics* **7(1)**: 2-12

Alm P, Elmer M. 1975. Adrenergic and cholinergic innervation of the rat urinary bladder. *Acta Physiol Scand* **94**: 36-45

Amato D, Maravilla A, Garcia-Contreras F, Paniagua R. 1997. Soft-drinks and health. *Revista de Investigacion Clinica* **49(5)**: 387-95

Ambache N, Zar MA. 1970. Non-cholinergic transmission by post-ganglionic motor neurones in the mammalian bladder. *J Physiol* **210(3)**: 761-83

An JY, Yun HS, Lee YP, Yang SJ, Shim JO, Jeong JH, Shin CY, Kim JH, Kim DS, Sohn UD. 2002. The intracellular pathway of the acetylcholine-induced contraction in cat detrusor muscle cells. *Br J Pharmacol* **137**: 1001-1010

Andersson KE, Holmquist F, Fovaeus M, Hedlund H, Sundler R. 1991. Muscarinic receptor stimulation of phosphoinositide hydrolysis in the human isolated urinary bladder. *J Urol* **146**: 1156-1159

Andersson KE. 1992. Clinical pharmacology of potassium channel openers. *Pharmacol Toxicol* **70**: 244-254

Andersson KE. 1993. Pharmacology of lower urinary tract smooth muscles and penile erectile tissues. *Pharmacol Rev* **45**: 253-308

Andersson KE, Persson K. 1993. The L-arginine/nitric oxide pathway and non-adrenergic, non-cholinergic relaxation of the lower urinary tract. *Gen Pharmacol Rev* **45**: 253-308

Andersson KE, Persson K. 1995. Nitric oxide synthase and the lower urinary tract: possible implications for physiology and pathophysiology. *Scand J Urol Nephrol* **29 (*Suppl* 175)**: 43-53

Andersson KE, Appell R, Awad S, Chapple C, Drutz H, Fourcroy J, Finkbeiner AE, Haab F, Wein A. 2002. Pharmacological treatment of urinary incontinence. In: *Incontinence, Second International Consultation on Incontinence*, edited by Abrams P, Khoury S, and Wein A. Plymouth, MA: Plymouth Distributors, p. 479-511

Andersson, KE. 2004. Detrusor contraction – focus on muscarinic receptors. *Scand J Urol Nephrol Suppl* **215**: 54-7

Andersson KE, Arner A. 2004. Urinary bladder contraction and relaxation: physiology and pathophysiology. *Physiological Reviews* **84(3)**: 935-86

Andersson KE, Wein AJ. 2004. Pharmacology of lower urinary tract: basis for current and future treatments of urinary incontinence. *Pharmacol Rev* **56(4)**: 581-631

Andersson, KE. 2006. Pharmacology of the bladder in in Textbook of Urology and Urogynecology, edited by Cardozo L and Statskin D, Infirma Healthcare, Vol 1, pp 158

Anger JT, Saigal CS, Litwin MS. 2006a. The prevalence of urinary incontinence among community dwelling adult women: results from the National Health and Nutrition Examination Survey. *J Urol* **175(2)**: 601-4

Anger JT, Saigal CS, Stothers L, Thom DH, Rodriguez LV, Litwin MS, Urologic Diseases of America Project. 2006b. The prevalence of urinary incontinence among community dwelling men: results from the National Health and Nutrition Examination survey. *J Urol* **176(5)**: 2103-8

Apostolidis A. Popat R. Yiangou Y. Cockayne D. Ford AP. Davis JB. Dasgupta P. Fowler CJ. Anand P. 2005. Decreased sensory receptors P2X3 and TRPV1 in suburothelial nerve fibers following intradetrusor injections of botulinum toxin for human detrusor overactivity. *J Urol* **174(3)**: 977-82

Arafat HA, Kim GS, DiSanto ME, Wein AJ, Chacko S. 2001. Heterogeneity of bladder myocytes in vitro: modulation of myosin isoform expression. *Tissue Cell* **33**: 219-232

Araki H, Watanabe H, Mishina T, Nakao M. 1983. High-risk group for benign prostatic hypertrophy. *Prostate* **4**: 253-64

Ariyasu T, Matsumoto S, Kyono F, Hanaya T, Arai S, Ikeda M, Kurimoto M. 2003. Taste receptor T1R3 is an essential molecule for the cellular recognition of the disaccharide trehalose. *In Vitro Cell Dev Biol Anim* **39**: 80-88

Armstrong B, Doll R. 1974. Bladder cancer mortality in England and Wales in relation to cigarette smoking and saccharin consumption. *Br J Prev Soc Med* **28**: 233-240

Armstrong B, Doll R. 1975. Bladder cancer mortality in diabetics in relation to saccharin consumption and smoking habits. *Br J Prev Soc Med* **29**: 73-81

Arner A, Hellstrand P. 1983. Activation of contraction and ATPase activity in intact and chemically skinned smooth muscle of rat portal vein. Dependence on Ca^{2+} and muscle length. *Circ Res* **53**: 695-702

Arner A, Mattiasson A, Radzizewski P, Uvelius B. 1998. Shortening velocity is different in longitudinal and circular muscle layers of the rabbit urethra. *Urol Res* **26**: 423-426

Arya LA, Myers DL, Jackson ND. 2000. Dietary caffeine intake and the risk for detrusor instability: A case-control study. *Obstet Gynecol* **96**: 85-89

Arya LA, Jackson ND, Myers DL, Verma A. 2001. Risk of new-onset urinary incontinence after forceps and vacuum delivery in primiparous women. *Am J Obstet Gynecol* **185**: 1318-1323

Asamoto M, Imaida K, Hasegawa R, Hotta K, Fukushima S. 1992. Change of membrane potential in rat urinary bladder epithelium treated with sodium L ascorbate. *Cancer Lett* **63**: 1-5

Ashby RA, Sleet RJ. 1992. The role of citrate complexes in preventing urolithiasis. *Clinica Chimica Acta* **210(3)**:157-65

Ashcroft SJH, Ashcroft FM. 1990. Properties and functions of ATP-sensitive K-channels. *Cell Signalling* **2**: 197-214

Athwal BS, Berkley KJ, Hussain I, Brennan A, Craggs M, Sakakibara R, Frackowiak RS, Fowler CJ. 2001. Brain responses to changes in bladder volume and urge to void in healthy men. *Brain* **124**: 369-77

Avenet P, Hofmann F, Lindemann, B. 1988. Transduction in taste receptor cells requires cAMPdependent protein kinase. *Nature* **331**: 351-354

Azadzoi KM, Pontari M, Vlachiotis J, Siroky MB. 1996. Canine bladder blood flow and oxygenation: changes induced by filling, contraction and outlet obstruction. *J Urol* **155**: 1459-1465

Babij P, Kelly C, Periasamy M. 1991. Characterization of a mammalian smooth muscle myosin heavy-chain gene: complete nucleotide and protein coding sequence

and analysis of the 5' end of the gene. *Proc Natl Acad Sci USA* **88**: 10676-10680

Babu GJ, Warshaw DM, Periasamy M. 2000. Smooth muscle myosin heavy chain isoforms and their role in muscle physiology. *Microsc Res Tech* 50: 532–540

Bachmanov AA, Reed DR, Ninomiya Y, Inoue M, Tordoff MG, Price RA, Beauchamp GK. 1997. Sucrose consumption in mice: major influence of two genetic loci affecting peripheral sensory responses. *Mamm Genome* **8**: 545-548

Badawi JK, Uecelehan H, Hatzinger M, Michel MS, Haferkamp A, Bross S. 2005. Relaxant effects of beta-adrenergic agonists on porcine and human detrusor muscle. *Acta Physiologica Scandinavica* **185(2)**:151-9

Barger-Lux MJ, Heaney RP. 1995. Caffeine and the calcium economy revisited. *Osteoporos Int* **5**: 97-102

Barstead RJ, Kleiman L, Waterston RH. 1991. Cloning, sequencing and mapping of an alpha-actinin gene from the nematode Caenorhabditis Elegans. *Cell Motil Cytoskelet* **20**: 69-78

Barrington RJ. 1925. The effect of lesions of the hind and midbrain on micturition in the cat. *Q J Exp Physiol* **15**: 81-102

Barzel US. 1995. The skeleton as an ion exchange system: implications for the role of acid-base imbalance in the genesis of osteoporosis. *J Bone Miner Res* **10**: 1431-6

Barzel US, Massey LK. 1998. Excess dietary protein can adversely affect bone. *J Nutr* **128(6)**: 1051-53

Baskin L, DiSandro M, Li Y. 2001. Mesenchymal-epithelial interactions in bladder smooth muscle development: effects of the local tissue environment. *J Urol* **165(4)**: 1283-8

Basu NM, Biswas P. 1940. The influence of ascorbic acid on contractions and the incidence of fatigue of different types of muscles. *Indian J Med Res* **28**: 405-417

Bates CP, Whiteside CG, Turner-Warwick R. 1970. Synchronous cine-pressure-flow-cysto-urethrography with special reference to stress and urge incontinence. *Br J Urol* **42:** 714-723

Bates P, Bradley WE, Glen E, Griffiths D, Melchior H, Rowan D, Sterling A, Zinner N, Hald T. 1979. The standardization of terminology of lower urinary tract function. *J Urol* **121**: 551-554

Bayliss M, Wu C, Newgreen D, Mundy AR, Fry CH. 1999. Quantitative study of atropine-resistant contractile responses in human detrusor smooth muscle, from stable, unstable and obstructed bladders. *J Urol* **162**:1833-1839

Beggs AH, Byers TJ, Knoll JHM, Boyce FM, Bruns GAP, Kunkel LM. 1992. Cloning and characterization of two human skeletal muscle alpha-actinin genes located on chromosomes 1 and 11. J. *Biol. Chem* **267**: 9281-9288

Bender DA. 2003. Vitamin C (Ascorbic acid) In: *Nutritional Biochemistry of the Vitamins*, 2nd edition, by Cambridge University Press. pp 357

Bendich A, Machlin LJ, Scandurra O. 1986. The antioxidant role of vitamin C. *Adv Free Radic Biol Med* **2**: 419-444

Bennet BC, Vizzard MA, Booth AM. 1993. Role of nitric oxide in reflex urethral sphincter relaxation during micturition. *Soc Neurosci Abstr* **19**: 511-512

Benowitz NL, Osterloh J, Goldschlager N, Kaysen G, Pond S, Forhan S. 1982. Massive catecholamine release from caffeine poisoning. *JAMA* **248**: 1097–8

Bergsten P, Moura AS, Atwater I, Levine M. 1994. Ascorbic acid and insulin secretion in pancreatic islets. *J Biol Chem* **269**:1041-1045

Berkow S G. 1953. The corpus spongiosum of the urethra: its possible rolein urinary control and stress incontinence in women. *Am J Obstet Gynecol* **65**: 346-51

Berne RM, Levy MN. 2003. Principles of Physiology. 2nd Edn. Part 3. Chapter 14: 200

Bernhardt SJ, Naim M, Zehavi U, Lindemann B. 1996. Changes in IP3 and cytosolic Ca^{2+} in response to sugars and non sugar sweeteners in transduction of sweet taste in the rat. *J Physiol* **490**: 325-336

Berridge MJ, Bootman MD, Roderick HL. 2003. Calcium signalling: dynamics,

homeostasis and remodelling. *Nat Rev Mol Cell Biol* **4**: 517-529

Berrocal T, Lopez-Pereira P, Arjonilla A, Gutierrez J. 2002. Anomalies of the distal ureter, bladder, and urethra in children: embryologic, radiologic, and pathologic features. *Radiographics* **22(5)**: 1139-64

Bing W, Chang S, Hypolite JA, DiSanto ME, Zderic SA, Rolf L, Wein AJ, Chacko S. 2003. Obstruction-induced changes in urinary bladder smooth muscle contractility: a role for Rho-kinase. *Am J Physiol Renal Physiol* **285**: F990-F997

Birder LA, Nealen ML, Kiss S, de Groat WC, Caterina MJ, Wang E, Apodaca G, Kanai AJ. 2002. Beta-adrenoceptor agonists stimulate endothelial nitric oxide synthase in rat urinary bladder urothelial cells. *J Neurosci* **22(18)**: 8063-70

Bizri JN, Wahem IA. 1994. Citric Acid and Antimicrobials Affect Microbiological Stability and Quality of Tomato Juice. *J Food Sci* **59 (1)**: 130

Black JW, Leff P. 1983. **Operational models of pharmacological agonism.** *Proc R Soc Lond* **220(1219)**: 141-62

Blanchard A, Ohanian V, Critchley D. 1989. The structure and function of alpha-actinin. *J Muscle Res Cell Motil* **10**: 280-289

Blok BF, Sturms LM, Holstege G. 1998. Brain activation during micturition in women. *Brain* **121**: 2033-42

Blok BF, Holstege G. 1999. The central control of micturition and continence: implications for urology. *BJU Int* **83(2)**: 1-6

Blot WJ, Li JY, Taylor PR, Guo W, Dawsey S, Wang GQ, Yang CS, Zheng SF, Gail M, Li GY. 1993. Nutrition intervention trials in Linxian, China: supplementation with specific vitamin/mineral combinations, cancer incidence, and disease-specific mortality in the general population. *J Natl Cancer Inst* **185**: 1483-92

Blundell JE, Hill AJ. 1986. Paradoxical effects of an intense sweetener (aspartame) on appetite. *Lancet* **l**: 1092-3

Bolton TB, Prestwic SA, Zholos AV, Gordienko DV. 1999. Excitation-contraction coupling in gastrointestinal and other smooth muscles. *Annu Rev Physiol* **61**: 85-115

Bo XN, Burnstock G. 1989. [3H] –alpha, beta-methylene ATP, a radioligand labelling P2-purinoceptors. *J Auton Nerv Syst* **28**: 85-88

Bo XN, Burnstock G. 1990. The effects of Bay K 8644 and nifedipine on the responses of rat urinary bladder to electrical field stimulation, α, β methylene ATP and acetylcholine. *Br J Pharmacol* **101**: 494-498

Bo XN, Burnstock G. 1993. Triphosphate, the key structure of the ATP molecule responsible for the interaction with P2X-purinoceptors. *Gen Pharmacol* **24(3)**: 637-40

Bo K, Borgen JS. 2001. Prevalence of stress and urge urinary incontinence in elite athletes and controls. *Med Sci Sports Exerc* **33**: 1797-1802

Boos K, Hextall A, Cardozo L, Toozs-Hobson P, Anders K, Treasure J. 1999. Lower urinary tract symptoms and their impact on women with anorexia nervosa. *Br J Obstets Gynaecol* **106(5)**: 501-4

Bonev AD, Nelson MT. 1993. ATP-sensitive potassium channels in smooth muscle cells from guinea pig urinary bladder. *Am J Physiol Cell Physiol* **264**: C1190-C1200

Bonner TI, Buckley NJ, Young AC, Brann MR. 1987. Identification of a family of muscarinic acetylcholine receptor genes. *Science* **237**: 527-532

Bonner TI. 1989. The molecular basis of muscarinic receptor diversity. *Trends Neurosci* **12**: 148-51

Bond M, Somlyo AV. 1982. Dense bodies and actin polarity in vertebrate smooth muscle. *J Cell Biol* **95**: 403-413

Bouchelouche K, Andersen L, Alverez S. 2005. Increased contractile response to phenylephrine in detrusor of patients with bladder outlet obstruction: effect of the alpha 1A and alpha 1D- adrenergic receptor antagonist tamsulosin. *J Urol* **173(2)**: 657-61

Bozler E. 1941. Action potentials and conduction of excitation in muscle. *Biol Symp* **3**: 95-109

Bozler E. 1969. Role of calcium in initiation of activity of smooth muscle. *Am J Physiol* **216**: 671-674

Brading AF, Sibley GNA. 1983. A superfusion apparatus to study field stimulation of smooth muscle from mammalian urinary bladder. *J Physiol* **334**: 11-13

Brading AF. 1987. Physiology of urinary tract smooth muscle. In: Webster G, Kirby R, King L, Goldwasser B (eds) Reconstructive Urology. Boston: Blackwell Scientific, 15-26

Brading AF, Mostwin JL. 1989. Electrical and mechanical responses of guinea-pig bladder muscle to nerve stimulation. *Br J Pharmacol* **98**: 1083-1090

Brading AF, Williams JH. 1990. Contractile responses of smooth muscle strips from rat and guinea-pig urinary bladder to transmural stimulation: effects of atropine and alpha, beta-methylene ATP. *Br J Pharmacol* **99**: 493-498

Brading AF, Inoue R. 1991. Ion channels and excitatory transmission in the smooth muscle of the urinary bladder. *Zeitschrift fur Kardiologie* **80 Suppl 7**: 47-53

Brading AF. 1992. Ion channels and control of contractile activity in urinary bladder smooth muscle. *Jpn J Pharmacol* **58 Suppl 2**: 120–127

Brading AF. 2002. The sarcoplasmic reticulum in disease and smooth muscle dysfunction: therapeutic potential. *Novartis Found Symp* **246**: 244-254

Brading AF. 2006. Spontaneous activity of the lower urinary tract smooth muscles: correlation between ion channels and tissue function. *J. Physiol* **570**: 13-22

Brady CM, Apostolidis A, Harper M, Yiangou Y, Beckett A, Jacques TS. 2004. Parallel changes in bladder suburothelial vanilloid receptor TRPV1 and pan-neuronal marker PGP9.5 immunoreactivity in patients with neurogenic detrusor overactivity following intravesical resiniferatoxin treatment. *BJU Int* **93:** 770-776

Bramich NJ, Brading AF. 1996. Electrical properties of smooth muscle in the guinea-pig urinary bladder. *J Physiol* **492**: 185-198

Branen AL, Davidson PM, Salminen S. 1990. *Food additives.* New York: Marcel Dekker, Inc.

Braverman AS, Luthin GR, Ruggieri MR. 1998. M2 muscarinic receptor contributes to contraction of the denervated rat bladder. *Am J Physiol* **275**: 1654-60

Braverman AS, Ruggieri MR. 2003. Hypertrophy changes the muscarinic receptor subtype mediating bladder contraction from M_3 toward M_2. *Am J Physiol Regul Integr Comp Physiol* **285(3)**: 701-708

Bray GA, Nielsen SJ, Popkin BM. 2004. Consumption of high-fructose corn syrup in beverages may play a role in the epidemic of obesity. *Am J Clin Nutr* **79**: 537-43

Bridgewater M, Brading AF. 1993. Evidence for a non-nitrergic inhibitory innervation in the pig urethra. *Neurol Urodyn* **12**: 357-8

Bright G. 1999. Low-calorie sweeteners-from molecules to mass markets. *World Rev Nutr Diet* **85**: 3-9

Brink PR. 1998. Gap junctions in vascular smooth muscle. *Acta Physiol Scand* **164**: 349-356

Brown JS, Vittinghoff E, Wyman JF, Stane KL, Nevitt MC, Eusrud KE, Grady D. 2000. Urinary incontinence: does it increase risk of falls and fractures? *J Am Geriatr Soc* **48**: 721-725

Brown JS, Nyberg LM, Kusek JW, Burgio KL, Diokno AC, Foldspang A, Fultz NH, Herzog AR, Hunskaar S, Milsom I, Nygaard I, Subak LL, Thom DH. 2003. National Institute of Diabetes and Digestive Kidney Diseases International Research Working Group on Bladder Dysfunction. Proceedings of the National Institute of Diabetes and Digestive and Kidney Diseases International Symposium on Epidemiologic Issues in Urinary Incontinence in Women. *Am J Obstet Gynecol* **188(6)**: 77-88

Bryant CM, Dowell CJ, Fairbrother G. 2002. Caffeine reduction education to improve urinary symptoms. *B J Nurs* **11(8)**: 560-565

Buchsbaum GM, Chin M, Glantz C, Guzick D. 2002. Prevalence of urinary incontinence and associated risk factors in a cohort of nuns. *Obstet Gynecol* **100(2)**: 226-9

Bump RC, McClish DK. 1992. Cigarette smoking and urinary incontinence in women. *Am J Obstet Gynecol* **167**: 1213-1218

Bump RC. 1993. Racial comparisons and contrasts in urinary incontinence and pelvic organ prolapse. *Obstet Gynecol* **81**: 421-5

Bump RC, Norton PA. 1998. Epidemiology and natural history of pelvic floor dysfunction. *Obstet Gynecol Clin North Am* **25**: 723-746

Bunker JP, Bendixen HH, Murphy AJ. 1962. Hemodynamic effects of intravenously administered sodium citrate. *N Engl J Med* **266**: 372-377

Burdock GA. 1999. Fenaroli's Handbook of Flavour Ingredients. Vol 1 ad II. 3rd Edition. CRC Press Boca Roton, FL

Burgio K, Ouslander JG. 1999. Effects of urge urinary incontinence on quality of life in older people. *J Am Geriatr Soc* **47**: 1032-1033

Burnette WN. 1981. Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem* **112(2)**: 195-203

Burnett A. 1995. Nitric oxide control of lower genitourinary tract functions: a review. *Urology* **45**: 1071-1083

Burns JJ. 1975. Metabolism of ascorbic acid. Introduction: overview of ascorbic acid metabolism. *Ann NY Acad Sci* **258**: 5-6

Burnstock G. Dumsday B. Smythe A. 1972. Atropine resistant excitation of the urinary bladder: the possibility of transmission via nerves releasing a purine nucleotide. *B J Pharmacol* **44(3)**: 451-61

Burnstock G. 1978. A basis for distinguishing two types of purinergic receptor. In Straub RW, Bolis L, eds, *Cell Membrane Receptors for Drugs and Hormones; a Multidisciplinary Approach* New York: Raven Press, 107-18

Burnstock G, Williams M. 2000. P2 purinergic receptors: modulation of cell function and therapeutic potential. *J Pharmacol Exp Ther* **295**: 862-869

Burnstock G. 2001. Purinergic signalling in lower urinary tract. In: Purinergic and Pyrimidinergic Signalling. I. Molecular, Nervous and Urogenitary System Function, edited by Abbracchio MP and Williams M. Berlin: Springer. Verlag, 423-515

Butchko HH, Stargel WW. 2001. Aspartame: scientific evaluation in the postmarketing period. *Reg Toxic Pharma* **34**: 221-233

Byer K, Khan SR. 2005. Citrate provides protection against oxalate and calcium oxalate crystal induced oxidative damage to renal epithelium. *J. Urol* **173(2)**: 640-6

Calvert RC, Thompson CS, Khan MA, Mikhailidis DP, Morgan RJ, Burnstock G. 2001. Alterations in cholinergic and purinergic signaling in a model of the obstructed bladder. *J Urol* **166**: 1530-1533

Calvert RC, Mumtaz FH, Dashwood MR, Khan MA, Morgan RJ, Mikhailidis D P, Thompson CS. 2002. Reduction of endothelin-1 binding and inhibition of endothelin-1-mediated detrusor contraction by naftidrofuryl. *Clin Sci* **103(48)**: 459S-463S

Canty DJ, Chan MM. 1991. Effects of consumption of caloric vs noncaloric sweet drinks on indices of hunger and food consumption in normal adults. *Am J Clin Nutr* **53(5)**: 1159-64

Cartwright RA, Adib R, Glashan R, Gray BK. 1981. The epidemiology of bladder cancer in West Yorkshire. A preliminary report on non-occupational aetiologies. *Carcinogenesis* **2**: 343-347

Castro D, Espuna M, Prieto M, Badia X. 2005. Prevalence of overactive bladder in Spain: a population-based study. *Arch Esp Urol* **58(2)**: 131-8

Caulfield MP, Birdsall NJM. 1998. International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol Rev* **50**: 279-290

Cavaille F, Janmot C, Ropert S, d'Albis A. 1986. Isoforms of myosin and actin in human, monkey and rat myometrium. Comparison of pregnant and non-pregnant uterus proteins. *Eur J Biochem* **160**: 507-513

Chacko S, Longhurst PA. 1994. Regulation of actomyosin and contraction in smooth muscle. *World J Urol* **12**: 292-7

Chalovich JM, Cornelius P, Benson CE. 1987. Caldesmon inhibits skeletal actomyosin subfragment-1 ATPase activity and the binding of myosin subfragment-1 to actin. *J Biol Chem* **262**: 5711-5716

Chalmers AH, Cowley DM, Brown JM. 1986. A possible etiological role for ascorbate in calculi formation. *Clin Chem* **32(2)**: 333-6

Chambers P, Neal DE, Gillespie JI. 1997. Ca^{2+} mobilization in cultured human bladder smooth muscle cells in response to hypotonic stimuli. *Exp Physiol* **82**: 677-686

Chancellor MB, Kaplan SA, Blaivas JG. 1992. The cholinergic and purinergic components of detrusor contractility in a whole rabbit bladder model. *J Urol* **148**: 906-909

Chandrashekar J, Mueller KL, Hoon MA, Adler E, Feng L, Guo W, Zuker CS, Ryba NJP. 2000. T2Rs function as bitter taste receptors. *Cell* **100**: 703-711

Chapple CR, Milner P, Moss HE, Burnstock G. 1992. Loss of sensory neuropeptides in the obstructed human bladder. *Br J Urol* **70**: 373-381

Chapple CR, Yamanishi T, Chess-Williams R. 2002. Muscarinic receptor subtypes and management of the overactive bladder. *Urology* **60(5 Suppl 1)**: 82-8

Chapple CR, Rechberger T, Al-Shukri S Meffan P. Everaert K, Huang M, Ridder A, YM-905 Study Group. 2004. Randomized, double-blind placebo- and tolterodine- controlled trial of the once-daily antimuscarinic agent solifenacin in patients with symptomatic overactive bladder. *BJU Int* **93(3)**: 303-310

Chapple CR, Patroneva A, Raines SR. 2006. Effect of an ATP-sensitive potassium channel opener in subjects with overactive bladder: a randomized, double-blind, placebo-controlled study (ZD0947IL/0004). *Eur Urol* **49(5)**: 879-86

Chaudhari N, Roper SD. 1998. Molecular and physiological evidence for glutamate (umami) taste transduction via a G protein-coupled receptor. *Ann NY Acad Sci* **855**: 398-406

Chess-Williams R, Chapple CR, Yamanishi T, Yasuda K, Sellers DJ. 2001. The minor population of M3-receptors mediate contraction of human detrusor muscle in vitro. *J Auton Pharmacol* **21**: 243-8

Chess-Williams R. 2002. Muscarinic receptors of the urinary bladder: detrusor, urothelial and prejunctional. *Auton Autacoid Pharmacol* **22**: 133-145

Chopra A, Morrison L. 1995. Resolution of caffeine-induced complex dysrhythmia with procainamide therapy. *J Emerg Med* **13**: 113-7

Chou TM, Benowitz NL. 1994. Caffeine and coffee: effects on health and cardiovascular disease. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* **109**: 173-89

Chyou P, Nomura A. 1993. A prospective study of alcohol, diet, and other lifestyle factors in relation to obstructive uropathy. *Prostate* **22**: 253-64

Cockayne DA, Hamilton SG, Zhu QM, Dunn PM, Zhong Y, Novakovic S. 2000. Urinary bladder hyporeflexia and reduced pain-related behaviour in P2X3-deficient mice. *Nature* **407**: 1011-5

Cohen DM, Murphy RA. 1979. Cellular thin filament protein contents and force generation in porcine arteries and veins. *Circ Res* **45**: 661-665

Cohen P. 1989. The structure and regulation of protein phosphatases. *Annu Rev Biochem* **58**: 453-508

Cohen SM. Lawson TA. 1995. Rodent bladder tumors do not always predict for humans. *Cancer Lett* **93(1)**: 9-16

Cohen SM, Anderson TA, de Oliveira LM, Arnold LL. 1998.Tumorigenicity of sodium ascorbate in male rats. *Cancer Res* **58**: 2557-2561

Cole P. 1971. Coffee drinking and cancer of the lower urinary tract. *Lancet* **1**: 1335-1337

Collier ML, Ji G, Wang Y, Kotlikoff MI. 2000. Calcium-induced calcium release in smooth muscle: loose coupling between the action potential and calcium release. *J Gen Physiol* **115(5)**: 653-62

COMA. 1991. Dietary Reference Values for Food Energy and Nutrients for the United Kingdom. Report of the Panel on Dietary Reference Values, Committee on Medical Aspects of Food and Nutrition Policy. HMSO, London. (Ref. URL 9)

Cooke R, Pate E. 1985. The effects of ADP and phosphate on the contraction of muscle fibers. *Biophys J* **48**: 789-798

Corcos J, Schick E. 2004. Prevalence of overactive bladder and incontinence in Canada. *Can J Urol* **11(3)**: 2278-84

Corna LM, Cairney J. 2005. The role of social support in the relationship between urinary incontinence and psychological distress in older adults. *Can J Aging* **24(3)**: 285-94

Coulson FR, Jacoby DB, Fryer AD. 2004. Insulin regulates neuronal M2 muscarinic receptor function in the ileum of diabetic rats. *J Pharmacol Exp Ther* **308(2)**: 760-6

Cowan WD, Daniel EE. 1983. Human female bladder and its noncholinergic contractile function. *Can J Physiol Pharmacol* **61**: 1236-1246

Coyne KS, Zhou Z, Bhattacharyya SK, Thompson CL, Dhawan R. Versi E. 2003. The prevalence of nocturia and its effect on health-related quality of life and sleep in a community sample in the USA. *BJU Int* **92**: 948-954

Coyne KS, Margolis MK, Brewster-Jordan J, Sutherland SE, Bavendam T, Rogers RG. 2007. Evaluating the impact of overactive bladder on sexual health in women: what is relevant? *J Sexual Medicine.* **4(1)**: 124-36

Creed KE. 1971. Membrane properties of the smooth muscle membrane of the guinea-pig urinary bladder. *Pflügers Arch* **326**: 115-126

Creed KE, Ishikawa S, Ito Y. 1983. Electrical and mechanical activity recorded from rabbit urinary bladder in response to nerve stimulation. *J Physiol* **338**: 149-164

Creed KE, Callahan SM. 1989. Prostaglandins and neurotransmission at the guinea pig and rabbit urinary bladder. *Pflügers Arch* **413**: 299-302

Creed KE, Malmgren A. 1993. The effect of cromakalim on the electrical properties of and ($^{86}Rb^{+}$) efflux from normal and hypertrophied rat bladder. *Clin Exp Pharmacol Physiol* **20**: 215-221

Creighton SM, Stanton SL. 1990. Caffeine: does it affect your bladder? *B J Urol* **66(6)**: 613-4

Crowe R, Moss H, Chapple CR, Light JK, Burnstock G. 1991. Patients with lower motor spinal cord lesions: a decrease of vasoactive intestinal polypeptide, calcitonin gene-related peptide and substance P but not neuropeptide Y and somatostatin-immunoreactive nerves in the detrusor muscle of the bladder. *J. Urol* **145**: 600-604

Cullen KW, Eagan J, Baranowski T, Owens E, deMoor C. 2000. Effects of a la carte and snack bar foods at school on children's lunchtime intake of fruits and vegetables. *J Am Diet Assoc* **100**: 1482-1486

Cummings TA, Daniels C, Kinnamon SC. 1996. Sweet taste transduction in hamster: sweeteners and cyclic nucleotides depolarize taste cells by reducing a K^+ current. *J Neurophysiol* **75**: 1256-1263

Cunningham MA, Marshall TA. 2003. Effectiveness of carbonated beverage education on dental students' knowledge and behavioral intent. *J Dent Educ* **67(9)**: 1011-5

Cuomo R, Grasso R, Sarnelli G, Capuano G, Nicolai E, Nardone G, Pomponi D, Budillon G, Ierardi E. 2002. Effects of carbonated water on functional dyspepsia and constipation. *Eur J Gastroenterol Hepatol* **14(9)**: 991-999

D'Agostino G, Kilbinger H, Chiari MC, Garna E. 1986. Presynaptic inhibitory muscarinic receptors modulating ['H]acetylcholine release in the rat urinary bladder. *J Pharrnacol Exp Ther* **239**: 522-528

D'Agostino G, Bolognesi ML, Lucchelli A, Vicini D, Balestra B, Spelta V, Melchiorre C, Tonini M. 2000. Prejunctional muscarinic inhibitory control of acetylcholine release in the human isolated detrusor: involvement of the M_4 receptor subtype. *Br J Pharmacol* **129(3)**: 493-500

Dallosso HM, McGrother CW, Matthews RJ, Donaldson MMK. 2003a. The association of diet and other lifestyle factors with overactive bladder and stress incontinence: a longitudinal study in women. *BJU Int* **92**: 69-77

Dallosso HM, Matthew RJ, McGrother CW, Clarke M, Perry SI, Shaw C, Jagger C, Leicestershire MRC Incontinence Study Team. 2003b. An investigation into non-response bias in a postal survey on urinary symptoms. *BJU Int* **91**: 631-6

Dallosso HM, Matthews RJ, McGrother CW, Donaldson MMK. 2004a. Diet as a risk factor for the development of stress urinary incontinence: a longitudinal study in women. *Eur J Clin Nutr* **58(6)**: 920-6

Dallosso HM, Matthews RJ, McGrother CW, Donaldson MMK, Shaw C, Leicestershire MRC Incontinence Study Group. 2004b. The association of diet and other lifestyle factors with the onset of overactive bladder: a longitudinal study in men. *Public Health Nutr* **7(7)**: 885-91

Damak S, Rong M, Yasumatsu K, Kokrashvili Z, Varadarajan V, Zou S, Jiang P, Ninomiya Y, Margolskee RF. 2003. Detection of sweet and umami taste in the absence of taste receptor T1r3. *Science* **301**: 850-853

Damaser MS, Haugaard N, Uvelius B. 1997. Partial obstruction of the rat urinary bladder: effects on mitochondria and mitochondrial glucose metabolism in detrusor smooth muscle cells. *Neurourol Urodyn* **16**: 601-607

Damaser MS. 1999. Whole bladder mechanics during filling. *Scand J Urol Nephrol Suppl* **201**: 51-58

Damaser MS, Whitbeck C, Barreto M, Horan P, Benno H, O'Connor LJ, Levin RM. 2000. Comparative physiology and biochemistry of rat and rabbit urinary bladder. *BJU Int* **85(4)**: 519-25

Danforth KN, Townsend MK, Lifford K, Curhan GC, Resnick NM, Grodstein F. 2006. Risk factors for urinary incontinence among middle-aged women. *Am J Obstet Gynecol* **194(2)**: 339-45

Danforth KN, Shah AD, Townsend MK, Lifford KL, Curhan GC, Resnick NM, Grodstein F. 2007. Physical activity and urinary incontinence among healthy, older women. *Obstet Gynecol* **109(3)**: 721-7

Daniel EEL, Cowan W, Daniel VP. 1983. Structural bases for neural and myogenic control of human detrusor muscle. *Can J Physiol Pharmacol* **61**: 1247-1273

Danuser H, Thor KB. 1995. Inhibition of central sympathetic and somatic outflow to the lower urinary tract of the cat by the alpha 1 adrenergic receptor antagonist prazosin. J Urol **153(4)**: 1308-12

Danuser H, Thor KB. 1996. Spinal 5-HT2 receptor-mediated facilitation of pudendal nerve reflexes in the anaesthetized cat. *B J Pharmacol* **118(1)**: 150-4

Davies MB, Austin J, Partridge DA. 1991. Discovery and structure of vitamin C in Vitamin C: Its Chemistry and Biochemistry, Royal Society of Chemistry. pp 26-36

Davies AM, Batchelor TJ, Eardley I, Beech DJ. 2002. Potassium channel KV alpha1 subunit expression and function in human detrusor muscle. *J Urol* **167**: 1881-1886

Debruyne FM, Heesakkers JP. 2004. Clinical and socioeconomic relevance of overactive bladder. *Urology* **63**: 42-44

De Groat WC, Saum WR. 1972. Sympathetic inhibition of the urinary bladder and of pelvic ganglionic transmission in the cat. *J Physiol* **220**: 297-314

De Groat WC, Booth AM. 1980. Inhibition and facilitation in parasympathetic ganglia of the urinary bladder. *Federation Proceedings* **39(12)**: 2990-6

De Groat WC. 1990. Central neural control of the lower urinary tract. *Ciba Foundation Symposium* **151**: 27-44

De Groat WC, Booth AM, Yoshimura N. 1993. Neurophysiology of micturition and its modification in animal models of human disease. In Maggi, C.A. (ed) Nervous control of the urogenital system. London: Harwood, 227-90

De Groat WC, Yoshimura N. 2001. Pharmacology of the lower urinary tract. *Annu Rev Pharmacol Toxicol* **41**: 691-721

De Groat WC. 2006. Integrative control of the lower urinary tract: preclinical perspective. *B J Pharmacol* **147 Suppl 2**: S25-40

De Lancey JOL. 2006. Anatomy in Textbook of Urology and Urogynecology, edited by Cardozo L and Statskin D, Infirma Healthcare, Vol 1, chapter 7, pp 116

De Tayrac R, Chevalier N, Chauveaud-Lambling A, Gervaise A, Fernandez H. 2004. Risk of urge and stress urinary incontinence at long-term follow-up after vaginal hysterectomy. *Am J Obstet Gynecol* **191**: 90-94

Diamant H, Oakley B, Stroem L, Wells C, Zotterman Y. 1965. A comparison of neural and psychophysical responses to taste stimuli in man. *Acta Physiol Scand* **64**: 67-74

Dickinson R L. 1989. Studies of the levator ani muscle. *Am J Dis Women* **22**: 897-917.

Dillon PF, Root-Bernstein RS, Sears PR, Olson LK. 2000. Natural electrophoresis of norepinephrine and ascorbic acid. *Biophys J* **79**: 370-376

Dillon PF, Root-Bernstein RS, Leider CM. 2004. Antioxidant-independent ascorbate enhancement of catecholamine-induced contractions of vascular smooth muscle. *Am J Physiol Heart Circ Physiol* **286**: H2353-H2360

Diokno AC, Brock BM, Brown MB, Herzog AR. 1986. Prevalence of urinary incontinence and other urological symptoms in the noninstitutionalized elderly. *J Urol* **136**: 1022-25

Dionex Corporation. Sunnyvale, CA Rapid Determination of Phosphate and Citrate in Carbonated Soft Drinks Using a Reagent-Free™ Ion Chromatography System, Application Note 169

Di Stefano M, Caserta D, Marci R, Moscarini M. 2000. Urinary incontinence in pregnancy and prevention of perineal complications of labor. Minerva Ginecologica. **52(7-8)**: 307-11

Dixon JS, Gosling JA. 1983. Histology and fine structure of the muscularis mucosae of the human urinary bladder. *J Anat* **136**: 265-271

Dixon JS, Gosling JA. 1987. Structure and innervation in the human. In: *The Physiology of the Lower Urinary Tract*, edited by Torrens M and Morrison JFB. Berlin: Springer-Verlag, p. 3-33

Dixon JS, Gosling JA. 1990. Ultrastructure of smooth muscle cells in the urinary system. In: *Ultrastructure of Smooth Muscle*, edited by Motta PM. London: Kluwer Academic, p. 153–169

Dixson JD, Forstner MJ, Garcia DM. 2003. The α-actinin gene family: A revised classification. *J. Mol. Evol* **56**: 1-10

Dollery C. 1991. *Therapeutic Drugs,* Vol 1, Churchill Livingstone, London, A181-A185

Donoso MV, Salas C, Sepulveda G, Lewin J, Fournier A, Huidobro-Toro JP. 1994. Involvement of ET_A receptors in the facilitation by endothelin-1 of non-adrenergic non-cholinergic transmission in the rat urinary bladder. *Br J Pharmacol* **111**: 473-482

Downie JW, Dean DM. 1977. The contribution of cholinergic postganglionic neurotransmission to contractions of rabbit detrusor. *J Pharmacol Exp Ther* **203(2)**: 417-25

Drake MJ, Hedlund P, Andersson KE, Brading AF, Hussain I, Fowler C, Landon DN. 2003a. Morphology, phenotype and ultrastructure of fibroblastic cells from normal and neuropathic human detrusor: absence of myofibroblast characteristics. *J Urol* **169**: 1573-1576

Drake MJ, Harvey IJ, Gillespie JI. 2003b. Autonomous activity in the isolated guinea pig bladder. *Exp Physiol* **88**: 19-30

Dubreuil RR. 1991. Structure and evolution of the actin crosslinking proteins. *Bioessays* **13**: 219-226

Dubyak GR, El Moatassim C. 1993. Signal transduction via P_2-purinergic receptors for extracellular ATP and other nucleotides. *Am J Physiol* **265**: C577-C606

Dudley EC. 1905. The expansion of gynecology and a suggestion for the surgical treatment of incontinence in women. *Transactions Am Gynecol Soc* **30**: 3

Dugan E, Cohen SJ, Bland DR, Preisser JS, Davis CC, Suggs PK, McGann P. 2000. The association of depressive symptoms and urinary incontinence among older adults. *J Am Geriatr Soc* **48**: 413-416

Dulac C. 2000. The physiology of taste, vintage 2000. *Cell* **100**: 607-610

Duong TH, Korn AP. 2001. A comparison of urinary incontinence among African American, Asian, Hispanic, and white women. *Am J Obstet Gynecol* **184(6)**:1083-6

Dyer J, Salmon KS, Zibrik L, Shirazi-Beechey SP. 2005. Expression of sweet taste receptors of the T1R family in the intestinal tract and enteroendocrine cells. *Biochem Soc Trans* **33(Pt 1)**: 302-5

Eddinger TJ, Murphy RA. 1988. Two smooth muscle myosin heavy chains differ in their light meromyosin fragment. *Biochemistry* **27**: 3807-3811

Eddinger TJ, Wolf JA. 1993. Expression of four myosin heavy chain isoforms with development in mouse uterus. *Cell Motil Cytoskeleton* **25**: 358-368

Eddinger TJ, Meer DP. 2001. Single rabbit stomach smooth muscle cell myosin heavy chain SMB expression and shortening velocity. *Am J Physiol Cell Physiol* **280**: C309–C316

Eglen RM, Reddy H, Watson N, Challiss RAJ. 1994. Muscarinic acetylcholine receptor subtypes in smooth muscle. *Trends Pharmacol Sci* **15:** 114-9

Eglen RM, Hegde SS, Watson N. 1996. Muscarinic receptor subtypes and smooth muscle function. *Pharmacol Rev* **48**: 531-565

Eitenmiller RR, Landen WO. 1999. Ascorbic acid in Vitamin analysis for the health and food sciences, Chap 6., pp 225

Ek A, Alm P, Andersson KE, Persson CGA. 1977. Adrenergic and cholinergic nerves of the human urethra and urinary bladder. A histochemical study. *Acta Physiol Scand* **99**: 345-352

Elbadawi A, Schenk EA. 1966. Dual innervation of the mammalian urinary bladder. A histological study of the distribution of cholinergic and adrenergic nerves. *Am J Anat* **119**: 405-427

Elbadawi A, Schenk EA. 1971. A new theory of the innervation of bladder musculature. The innervation apparatus of the ureterovesicular junction. *J Urol* **105**: 368-371

Elbadawi A. 1982. Ultrastructure of vesicourethral innervation. I. Neuroeffector and cell junctions in the male internal sphincter. *J Urol* **128(1)**: 180-8

Elcock M, Morgan RW. 1993. Update on artificial sweeteners and bladder cancer. *Regulatory Toxicol Pharmacol* **17(1)**: 35-43

Elliott RA, Norman RI, Parkar SG, Whitaker RP, Castleton CA. 1996. Effect of treatment with calcium antagonists in vitro and in vivo on the contractile response of isolated rat and human detrusor muscle. *Clin Sci* **91**: 467-474

Elneil S, Skepper JN, Kidd EJ, Williamson JG, Ferguson DR. 2001. Distribution of P2X(1) and P2X(3) receptors in the rat and human urinary bladder. *Pharmacol* **63**: 120-8

Elsas LJ II, Trotter JF. 1988. Changes in physiological concentrations of blood phenylalanine produce changes in sensitive parameters of human brain function. In: Wurtman RJ, Ritter-Walker E, eds. Dietary phenylalanine and brain function. Boston: Birkhauser, pp 187-95

Erol K, Ulak G, Donmez T, Cingi MI, Alpan RS, Ozdemir M. 1992. Effects of vasoactive intestinal polypeptide on isolated rat urinary bladder smooth muscle. *Urologia Internationalis* **49(3)**: 151-3

Ettinger B, Pak CYC, Citron JT, Thomas C, Adams-Huet B, Vangessel A. 1997. Potassium-magnesium citrate is an effective prophylaxis against recurrent calcium oxalate nephrolithiasis. *J Urol* **158**: 2069-73

Euromonitor International. 2002. Soft drinks in the UK 2001. London: Euromonitor plc

Evans RJ, Lewis C, Virginio C, Lundstrom K, Buell G, Surprenant A, North RA. 1996. Ionic permeability of, and divalent cation effects on, two ATP-gated cation channels (P2X receptors) expressed in mammalian cells. *J Physiol* 497: 413-422

Falconer C, Ekman G, Malmstrom A, Ulmsten U. 1994. Decreased collagen-synthesis in stress-incontinent women. *Obstet Gyneco* **84**: 583-6

Fantl JA, Newman, DK, Colling J, DeLancey JOL, Keeys C, Loughery R. 1996. Urinary incontinence in adults: acute and chronic management. In: Clinical Practice Guideline. Rockville, Maryland: United States Department of Health and Human Services, No. 2

Felsen D, Dardashti K, Ostad M, Lemer ML, Gross SS, Chen J, Vaughan ED Jr, Poppas DP. 2003. Inducible nitric oxide synthase promotes pathophysiological consequences of experimental bladder outlet obstruction. *J Urol* **169**: 1569-1572

Ferre S, Fuxe K, von Euler G, et al. 1992. Adenosine-dopamine interactions in the brain. *Neuroscience* **51**: 501-512

Ferrer M, Marin J, Encabo A, Alonso MJ, Balfagon G. 1999. Role of K^+ channels and sodium pump in the vasodilation induced by acetylcholine, nitric oxide, and cyclic GMP in the rabbit aorta. *Gen Pharmacol* **33**: 35-41

Fetscher C, Fleichman M, Schmidt M, Krege S, and Michel MC. 2002. M_3 muscarinic receptors mediate contraction of human urinary bladder. *Br J Pharmacol* **136**: 641-643

Finkbeiner AE. 1999. In vitro responses of detrusor smooth muscle to stretch and relaxation. *Scand J Urol Nephroly Suppl* **201**: 5-11

Finkielstein VA, Goldfarb DS. 2006. Strategies for preventing calcium oxalate stones. *Can Med Assoc J* **174(10)**: 1407-9

Fleming KH, Heimbach JT. 1994. Consumption of calcium in the U.S.: Food sources and intake levels. *J. Nutr* **124**: 1426S-1430S

Food and Drug Administration. 1988. A primer on food additives. *FDA Consumer* **22(8)**: 13-17

Food and Drug Administration and International Food Information Council. 1990. *Food additives.* Washington, DC: Food and Drug administration.

Food and Nutrition Board. 2005. Dietary Carbohydrates: Sugars and Starches in Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids (Macronutrients), p 266

Foresta C, Rossato M, Bordon P, Di Virgilio F. 1995. Extracellular ATP activates different signalling pathways in rat Sertoli cells. *Biochem J* **311**: 269-274

Forshee RA, Storey ML. 2003. Total beverage consumption and beverage choices among children and adolescents. *Int J Food Sci Nutr* **54(4)**: 297-307

Forshee RA, Storey ML. 2004. Evaluation of the association of demographics and beverage consumption with dental caries. *Food Chem Toxicol* **42(11)**: 1805-16

Fovaeus M, Andersson KE, Batra S, Morgan E, Sjögren C. 1987. Effects of calcium, calcium channel blockers and Bay K 8644 on contractions induced by muscarinic receptor stimulation of isolated bladder muscle from rabbit and man. *J Urol* **137**: 798-803

Frankel EN. 1998. Antioxidants. Pages 129–166 in Lipid Oxidation. The Oily Press Ltd, Dundee, Scotland

Fraumeni JF Jr, Scotto J, Dunham LF. 1971. Coffee drinking and bladder cancer. *Lancet* **2**: 1204

Frazier RA, Inns EL, Dossi N, Ames JM, Nursten HE. 2000. Development of a capillary electrophoresis method for the simultaneous analysis of artificial sweeteners, preservatives and colours in soft drinks. *J Chromatogr A* **876**: 213-220

Freeman RM, Adekanmi OA. 2005. Overactive bladder. *Best Practice & Research in Clinical Obstetrics & Gynaecology* **19(6)**: 829-41

Frei B, Stocker R, Ames BN. 1988. Antioxidant defenses and lipid peroxidation in human blood plasma. *Proceedings of the National Academy of Sciences of the United States of America.* **85(24)**: 9748-52

Fried EJ, Nestle M. 2002. The growing political movement against soft drinks in schools. *JAMA* **288(17)**: 2181

Friedman GJ, Sherry S, Ralli E. 1940. The mechanism of excretion of vitamin C by the human kidney at low and normal plasma levels of ascorbic acid. *J Clin Invest* **19**: 685-689

Frost L. Vestergaard P. 2005. Caffeine and risk of atrial fibrillation or flutter: the Danish Diet, Cancer, and Health Study. *Am J Clin Nutr* **81(3)**: 578-82

Fry CH, Wu C. 1998. The cellular basis of bladder instability. *Br J Urol* **81**: 1-8

Fry CH, Wu C, Mundy AR. 1999. Bladder instability and detrusor smooth muscle function. *Experimental Physiol* **84**: 161-169

Fry CH, Skennerton D, Wood D, Wu C. 2002. The cellular basis of contraction in human detrusor smooth muscle from patients with stable and unstable bladders. *Urol* **59 (5 Suppl 1):** 3-12

Fujii K, Foster CD, Brading AF, Parekh AB. 1990. Potassium channel blockers and the effects of cromakalim on the smooth muscle of the guinea-pig bladder. *Br. J. Pharmacol* **99**: 779-785

Fujii K. 1988. Evidence for adenosine triphosphate as an excitatory transmitter in guinea-pig, rabbit and pig urinary bladder. *J Physiol* **404**: 39-52

Fukushima S, Hagiwara A, Ogiso T, Shibata M, Ito N. 1983. Promoting effects of various chemicals in rat urinary bladder carcinogenesis initiated by N-nitroso-n-butyl-(4-hydroxybutyl)amine. *Food Chem Toxicol* **21(1)**: 59-68

Fukushima S, Imaida K, Sakata T, Okamura T, Shibata M, Ito N. 1983. Promoting effects of sodium L-ascorbate on two-stage urinary bladder carcinogenesis in rats. *Cancer Res* **43(9)**: 4454-7

Fukushima S, Thamavit W, Kurata Y, Ito N. 1986. Sodium citrate: A promoter of bladder carcinogenesis. *Jpn J Cancer Res* **77:** 1-4

Fukuyama H, Matsuzaki S, Ouchi Y, Yamauchi Y, Nagahama Y, Kimura J, Shibasaki H. 1996. Neural control of micturition in man examined with single photon emission computed tomography using 99mTc-HMPAO. *Neuroreport* **7**: 3009-12

Fultz NH, Herzog AR, Raghunathan TE, Wallace RB, Diokno AC. 1999. Prevalence and severity of urinary incontinence in older African American and Caucasian women. *J Gerontol* **54A**: M299-M303

Fultz N, Girts T, Kinchen K, Nygaard I, Pohl G, Sternfeld B. 2005. Prevalence, management and impact of urinary incontinence in the workplace. *Occ Med (Oxford)* **55(7)**: 552-7

Furst DO, Cross RA, De Mey J, Small JV. 1986. Caldesmon is an elongated, flexible molecule localized in the actomyosin domains of smooth muscle. *EMBO J* **5**: 251-257

Fyrberg E, Kelly M, Ball E, Fyrberg C, Reedy MC. 1990. Molecular genetics of

Drosophila alpha-actinin: mutant alleles disrupt Z disc integrity and muscle insertions. *J. Cell Biol* **110**: 1999-2011

Gabella G. 1977. A morphological study on the transmission of force along a smooth muscle. *J Physiol* **271(2)**: 10-11

Gabella G. 1995. The structural relations between nerve fibres and muscle cells in the urinary bladder of the rat. *J Neurocytol* **24:** 159-87

Gallagher PJ, Herring BP, Stull JT. 1997. Myosin light chain kinases. *J Muscle Res Cell Motil* **18**: 1-16

Gallegos CRR, Fry CH. 1994. Alterations to the electrophysiology of isolated human detrusor smooth muscle cells in bladder disease. *J Urol* **151**: 754-8

Ganitkevich VY, Isenberg G. 1992. Contribution of Ca^{2+}-induced Ca^{2+} release to the $[Ca^{2+}]_i$ transients in myocytes from guinea-pig urinary bladder. *J Physiol* **458**: 119-137

Garcia-Pascual A, Larsson B, Andersson KE. 1990. Contractile effects of endothelin-1 and localization of endothelin binding sites in rabbit lower urinary tract smooth muscle. *Acta Physiol Scand* **140**: 545-555

Garcia-Pascual A, Persson K, Holmquist F, Andersson KE. 1993.Endothelin-1-induced phosphoinositide hydrolysis and contraction in isolated rabbit detrusor and urethral smooth muscle. *Gen Pharmacol* **24**: 131-138

Garnett S, Abrams P. 2003. The natural history of overactive bladder and detrusor overactivity. A review of the evidence regarding the long-term outcome of the overactive bladder. *J Urol* **169**: 843-848

Geeves MA, Holmes K C. 1999. Structural mechanism of muscle contraction. *Annu Rev Biochem* **68**: 687-728

German K, Bedwani J, Davies J, Brading AF, Stephenson TP. 1995. Physiological and morphometric studies into the pathophysiology of detrusor hyperreflexia in neuropathic patients. *J Urol* **153**: 1678-1683

Giglio D, Delbro DS, Tobin G. 2001. On the functional role of muscarinic M2 receptors in cholinergic and purinergic responses in the rat urinary bladder. *Eur J*

Pharmacol **428**: 357-364

Gilbertson TA, Damak S, Margolskee RF. 2000. The molecular physiology of taste transduction. Curr. Opin. *Neurobiol* **10**: 519-527

Gillespie JI, Harvey IJ, Drake MJ. 2003. Agonist and nerve induced phasic activity in the isolated whole bladder of the guinea pig. Evidence for two types of bladder activity. *Exp Physiol* **88**: 343-57

Gillespie JI. 2004a. The autonomous bladder: a view of the origin of bladder overactivity and sensory urge. *BJU Int* **93**: 478-483

Gillespie JI. 2004b. Noradrenaline inhibits autonomous activity in the isolated guinea pig bladder. *BJU Int.* **93**: 401-409

Gilpin CJ, Dixon JS, Gilpin SA, Gosling JA. 1983. The fine structure of autonomic neurons in the wall of the human urinary bladder. *J Anat (Lond)* **137**: 705-713

GilVernet S. 1968. Morphology and function of the vesico-prostato-urethарl musculature. Italy: Edizioni Canova Treviso

Giuriato L, Borrione AC, Zanellato AM, Tonello M, Scatena M, Scannapieco G, Pauletto P, Sartore S. 1991. Aortic intimal thickening and myosin isoform expression in hyperthyroid rabbits. *Arterioscler Thromb* **11**: 1376-1389

Glazener CM, Herbison GP, MacArthur C, Lancashire R, McGee MA, Grant AM, Wilson PD. 2006. New postnatal urinary incontinence: obstetric and other risk factors in primiparae. *Br J Obstet Gynaecol* **113(2)**: 208-17

Glinsmann WH, Irausquin H, Park YK. 1986. Evaluation of health aspects of sugars contained in carbohydrate sweeteners; report of Sugars Task Force. *J Nutr* **116**: S1-S216

Goepel M, Wittmann A, Rubben H, Michel MC. 1997. Comparison of adrenoceptor subtype expression in porcine and human bladder and prostate. *Urol Res* **25**: 199-206

Goldberg RP, Sand PK. 2002. Pathophysiology of the overactive bladder. *Clin Obstet Gynaecol* **45(1)**: 182-192

Goldin BR, Adlercreutz H, Gorbach SL, Woods MN, Dwyer JT, Conlon T, Bohn E, Gershoff SN. 1986. The relationship between estrogen levels and diets of caucasian American and oriental immigrant women. *Am J Clin Nutr* **44**: 945-53

Gonce JE, Templeton HL. 1930. *Am J Dis Child* **39**: 265

Gopalakrishnan M, Whiteaker KL, Molinari EJ, Davis-Taber R, Scott VES, Shieh CC, Buckner SA, Milicic I, Cain JC, Postl S, Sullivan JP, and Brioni JD. 1999. Characterization of the ATP-Sensitive Potassium Channels (KATP) Expressed in Guinea Pig Bladder Smooth Muscle Cells. *J. Pharmacol Exp Ther* **289(1)**: 551-558

Gorbach SL. Goldin BR. 1987. Diet and the excretion and enterohepatic cycling of estrogens. *Preventive Medicine* **16(4)**: 525-31

Gosling JA, Dixon JS. 1975. The structure and innervation of smooth muscle in the wall of the bladder neck and proximal urethra. *Br J Urol* **47(5)**: 549-58

Gosling JA. Dixon JS. Lendon RG. 1977. The autonomic innervation of the human male and female bladder neck and proximal urethra. *J Urol* **118(2)**: 302-5

Gosling JA. 1979. The structure of the bladder and urethra in relation to function. *Urol Clin North Am* **6**: 31-38

Gosling JA, Dixon JS, Critchley HOD, Thompson SA. 1981. A comparative study of the human external sphincter and periurethral levator ani muscles. *Br J Urol* **53**: 35-41

Gosling JA, Dixon JS, Humpherson JA. 1983. Functional anatomy of the urinary tract: an integrated text and colour atlas. Churchill Livingstone, Edinburgh

Gosling JA. 1986. The distribution of noradrenergic nerves in the human lower urinary tract. *Clin Sci* **70** (Suppl. 14): 3s-6s

Gosling JA, Dixon JS, Jen PY. 1999. The distribution of noradrenergic nerves in the human lower urinary tract. A review. *Eur Urol* 36 *Suppl* **1**: 23-30

Grady D, Brown JS, Vittinghoff E, Applegate W, Varner E, Snyder T. 2001. Postmenopausal hormones and incontinence: The Heart and Estrogen/Progestin Replacement Study. *Obstet Gynecol* **97**: 116-20

Graham CA, Mallett VT. 2001. Race as a predictor of urinary incontinence and pelvic organ prolapse. *Am J Obstet Gynecol* **185**:116-120

Grant TL, Zuzack JS. 1991. Effects of K^+ channel blockers and cromakalim (BRL 34915) on the mechanical activity of guinea pig detrusor smooth muscle. *J Pharmacol Exp Ther* **259**: 1158-1164

Gray M. 2001. Caffeine and urinary continence. *J Wound Ostomy Continence Nur* **28(2)**: 66-9

Green DE, D Richter. 1937. *Biochem J* **31**: 596

Gregory J, Foster KTH, Wiseman M. 1990. *The Dietary and Nutritional Survey of British Adults (Part 2).* London: HMSO

Griffiths D, Holstege G, Dalm E, de Wall H. 1990. Control and coordination of bladder and urethral function in the brainstem of the cat. *Neurourol Urodyn* **9**: 63-82

Griffiths RR, Vernotica EM. 2000. Is caffeine a flavouring agent in cola soft drinks? *Arch Fam Med* **9(8)**: 727-34

Grindefjord M, Dahllof G, Nilsson B, Modéer T. 1995. Prediction of dental caries development in 1-year-old children. *Caries Res* **29**: 343-348

Grossmann M, Dobrev D, Himmel HM, Ravens U, Kirch W. 2001. Ascorbic acid-induced modulation of venous tone in humans. *Hypertension* **37(3)**: 949-54

Gu J, Restorick JM, Blank MA, Huang WM, Polak JM, Bloom SR, Mundy AR. 1983. Vasoactive intestinal polypeptide in the normal and unstable bladder. *Br J Urol* **55**: 645-647

Gu J, Blank MA, Huang WM, Aslam K N, McGregor GP, Christofides N, Allen JM, Bloom SR, Polak JM. 1984. Peptide containing nerves in human urinary bladder. *Urology* **24**: 353-7

Guilford WH, Warshaw DM. 1998. The molecular mechanics of smooth muscle myosin. *Comp Biochem Physiol B Biochem Mol Biol* **119**: 451-458

Gunasena KT, Nimmo AJ, Morrison JFB, Whitaker EM. 1995. Effects of

denervation on muscarinic receptors in the rat bladder. *Br J Urol* **76**: 291-296

Gup DI, Baumann M, Lepor H, Shapiro E. 1989. Muscarinic cholinergic receptors in normal pediatric and myelodysplastic bladders. *J Urol* **142**: 595-599

Gurney JG, Pogoda JM, Holly EA, Hecht SS, Preston-Martin S. 1997. Aspartame Consumption in Relation to Childhood Brain Tumor Risk: Results from a Case–Control Study. *J Nat Cancer Inst* **89(14)**: 1072-73

Habler HJ, Janig W, Koltzenburg M. 1990. Activation of unmyelinated afferent fibres by mechanical stimuli and inflammation of the urinary bladder in the cat. *J Physiol* **425**: 545-62

Hagiwara A, Fukushima S, Kitaori M, Shibata M, Ito N. 1984. Effects of three sweeteners on rat urinary bladder carcinogenesis initiated by N-butyl-N-(4-hydroxybutyl)-nitrosamine. *Gann* **75**: 763-768

Hale DS, Benson JT, Brubaker L, Heidkamp MC, Russell B. 1999. Histologic analysis of needle biopsy of urethral sphincter from women with normal and stress incontinence with comparison of electromyographic findings. *Am J Obstet Gynecol* **180**: 342-48

Hallberg L, Rossander L. 1984. Improvement of iron nutrition in developing countries: comparison of adding meat, soy protein, ascorbic acid, citric acid, and ferrous sulphate on iron absorption from a simple Latin American-type of meal. *Am J Clin Nutr* **39(4)**: 577-83

Hamberger B, Norberg KA. 1965. Studies on some systems of adrenergic synaptic terminals in the abdominal ganglia of the cat. *Acta Physiol Scand* **65**: 235-42

Hammarstrom M, Sjostrand NO. 1984. Comments on the atropine resistance of the neurogenic contractile response of the rat detrusor muscle. *Acta Physiol Scand* **122(4)**: 475-81

Hamoui N, Lord RV, Hagen JA, Theisen J, Demeester TR, Crookes PF. 2006. Response of the lower esophageal sphincter to gastric distention by carbonated beverages. *J Gastro Surg* **10(6)**: 870-7

Hampel C, Dolber PC, Smith MP, Savic SL, Thor RJ, Thor KB, Schwinn DA. 2002. Modulation of bladder alpha1-adrenergic receptor subtype expression by

bladder outlet obstruction. *J Urol* **167**: 1513-1521

Hampel C, Gillitzer R, Pahernik S, Hohenfellner M, Thuroff JW. 2003. Epidemiology and aetiology of overactive bladder. *Urology* **42**: 776-786

Han MO, Lee NY, Park HS. 2006. Abdominal obesity is associated with stress urinary incontinence in Korean women. *Int Urogynecol J* **17(1)**: 35-9

Hanna-Mitchell AT, Beckel JM, Barbadora S, Kanai AJ, de Groat WC, Birder LA. 2007. Non-neuronal acetylcholine and urinary bladder urothelium. *Life Sciences* **80(24-25)**: 2298-302

Hannestad YS, Rortveit G, Sandvik H and Hunskaar S. 2000. A community-based epidemiological survey of female urinary incontinence: the Norwegian EPINCONT study. Epidemiology of Incontinence in the County of Nord-Trondelag. *J Clin Epidemiol* **53(11)**:1150-7

Hannestad YS, Rortveit G, Hunskaar S. 2002. Help-seeking and associated factors in female urinary incontinence. The Norwegian EPINCONT Study. Epidemiology of Incontinence in the County of Nord-Trondelag. *Scand J Primary Health Care* **20(2)**: 102-7

Hannestad YS, Rortveit G, Daltveit A K, Hunskaar S. 2003. Are smoking and other lifestyle factors associated with female urinary incontinence? The Norwegian EPINCONT Study. *Br J Obstet Gynaecol* **110(3)**: 247-54

Harmar AJ, Arimura A, Gozes I, Journot L, Laburthe M, Pisegna JR, Rawlings SR, Robberecht P, Said SI, Sreedharan SP, Wank SA, Waschek JA. 1998. International Union of Pharmacology. XVIII. Nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. *Pharmacol Rev* **50**: 265-270

Harnack L, Stang J, Story M. 1999. Soft drink consumption among US children and adolescents: Nutritional consequences. *J. Am. Diet. Assoc* **99**: 436-441

Harrison SC, Hunnam GR, Farman P, Ferguson DR, Doyle PT. 1987. Bladder instability and denervation in patients with bladder outflow obstruction. *Br J Urol* **60**: 519-522

Harriss DR, Marsh KA, Birmingham AT, Hill SJ. 1995. Expression of muscarinic

M3-receptors coupled to inositol phospholipid hydrolysis in human detrusor cultured smooth muscle cells. *J Urol* **154**: 1241-1245

Hartge P, Hoover R, West DW, Lyon JL. 1983. Coffee drinking and risk of bladder cancer. *J Nati Cancer Inst* **70**: 1021-1026

Harvey RA, Skennerton DE, Newgreen D, Fry CH. 2002. The contractile potency of adenosine triphosphate and ecto-adenosine triphosphatase activity in guinea pig detrusor and detrusor from patients with a stable, unstable or obstructed bladder. *J Urol* **168**: 1235-1239

Hashitani H, Suzuki H. 1996. Altered electrical properties of bladder smooth muscle in streptozotocin-induced diabetic rats. *Br J Urol* **77**: 798-804

Hashitani H, Edwards FR. 1999. Spontaneous and neurally activated depolarizations in smooth muscle cells of the guinea-pig urethra. *J Physiol* **514:** 459-70

Hashitani H, Bramich NJ, Hirst GD. 2000. Mechanisms of excitatory neuromuscular transmission in the guinea-pig urinary bladder. *J Physiol* **524**: 565-579

Hathaway DR, Konicki MV, Coolical SA. 1985. Phosphorylation of myosin light chain kinase from vascular smooth muscle by cAMP- and cGMP-dependent protein kinases. *J Mol Cell Cardiol* **17**: 841-50

Haugaard N, Wein AJ, Chandy B, Soyupak B, Zderic SA, Levin RM. 1996. Properties of Ca^{2+}-Mg^{2+} ATP-ase in rabbit bladder muscle and mucosa: effect of urinary outlet obstruction. *Neurourol Urodyn* **15(5)**: 555-61

Haylen BT, Chetty N, Logan V, Schulz S, Verity L, Law M, Zhou J. 2007. Is sensory urgency part of the same spectrum of bladder dysfunction as detrusor overactivity? *Int Urogynecol J* **18(2)**:123-8

Hazan R. Levine A. Abeliovich H. 2004. Benzoic acid, a weak organic acid food preservative, exerts specific effects on intracellular membrane trafficking pathways in Saccharomyces cerevisiae. *Appl Environ Microbiol* **70(8)**: 4449-57

Heaney RP, Rafferty K. 2001. Carbonated beverages and urinary calcium excretion. *Am J Clin Nutr* **74**: 343-7

Hegde SS, Choppin A, Bonhaus D, Briaud S, Loeb M, Moy TM, Loury D, Eglen RM. 1997. Functional role of M2 and M3 muscarinic receptors in the urinary bladder of rats in vitro and in vivo. *Br J Pharmacol* **120**: 1409-1418

Hegde SS, Eglen RM. 1999. Muscarinic receptor subtypes modulating smooth muscle contractility in the urinary bladder. *Life Sci* **64**: 419-428

Hegde SS. 2006. Muscarinic receptors in the bladder: from basic research to therapeutics. *Br J Pharmacol* **147 Suppl 2**: S80-7

Heidrich SM, Wells TJ. 2004. Effects of urinary incontinence: psychological well-being and distress in older community-dwelling women. *J Gerontol Nurs* **30**: 47-54

Heller R, Munscher-Paulig F, Grabner R, Till U. 1999. L-Ascorbic acid potentiates nitric oxide synthesis in endothelial cells. *J Biol Chem* **274(12)**: 8254-60

Heller KE, Burt BA, Eklund SA. 2001. Sugared soda consumption and dental caries in the United States. *J Dent Res* **80**:1949-1953

Henson DE, Block G, Levine M. 1991. Ascorbic acid: biologic functions and relation to cancer. *J Natl Cancer Inst* **83**: 547-52

Heppner TJ, Bonev AD, Nelson MT. 1997. Ca^{2+}-activated K^+ channels regulate action potential repolarization in urinary bladder smooth muscle. *Am J Physiol Cell Physiol* **273**: C110-C117

Heppner TJ, Bonev AD, Nelson MT. 2005. Elementary purinergic Ca2+ transients evoked by nerve stimulation in rat urinary bladder smooth muscle. *J Physiol* **564(Pt 1)**: 201-12

Herbrandson C. Learning the Urinary System (Chapter 27) in Human Anatomy. Website in URL section (URL 7)

Herness MS, Gilbertson TA. 1999. Cellular mechanisms of taste transduction. *Annu Rev Physiol* **61**: 873-900

Herrera GM, Heppner TJ, Nelson MT. 2000. Regulation of urinary bladder smooth muscle contractions by ryanodine receptors and BK and SK channels. *Am J Physiol Regul Integr Comp Physiol* **279**: R60-R68

Herrera GM, Nelson MT. 2002. Differential regulation of SK and BK channels by

Ca^{2+} signals from Ca^{2+} channels and ryanodine receptors in guinea-pig urinary bladder myocytes. *J Physiol* **541**: 483-492

Herrera AM, McParland BE, Bienkowska A, Tait R, Pare PD, Seow CY. 2005. 'Sarcomeres' of smooth muscle: functional characteristics and ultrastructural evidence. *J Cell Sci* **118(11)**: 2381-92

Hewett TE, Martin AF, Paul RJ. 1993. Correlations between myosin heavy chain isoforms and mechanical parameters in rat myometrium. *J Physiol* **460**: 351-364

Hill AV. 1938. The heat of muscle shortening and the dynamic constants of muscle. *Proc R Soc Lond B Biol Sci* **126**: 136-195

Hille B. 1992. Ion channels of excitable membranes. 2nd Edn. Sunderland, Mass.

Hinata N, Shirakawa T, Okada H, Achaya B, Kamidono S, Gotoh A. 2004. Quantitative analysis of the levels of expression of muscarinic receptor subtype RNA in the detrusor muscle of patients with overactive bladder. *Mol Diag* **8(1)**: 17-22

Hirst GDS, Choate J K, Cousins HM, Edwards FR, Klemm MF. 1996. Transmission by post-ganglionic axons of the autonomic nervous system: the importance of the specialized neuroeffector junction. *Neurosci* **73**: 7-23

Hodgkinson CP. 1953. Relationships of female urethra in urinary incontinence. Am *J Obstet Gynecol* **65**: 560-73

Hogaboom GK. O'Donnell JP. Fedan JS. 1980. Purinergic receptors: photoaffinity analog of adenosine triphosphate is a specific adenosine triphosphate antagonist. *Science* **208(4449)**: 1273-6

Hogg RC, Wang Q, Large WA. 1994. Action of niflumic acid on evoked and spontaneous calcium-activated chloride and potassium currents in smooth muscle cells from rabbit portal vein. *Br J Pharmacol* **112**: 977-84

Holroyd-Ledue JM, Straus SE. 2004. Management of urinary incontinence in women: Scientific review. *JAMA* **291**: 986-995

Holstege G, Kuypers HG, Boer RC. 1979. Anatomical evidence for direct brain stem projections to the somatic motoneuronal cell groups and autonomic preganglionic cell groups in cat spinal cord. *Brain Res* **171**: 329-33

Holstege G, Griffiths D, de Wall H, Dalm E. 1986. Anatomical and physiological observations on supraspinal control of bladder and urethral sphincter muscles in the cat. *J Comp Neurol* **250**: 449-61

Holtedahl K, Hunskaar S. 1998. Prevalence, 1-year incidence and factors associated with urinary incontinence: a population based study of women 50–74 years of age in primary care. *Maturitas* **28**: 205-11

Homma Y, Yamaguchi O, Hayashi K. 2005. An epidemiological survey of overactive bladder symptoms in Japan. *BJU Int* **96(9)**: 1314-8

Hoon MA, Adler E, Lindemeier J, Battey JF, Ryba NJP, Zuker CS. 1999. Putative mammalian taste receptors: a class of taste-specific GPCRs with distinct topographic selectivity. *Cell* **96**: 541-551

Horowitz A, Menice CB, Laporte R, Morgan KG. 1996. Mechanisms of smooth muscle contraction. *Physiol Rev* **76**: 967-1003

Hosokawa H, Kaseda M. 1993. Experimental studies on VIP as non-cholinergic and non-adrenergic neurotransmitter in bladder neck and posterior urethra. *Jap J Urol* **84(3)**: 440-9

Howe GR, Burtch JD, Miller AB, Cook GM, Esteve J, Morrison B, Goron P, Chambers LW, Fodor G, Winsor GM. 1980. Tobacco use, occupation, coffee, various nutrients, and bladder cancer. *J Nati Cancer Inst* **64**: 701-713

Hoyle CHV, Burnstock G. 1985. Atropine-resistant excitatory junction potentials in rabbit bladder are blocked by α, β-methylene ATP. *Eur J Pharmacol* **114**: 239-240

Hoyle CHV, Burnstock G. 1993. Postganglionic efferent transmission in the bladder and urethra. *Autonom Nerv Sys* **6(10)**: 349-382

Hoyle CHV. 1994. Non-adrenergic, non-cholinergic control of the urinary bladder. *World J. Urol* **12**: 233

Hu TW, Wagner TH. 2000. Economic considerations in overactive bladder. *Am J Manag Care* **6**: S591-S598

Hu TW, Wagner TH, Bentkover J D, Leblanc K, Zhou S, Hunt T. 2004. Costs of urinary incontinence and overactive bladder in the United States: a comparative study.

Urology **63**: 461-5

Hudman D, Elliott RA, Norman R I. 2000. Inhibition of the contractile response of the rat detrusor muscle by the β2 adrenoceptor agonist clenbuterol. *Eur J Pharmacol* **392**: 79-85

Hudman D, Elliott RA, Whitaker P, Terry TR, Sandhu DP, Norman RI. 2001. Inhibition of the contractile responses of isolated human and rat bladders by clenbuterol. *J Urol* **166**: 1969-1973

Huisman AB. 1983. Aspects on the anatomy of the female urethra with special relation to urinary incontinence. *Contrib Gynecol Obstet* **10**: 1-31

Hunskaar S, Arnold EP, Burgio K, Diokno AC, Herzog AR, Mallett VT. 2000. Epidemiology and natural history of urinary incontinence. *Int Urogynecol J Pelvic FloorDysfunct* **11**: 301-19

Hunskaar S, Burgio K, Diokno A, Herzog R, Hjalmas K, Lapitan M. 2003. Epidemiology and natural history of urinary incontinence in women. *Urology* **62 (4 Suppl 1)**: 16-23

Hunskaar S, Lose G, Sykes D, Voss S. 2004. The prevalence of urinary incontinence in women in four European countries. *BJU Int* **93(3)**: 324-30

Husted S, Sjögren C, Andersson KE. 1983. Direct effects of adenosine and adenine nucleotides on isolated human urinary bladder and their influence on electrically induced contractions. *J Urol* **130**: 392-8

Hutch JA. 1972. Anatomy and physiology of the bladder, trigone and urethra. New York: Appleton Century Crofts.

Iacovou JW, Hill SJ, Birmingham AT, Bates CP. 1989. Calcium dependence and inositol phosphate accumulation in human detrusor smooth muscle. *Neurourol Urodyn* **8**: 418-9

Iacovou JW, Hill SJ, Birmingham AT. 1990. Agonist-induced contraction and accumulation of inositol phosphates in the guinea-pig detrusor: evidence that muscarinic and purinergic receptors raise intracellular calcium by different mechanisms. *J Urol* **144**: 775-779

Igawa Y, Mattiasson A, Andersson KE. 1993. Functional importance of cholinergic

and purinergic neurotransmission for micturition contraction in the normal, unanaesthetized rat. *Br J Pharmacol* **109**: 473-479

Igawa Y, Yamazaki Y, Takeda H, Hayakawa K, Akahane M, Ajisawa Y, Yoneyama T, Nishizawa O, Andersson KE. 1999. Functional and molecular biological evidence for a possible beta3-adrenoceptor in the human detrusor muscle. *Br J Pharmacol* **126**: 819-825

Igawa Y, Yamazaki Y, Takeda H, Kaidoh K, Akahane M, Ajisawa Y, Yoneyama T, Nisizawa O, Andersson KE . 2001. Relaxant effects of isoproterenol and selective beta3-adrenoceptor agonists on normal, low compliant and hyperreflexic human bladders. *J Urol* **165**: 240-244

Ilback NG, Alzin M, Jahrl S, Enghardt-Barbieri H, Busk L. 2003. Estimated intake of the artificial sweeteners acesulfame-K, aspartame, cyclamate and saccharin in a group of Swedish diabetics. *Food Addit Contam* **20(2)**: 99-114

Imaizumi Y, Torii Y, Ohi Y, Nagano N, Atsuki K, Yamamura H, Muraki K, Watanabe M, Bolton TB. 1998. Ca^{2+} images and K^{+} current during depolarization in smooth muscle cells of the guinea-pig vas deferens and urinary bladder. *J Physiol* **510**: 705-719

Inoue R, Brading AF. 1990. The properties of the ATP-induced depolarization and current in single cells isolated from the guinea-pig urinary bladder. *Br J Pharmacol* **100(3)**: 619-25

Inoue R, Brading AF. 1991. Human, pig and guinea-pig bladder smooth muscle cells generate similar inward currents in response to purinoceptor activation. *Br J Pharmacol* **103**: 1840-1841

Inoue T, Gabella G. 1992. The interface between epithelium and lamina propria in the rat urinary bladder. *Arch Hist Cytol* **55**: 157-63

Irwin DE, Milsom I, Hunskaar S, Reilly K, Kopp Z, Herschorn S, Coyne K, Kelleher C, Hampel C, Artibani W, Abrams P. 2006. Population-based survey of urinary incontinence, overactive bladder, and other lower urinary tract symptoms in five countries: results of the EPIC study. *Eur Urol* **50(6)**: 1306-15

Ishii H. 1981. Incidence of brain tumors in rats fed aspartame. *Toxicol Lett* **7**: 433-437

Ismail AI, Burt BA, Eklund SA. 1984. The cariogenicity of soft drinks in the United States. *J Am Dent Assoc* **109**: 241-245

Iwata H, Yamamoto S, Yano Y, Ohtani S, Fukushima S. 1997. Dose-dependent amplification by L-ascorbic acid of NaHCO3 promotion of rat urinary bladder carcinogenesis. *Toxicologic Patholog* **25(3)**: 284-90

Jackson RA, Vittinghoff E, Kanaya AM, Miles TP, Resnick HE, Kritchevsky SB, Simonsick EM, Brown JS. 2004. Urinary Incontinence in Elderly Women: Findings from the Health, Aging, and Body Composition Study. *Obstet Gynecol* **104**: 301-307

Jackson SL, Scholes D, Boyko EJ, Abraham L, Fihn SD. 2005. Urinary incontinence and diabetes in postmenopausal women. *Diabetes Care* **28(7)**: 1730-8

Jacobson MF, Farber E, Clapp R. 1998. Re: Long-term feeding of sodium saccharin to nonhuman primates: implications for urinary tract cancer. *J Natl Cancer Inst* **90**: 934-936

James MJ, Birmingham AT, Hill SJ. 1993. Partial mediation by nitric oxide of the relaxation of human isolated detrusor strips in response to electrical field stimulation. *Br J Clin Pharmacol* **35**: 366-372

Janig W, Morrison JF. 1986. Functional properties of spinal visceral afferents supplying abdominal and pelvic organs, with special emphasis on visceral nociception. *Prof Brain Res* **67**: 87-114

Jee SH, He J, Whelton PK, Suh I, Klag MJ. 1999. The effect of chronic coffee drinking on blood pressure: a meta-analysis of controlled clinical trials. *Hypertension.* **33(2)**: 647-52

Jeffrey AM, Williams GM. 2000. Lack of DNA-damaging activity of five non-nutritive sweeteners in the rat hepatocyte/DNA repair assay. *Food Chem Toxicol* **38**: 335-338

Jensen OM, Kamby C. 1982. Intra-uterine exposure to saccharin and risk of bladder cancer in man. *Int J Cancer* **29**: 507-509

Jensen PE, Gong MC, Somlyo AV, Somlyo AP. 1996. Separate upstream and convergent downstream pathways of G-pro. *Biochem J* **318**: 469-475

Jeremy JY, Tsang V, Mikhailidis DP, Rogers H, Morgan RJ, Dandona P. 1987.

Eicosanoid synthesis by human urinary bladder mucosa: pathological implications. *Br J Urol* **59**: 36-39

Jezior JR, Brady JD, Rosenstein DI, McCammon KA, Miner AS, Ratz PH. 2001. Dependency of detrusor contractions on calcium sensitization and calcium entry through LOE-908-sensitive channels. *Br J Pharmacol* **134**: 78-87

Jiang K, Novi JM, Darnell S, Arya LA. 2004. Exercise and urinary incontinence in women. *Obstet Gynecol Survey* **59(10)**: 717-21

Jiang P, Cui M, Zhao B, Snyder LA, Benard LM, Osman R, Max M, Margolskee RF. 2005a. Identification of the cyclamate interaction site within the transmembrane domain of the human sweet taste receptor subunit T1R3. *J Biol Chem* **280(40)**: 34296-305

Jiang P, Cui M, Zhao B, Liu Z, Snyder LA, Benard LMJ, Osman R, Margolskee RF, Max M. 2005b. Lactisole Interacts with the Transmembrane Domains of Human T1R3 to Inhibit Sweet Taste. *J Biol Chem* **280**: 15238-15246

John H, Hauri D, Bangerter U, Elbadawi A. 2001. Ultrastructure of the trigone and its functional implications. *Urologia Internationalis* **67(4)**: 264-71

Johnson LR, ed. 1993. Physiology of the Gastrointestinal Tract, vols. 1 and 2, Raven, New York

Joiner PD. 1973. Effects of ascorbate on contractile activity of ileal smooth muscle. *J Pharmacol Exp Ther* **185(1)**: 84-93

Jones C, Woods K, Whittle G, Worthington H, Taylor G. 1999. Sugar, drinks, deprivation and dental caries in 14-year-old children in the north west of England in 1995. *Commun Dent Health* **16**: 68-71

Jorgensen S, Hein HO, Gyntelberg F. 1994. Heavy lifting at work and risk of genital prolapse and herniated lumbar disc in assistant nurses. *Occup Med (Lond)* **44(1)**: 47-9

Junqueira LC, Carneiro J. 2001. Basic Histology Text & Atlas. Lange Medical Book, McGraw-Hill. 10th Edn. Chapter10: 207-208

Jurgens H, Haass W, Castaneda TR, Schurmann A, Koebnick C, Dombrowski F, Otto B, Nawrocki AR, Scherer PE, Spranger J, Ristow M, Joost HG, Havel

PJ, Tschop MH. 2005. Consuming fructose-sweetened beverages increases body adiposity in mice. *Obesity Res* **13(7)**: 1146-56

Kahn SG. 1951. Taste perception-individual reactions to different substances. *IL Acad Sci Trans* **44**: 263-269

Kajioka S, Nakayama S, McMurray G, Abe K, Brading AF. 2002. Ca(2+) channel properties in smooth muscle cells of the urinary bladder from pig and human. *Eur J Pharmacol* **443**: 19-29

Kamm EK, Stull JT. 1989. Regulation of smooth muscle contractile elements by second messengers. *Ann Rev Physiol* **51**: 299-313

Kantor AF, Hartge P, Hoover RN, Fraumeni JF Jr. 1988. Epidemiological characteristics of squamous cell carcinoma and adenocarcinoma of the bladder. *Cancer Res* **48(13)**: 3853-5

Kaplan SA, Te AE, Blaivas JG. 1995. Urodynamic findings in patients with diabetic cystopathy. *J Urol* **153**: 342-344

Kapicioglu S, Baki A, Reis A, Tekelioglu Y. 1999. Cola drinks consumption and oesophagitis. *Diseases of the Esophagus.* **12(4)**: 306-8

Karaki H, Urakawa N, Kutsky P. 1984. Potassium-induced contraction in smooth muscle. *Nippon Heikatsukin Gakkai Zasshi.* **20(6)**: 427-44

Karicheti V, Christ GJ. 2001. Physiological roles for K^+ channels and gap junctions in urogenital smooth muscle: implications for improved understanding of urogenital function, disease and therapy. *Curr Drug Targets* **2**: 1-20

Karikas GA, SchulpisKH, Reclos G, Kokotos G . 1998. Measurement of molecular interaction of aspartame and its metabolites with DNA. *Clin Biochem* **31**: 405-407

Kasakov L, Burnstock G. 1982. The use of the slowly degradable analog, alpha, beta-methylene ATP, to produce desensitisation of the P2-purinoceptor: effect on non-adrenergic, non-cholinergic responses of the guinea-pig urinary bladder. *Eur J Pharmacol* **86(2)**: 291-4

Katsuragawa Y, Yanagisawa M, Inoue A, Masaki T. 1989. Two distinct

nonmuscle myosin-heavy-chain mRNAs are differentially expressed in various chicken tissues. Identification of a novel gene family of vertebrate non-sarcomeric myosin heavy chains. *Eur J Biochem* **184**: 611-616

Katsuragi T, Usune S, Furukawa T. 1990. Antagonism by nifedipine of contraction and Ca^{2+}-influx evoked by ATP in guinea-pig urinary bladder. *Br J Pharmacol* **100**: 370-374

Kaufmann PA, Gnecchi-Ruscone T, di Terlizzi M, Schafers KP, Luscher TF, Camici PG. 2000. Coronary heart disease in smokers: vitamin C restores coronary microcirculatory function. *Circulation.* **102(11)**: 1233-8

Kearney R, Sawhney R, DeLancey JO. 2004. Levator ani muscle anatomy evaluated by origin-insertion pairs. *Obstet Gynecol* **22**: 897-917

Kelley CA, Takahashi M, Yu JH, Adelstein RS. 1993. An insert of seven amino acids confers functional differences between smooth muscle myosins from the intestines and vasculature. *J Biol Chem* **268**: 12848-12854

Khakh BS, Burnstock G, Kennedy C, King BF, North RA, Seguela P, Voigt M, Humphrey PP. 2001. International union of pharmacology. XXIV. Current status of the nomenclature and properties of P2X receptors and their subunits. *Pharmacol Rev* **53**: 107-118

Khakh BS, Egan TM. 2005. Contribution of transmembrane regions to ATP-gated P2X2 channel permeability dynamics. *Journal of Biological Chemistry.* **280(7)**: 6118-29

Khan MA, Thompson CS, Mumtaz FH, Jeremy JY, Morgan RJ, Mikhailidis DP. 1998. Role of prostaglandins in the urinary bladder: an update. *Prostaglandins Leukot Essent Fatty Acids* **59**: 415-422

Khan MA, Dashwood MR, Thompson CS, Mumtaz FH, Mikhailidis DP, Morgan RJ. 1999. Up-regulation of endothelin [ET(A) and ET(B)] receptors and down-regulation of nitric oxide synthase in the detrusor of a rabbit model of partial bladder outlet obstruction. *Urol Res* **27**: 445-453

Khaw KT, Bingham S, Welch A. 2001.Relation between plasma ascorbic acid and mortality in men and women in EPIC-Norfolk prospective study: a prospective

population study. *Lancet* **357**: 657-63

Khromov A, Somlyo AV, Somlyo AP. 1996. MgADP promotes a catch-like state developed through force-calcium hysteresis in tonic smooth muscle. *Biophys J* **75**: 1926-1934

Kim JC, Yoon JY, Seo SI, Hwang TK, Park YH. 2000. Effects of partial bladder outlet obstruction and its relief on types I and III collagen and detrusor contractility in the rat. *Neurourol Urodyn* **19**: 29-42

Kinchen K. 2002. Prevalence and frequency of stress urinary incontinence among community-dwelling women. *Eur Urol* (Suppl 1): 85

Kinder RB, Mundy AR. 1985. Atropine blockade of nerve-mediated stimulation of the human detrusor. *Br J Urol* **57**: 418-421

Kinder RB, Restorick JM, Mundy AR. 1985. Vasoactive intestinal polypeptide in the hyper-reflexic neuropathic bladder. *Br J Urol* **57**: 289-291

Kinder RB, Mundy AR. 1987. Pathophysiology of idiopathic detrusor instability and detrusor hyper-reflexia. An in vitro study of human detrusor muscle. *Br J Urol* **60**: 509-515

Kinnamon SC, Margolskee RF. 1996. Mechanisms of taste transduction. *Curr Opin Neurobiol* **6**: 506-513

Kitagawa M, Kusakabe Y, Mihura H, Ninomiya Y, Hino A. 2001. Molecular genetic identification of a candidate receptor gene for sweet taste. *Biochem Biophy Res Comm* **283**: 236-242

Kitazawa T, Somlyo AP. 1990. Desensitization and muscarinic re-sensitization of force and myosin light chain phosphorylation to cytoplasmic Ca^{2+} in smooth muscle. *Biochem Biophys Res Commun* **172**: 1291-1297

Kitto HJ, Bohr D F. 1953. Some Agents Which Potentiate the Inotropic Response of the Papillary Muscle to Epinephrine. **175**: 343-348

Klarskov P. 1987. Non-cholinergic, non-adrenergic inhibitory nerve responses of bladder outlet smooth muscle in vitro. *Br J Urol* **60**: 337-342

Klockner U, Isenberg G. 1985. Action potentials and net membrane currents of isolated smooth muscle cells (urinary bladder of the guinea-pig). *Pflugers Arch* **405**: 329-339

Koskimaki J, Hakama M, Huhtala H, Tammela TL. 2000. Association of dietary elements and lower urinary tract symptoms. *Scand J Urol Nephrol* **34**: 46-50

Kotlikoff MI, Dhulipala P, Wang YX. 1999. M2 signaling in smooth muscle cells. *Life Sci* **64**: 437- 442

Kozlowski, R, Choi M, Krane RJ, Siroky MB, Azadzoi KM. 1999. Neuroregulation of bladder blood flow during filling and contraction in the rabbit (Abstract). *J Urol* **161**: 137A

Kuh D, Cardozo L, Hardy R. 1999. Urinary incontinence in middle aged women: childhood enuresis and other lifetime risk factors in a British prospective cohort. *J Epidemiol Com Health* **53(8)**: 453-8

Kuhn C, Bufe B, Winnig M, Hofmann T, Frank O, Behrens M, Lewtschenko T, Slack JP, Ward CD, Meyerhof W. 2004. Bitter taste receptors for saccharin and acesulfame K. *J Neurosci* **24(45)**: 10260-5

Kuntz LA. 1993. *Food Product Design*

Kuo KH, Seow CY. 2004. Contractile filament architecture and force transmission in swine airway smooth muscle. *J Cell Sci* **117**: 1503-1511

Kurihara S, Creed KE. 1972. Changes in membrane potential of the smooth muscle cells of the guinea-pig urinary bladder in the various environments. *Jpn J Physiol* **22**: 667-683

Kuru M. 1965. Nervous control of micturition. *Physiol Rev* **45**: 425-94

Kuyper AC, Mattill HA. 1933. Some aspects of citric acid metabolism. *J Biol Chem* **103**: 51-60

Kvaavik E, Andersen LF, Klepp KI. 2005. The stability of soft drinks intake from adolescence to adult age and the association between long-term consumption of soft drinks and lifestyle factors and body weight. *Pulic Health Nutr* **8(2)**: 149-57

Kynast-Gales SA, Massey LK. 1994. Effect of caffeine on circadian excretion of urinary calcium and magnesium. *J Am Coll Nutr* **13**: 467-72

Lagergren J, Viklund P, Jansson C. 2006. Carbonated soft drinks and risk of esophageal adenocarcinoma: a population-based case-control study. *J Natl Cancer Inst* **98(16)**: 1158-61

Lagiou P, Wuu J, Trichopoulou A, Hsieh CC, Adami HO, Trichopoulos D. 1999. Diet and benign prostatic hyperplasia: a study in Greece. *Urology* **54**: 284-90

Lambert RJ, Stratford M. 1999. Weak acid preservatives: modeling microbial inhibition and response. *J Appl Microbiol* **86**: 157-164

Lam GW, Foldspang A, Elving LB, Mommsen S. 1992. Social context, social abstention, and problem recognition correlated with adult female urinary incontinence. *Dan Med Bulletin* **39(6)**: 565-70

Lane JD, Adcock RA, Williams RB, Kuhn CM. 1990. Caffeine effects on cardiovascular and neuroendocrine responses to acute psychosocial stress and their relationship to level of habitual caffeine consumption. *Psychosom Med* **52**: 320-336

Langley JN, Anderson HS. 1895. The innervation of the pelvic and adjoining viscera. Part II The bladder. *J Physiol (Lond)* **19**: 71-84

Lapitan MC, Chye PL. 2001. The epidemiology of overactive bladder among females in Asia: a questionnaire survey. *Int Urogynecol J* **12(4)**: 226-31

Larrieu S, Peres K, Letenneur L, Berr C, Dartigues JF, Ritchie K, Fevrier B, Alperovitch A, Barberger-Gateau P. 2004. Relationship between body mass index and different domains of disability in older persons: the 3C study. *Int J Obes Rel Metab Dis: J Int Ass Stud Obes* **28(12)**: 1555-60

Larsen JJ. 1979. α - and β-Adrenoceptors in the detrusor muscle and bladder base of the pig and -adrenoceptors in the detrusor of man. *Br J Urol* **65**: 215-222

Lassmann J, Zderic SA. 2004. Embryology of the female urogenital system with clinical applications. Textbook of female urology and urogynecology. 2nd edition.

Latifpour J, Gousse A, Kondo S, Morita T, Weiss RM. 1989. Effects of

experimental diabetes on biochemical and functional characteristics of bladder muscarinic receptors. *J Pharmacol Exp Ther* **248**: 81-88

Latifpour J, Fukumoto Y, Weiss RM. 1995. Regional differences in the density and subtype specificity of endothelin receptors in rabbit urinary tract. *Naunyn-Schmiedebergs Arch Pharmacol* **352**: 459-468

Laube N, Jansen B, Hesse A. 2002. Citric acid or citrates in urine: which should we focus on in the prevention of calcium oxalate crystals and stones? *Urol Res* **30**: 336-41

Lawson JON. 1974. Pelvic Anatomy. I. Pelvic floor mucles. *Ann R Coll Surg Engl* **54**: 244-52

Lean MEJ, Hankey CR. 2004. Aspartame and its effects on health. *BMJ* **329**: 755-756

Lee BL, Auerbach PS, Olson KR, Osterloh JD, Carmona R. 1986. Cardiac arrest following direct intravenous administration of a citrate anticoagulant solution. *Ann Emerg Med* **15**: 1353-1356

Lee JG, Wein AJ, Levin RM. 1993. The effect of caffeine on the contractile response of the rabbit urinary bladder to field stimulation. *General Pharmacology* **24(4)**: 1007-11

Lee JG, Wein AJ, Levin RM. 1994. Comparative pharmacology of the male and female rabbit bladder neck and urethra: involvement of nitric oxide. *Pharmacol* **48**: 250-9

Lee HY, Huang WC, Tsai JY, Huang JK. 1999. The efficacy of potassium citrate based medical prophylaxis for preventing upper urinary tract calculi: a midterm followup study. *J Urol* **161**: 1453

Lee HY, Bardini M, Burnstock G. 2000. Distribution of P2X receptors in the urinary bladder and the ureter of the rat. *J Urol* **163**: 2002-2007

Lee SH, Oe T, Blair IA. 2001. Vitamin C-induced decomposition of lipid hydroperoxides to endogenous genotoxins. *Science* **292**: 2083-6

Lee KW, Lee HJ, Surh YJ, Lee CY. 2003. Vitamin C and cancer chemoprevention: reappraisal. *Am J Clin Nutr* **78(6)**: 1074-8

LeLievre S. 2001. The management and prevention of incontinence dermatitis. *Br J Commun Nurs* **6(4)**: 180-5

Lemack GE, Szabo Z, Urban Z, Boyd CD, Csiszar K, Vaughan ED Jr, Felsen D. 1999. Altered bladder function in transgenic mice expressing rat elastin. *Neurourol Urodyn* **18**: 55-68

Lenton KJ, Sane AT, Therriault H, Cantin AM, Payette H, Wagner JR. 2003. Vitamin C augments lymphocyte glutathione in subjects with ascorbate deficiency. *Am J Clin Nutr* **77**: 189-95

Lesh RE, Nixon GF, Fleischer S, Airey JA, Somlyo AP, Somlyo AV. 1998. Localization of ryanodine receptors in smooth muscle. *Circ Res* **82**: 175-185

Levin RM, Shofer FS, Wein AJ. 1980. Cholinergic, adrenergic and purinergic response of sequential strips of rabbit urinary bladder. *J Pharmacol Exp Ther* **212**: 536-40

Levin RM. Wein AJ. 1982. Response of the in vitro whole bladder (rabbit) preparation to autonomic agonists. *J Urol* **128(5)**: 1087-90

Levin RM, Malkowicz SB, Wein AJ. 1984. Laboratory models for neuropharmacologic studies. *World J Urol* **2**: 222-33

Levin RM, Ruggieri MR, Wein AJ. 1986. Functional effects of the purinergic innervation of the rabbit urinary bladder. *J Pharmacol Exp Ther* **236**: 452-457

Levine M, Conry-Cantilena C, Wang Y, Welch RW, Washko PW, Dhariwal KR, Park JB, Lazarev A, Graumlich JF, King J, Cantilena LR. 1996. Vitamin C pharmacokinetics in healthy volunteers: evidence for a recommended dietary allowance. *Proceedings of the National Academy of Sciences of the United States of America.* **93(8)**: 3704-9

Levine M, Rumsey SC, Daruwala R, Park JB, Wang Y. 1999. Criteria and recommendations for vitamin C intake. *JAMA* **281(15)**: 1415-23

Levine M, Wang Y, Padayatty SJ, Morrow J. 2001. A new recommended dietary allowance of vitamin C for healthy young women. *Proc Natl Acad Sci USA* **98**: 9842-6

Levy SM, Warren JJ, Broffitt B, Hillis SL, Kanellis MJ. 2003. Fluoride, beverages

and dental caries in the primary dentition. *Caries Res* **37**: 157-165

Lewis SA. 2000. Everything you wanted to know about the bladder epithelium but were afraid to ask. *Am J Physiol - Renal Physiol* **278(6)**: F867-74

Li X, Staszewski L, Xu H, Durick K, Zoller M, Adler E. 2002. Human receptors for sweet and umami taste. *Proc Natl Acad Sci USA* **99**: 4692-4696

Liao J, Schultz PG. 2003. Three sweet receptor genes are clustered in human chromosome 1. *Mamm Genome* **14**: 291-301

Lincoln J, Burnstock G. 1993. Autonomic innervation of the urinary bladder and urethra. In: *The Autonomic Nervous System*, edited by Maggi CA. London: Harwood Academic **6**: 33-68

Lindemann B. 1996. Taste reception. *Physiol Rev* **76**: 718-766

Lindemann B. 1999. Receptor seeks ligand: on the way to cloning the molecular receptors for sweet and bitter taste. *Nature Med* **5**: 381-382

Lindemann B. 2000. A taste for umami. *Nature Neurosci* **3**: 99-100

Lindemann B. 2001. Receptors and transduction in taste. *Nature (Lond)* **413**: 219-225

Lindholm P, Lose G. 1986. Terbutaline (Bricanyl) in the treatment of female urge incontinence. *Urol Int* **41**: 158-60

Lindley MG. 1999. New developments in low-calorie sweeteners. *World Rev Nutr Diet* **85**: 44-51

Lips KS, Wunsch J, Zarghooni S, Bschleipfer T, Schukowski K, Weidner W, Wessler I, Schwantes U, Koepsell H, Kummer W. 2007. Acetylcholine and molecular components of its synthesis and release machinery in the urothelium. *European Urology* **51(4)**: 1042-53

Lluel P, Barras M, Palea S. 2002. Cholinergic and purinergic contribution to the micturition reflex in conscious rats with long-term bladder outlet obstruction. *Neurourol Urodyn* **21**: 142-153

Lofgren M, Malmqvist U, Arner A. 2001. Substrate and product dependence of force and shortening in fast and slow smooth muscle. *J Gen Physiol* **117**: 407-418

Longhurst PA, Leggett RE, Briscoe JA. 1995. Influence of strip size and location on contractile responses of rat urinary bladder body strips. *Gen Pharmacol* **26(7)**: 1519-27

Longhurst PA, Levendusky M. 1999. Pharmacological characterization of beta-adrenoceptors mediating relaxation of the rat urinary bladder in vitro. *Br J Pharmacol* **127**: 1744-1750

Lovallo WR, Pincomb GA, Sung BH, Passey RB. Sausen KP, Wilson MF. 1989. Caffeine may potentiate adrenocortical stress response in hypertension-prone men. *Hypertension* **14**: 170-176

Ludwig DS, Peterson KE, Gortmaker SL. 2001. Relation between consumption of sugar-sweetened drinks and childhood obesity; a prospective, observational analysis. *Lancet* **357**: 505-508

Lugaz O, Pillias AM, Faurion A. 2002. A new specific ageusia: some humans cannot taste L-glutamate. *Chem Senses* **27**: 105-115

Luheshi GN, Zar MA. 1990. Presence of non-cholinergic motor transmission in human isolated bladder. *J Pharm Pharmacol* **42**: 223-224

Mabuchi K, Li B, Ip W, Tao T. 1997. Association of calponin with desmin intermediate filaments. *J Biol Chem* **272**: 22662-22666

MacLennan AH, Taylor AW, Wilson DH, Wilson D. 2000. The prevalence of pelvic floor disorders and their relationship to gender, age, parity and mode of delivery. *BrJ Obstet Gynecol* **107**: 1460-70

MacKenzie I, Burnstock G, Dolly JO. 1982. The effects of purified botulinum neurotoxin type A on cholinergic, adrenergic and non-adrenergic, atropine-resistant autonomic neuromuscular transmission. *Neurosci* **7**: 997-1006.

Madersbacher S, Mohrle K, Burkhard F, Studer UE. 2002. Long-term voiding pattern of patients with ileal orthotopic bladder substitutes. *J Urol* **167(5)**: 2052-7

Madhavi DL, Deshpande SS, Salunkhe DK. 1996. Introduction. Pages 1–4 in Food Antioxidants: Technological, Toxicological, and Health Perspectives. D. L. Madhavi, S. S. Deshpande, and D. K. Salunkhe, ed. Marcel Dekker, Inc. New York, NY

Maggi CA, Santicioli P, Meli A. 1985. Pharmacological evidence for the existence of two components in the twitch response to field stimulation of detrusor strips from the rat urinary bladder. *J Auton Pharmacol* **5**: 221-229

Maggi CA, Giuliani S, Patacchini R, Turini D, Barbanti G, Giachetti A, Meli A. 1989a. Multiple sources of calcium for contraction of the human urinary bladder muscle. *Br. J. Pharmacol* **98**: 1021-1031

Maggi CA, Giuliani S, Patacchini R, Santicioli P, Turini D, Barbanti G, Meli A. 1989b. Potent contractile activity of endothelin on the human isolated urinary bladder. *Br J Pharmacol* **96**: 755-757

Maggi CA. 1991. The role of peptides in the regulation of the micturition reflex: an update. *Gen Pharmacol* **22**: 1-24

Maggi CA. 1992. Prostanoids as local modulators of reflex micturition. *Pharmacol Res* 25: 13-20

Majewski RF. 2001. Dental caries in adolescents associated with caffeinated carbonated beverages. *Pediatric Dentistry* **23(3)**: 198-203

Makalowska I, Sood R, Faruque MU, Hu P, Robbins CM, Eddings EM, Mestre JD, Baxevanis AD, Carpten JD. 2002. Identification of six novel genes by experimental validation of GeneMachine predicted genes. *Gene* **284**: 203-213

Mallet R, Sun J. 2003. Antioxidant properties of myocardial fuels. *Molecular Cell Biochem* **253(1-2)**: 103-11

Malloy BJ, Price DT, Price RR, Bienstock AM, Dole MK, Funk BL, Rudner XL, Richardson CD, Donatucci CF, Schwinn DA. 1998. Alpha1-adrenergic receptor subtypes in human detrusor. *J Urol* **160**: 937-943

Malmgren A, Andersson KE, Sjogren C, Andersson PO. 1989. Effects of pinacidil and cromakalim (BRL 34915) on bladder function in rats with detrusor instability. *J Urol* **142**: 1134-1138

Malmqvist U and Arner A. 1991. Correlation between isoform composition of the 17 kDa myosin light chain and maximal shortening velocity in smooth muscle. *Pflügers Arch* **418**: 523-530

Malmqvist U, Arner A, Uvelius B. 1991a. Cytoskeletal and contractile proteins in detrusor smooth muscle from bladders with outlet obstruction—a comparative study in rat and man. *Scand J Urol Nephrol* **25**: 261-267

Malmqvist U, Arner A, Uvelius B. 1991b. Contractile and cytoskeletal proteins in smooth muscle during hypertrophy and its reversal. *Am J Physiol Cell Physiol* **260**: C1085–C1093

Malo C, Wilson JX. 2000. Glucose modulates vitamin C transport in adult in adult human small instestinal brush border membrane vesicles. *J Nutr* **130**: 63-9

Maone TR, Mattes RD, Bernbaum JC, Beauchamp GK. 1990. A new method for delivering a taste without fluids to preterm and term infants. *Dev Psychobiol* **23**: 179-191

Margolskee RF. 2002. Molecular mechanisms of bitter and sweet taste transduction. *J Biol Chem* **277**: 1-4

Marston SB, Redwood CS. 1993. The essential role of tropomyosin in cooperative regulation of smooth muscle thin filament activity by caldesmon. *J Biol Chem* **268**: 12317-12320

Martin- Villa MC, Vidal-Valverde C, Rojas-Hidalgo E. 1981. Soluble sugars in soft drinks. *The Am J Clin Nutr* **34**: 2151-2153

Masters JG, Neal DE, Gillespie JI. 1997. The effects of thapsigargin on agonist-induced contractile responses in isolated human detrusor smooth muscle. *Proc Physiol Soc* **507**: 111-112

Matsui T, Amano M, Yamamoto T, Chihara K, Nakafuku M, Ito M, Nakano T, Okawa K, Iwamatsu A, Kaibuchi K. 1996. Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho. *EMBO J* **15**: 2208-2216

Matsunami H, Montmayeur JP, Buck LB. 2000. A family of candidate taste receptors in human and mouse. *Nature* **404(6778)**: 601-4

Mattes RD. 1996. Dietary compensation by humans for supplemental energy provided as ethanol or carbohydrate in fluids. *Physiol. Behav* **53**: 1133-1144

Mattiasson A, Ekblad E, Sundler F, Uvelius, B. 1985. Origin and distribution of neuropeptide Y-, vasoactive intestinal polypeptide- and substance P-containing nerve fibers in the urinary bladder of the rat. *Cell Tissue Res* **239(1)**: 141-6

Mattiasson A, Andersson KE, Elbadawi A, Morgan E, Sjogren C. 1987. Interaction between adrenergic and cholinergic nerve terminals in the urinary bladder of rabbit, cat and man. *J Urol* **137**: 1017-1019

Max M, Shanker YG, Huang L, Rong M, Liu Z, Campagne F, Weinstein H, Damak S, Margolskee RF. 2001. Tas1r3, encoding a new candidate taste receptor, is allelic to the sweet responsiveness locus Sac. *Nature Genetics* **28**: 58-63

Maxwell LC, Herlihy JT, Riedel GL. 1983. Effects of ascorbic acid and EDTA on vascular concentration-response to catecholamines. *Microvasc Res* **26**: 81-88

May JM. 1999. Is ascorbic acid an antioxidant for the plasma membrane? *The FASEB J* **13**: 995-1005

Mayne ST, Risch HA, Dubrow R, Chow WH, Gammon MD, Vaughan TL, Borchardt L, Schoenberg JB, Stanford JL, West AB, Rotterdam H, Blot WJ, Fraumeni JF Jr. 2006. Carbonated soft drink consumption and risk of esophageal adenocarcinoma. *J Nat Cancer Inst* **98(1)**: 72-5

Mazur RH. 1984. Discovery of aspartame. In: Stegink LD, Filer LJ Jr, editors. Aspartame physiology and biochemistry; vol 12. New York: Marcel Dekker; p. 3-9

McConnell J, Benson GS, Wood JG. 1982. Autonomic innervation of the urogenital system. Adrenergic and cholinergic elements. *Brain Res Bull* **9**: 679-694

McDaniel AH, Reed DR. 2004. The human sweet tooth and its relationship to obesity. In: Genomics and proteomics in nutrition. Moustaid-Moussa N, Berdanier S editors. New York: Marcel Dekker, Inc., pp. 49-67

McEvoy GK. 1993. Drug Information The American Hospital Formulary Service, American Society of Health-System Pharmacists, Inc.,MD

McFadzean I, Gibson A. 2002. The developing relationship between receptor-operated and store-operated calcium channels in smooth muscle. *Br J Pharmacol* **135**: 1-13

McGartland C, Robson PJ, Murray L, Cran G, Savage MJ, Watkins D, Rooney M, Boreham C. 2003. Carbonated soft drink consumption and bone mineral density in adolescence: the Northern Ireland Young Hearts project. *J Bone Min Res* **18(9)**: 1563-9

McGrother CW, Donaldson MM, Shaw C, Matthews RJ, Hayward TA, Dallosso HM, Jagger C, Clarke M, Castleden CM. MRC Incontinence Study Team. 2004. Storage symptoms of the bladder: prevalence, incidence and need for services in the UK. *BJU Int* **93**: 763-769

Meier-Kriesche H-U, Finkel KW, Gitomer JJ, DuBose TD Jr. 1999. Unexpected severe hypocalcemia during continuous venovenous hemodialysis with regional citrate anticoagulation. *Am J Kidney Dis* **33**: E8-E11

Meiss RA, Pidaparti RM. 2005. Active and passive components in the length-dependent stiffness of tracheal smooth muscle during isotonic shortening. *J Appl Physiol* **98**: 234-241

Mills IW, Greenland JE, McMurray G, McCoy R, Ho KM, Noble JG, Brading AF. 2000. Studies of the pathophysiology of idiopathic detrusor instability: the physiological properties of the detrusor smooth muscle and its pattern of innervation. *J Urol* **163(2)**: 646-51

Mills M, Yang N, Weinberger R, Vander Woude DL, Beggs AH, Easteal S, North K. 2001. Differential expression of the actin-binding proteins, alpha-actinin-2 and -3, in different species: implications for the evolution of functional redundancy. *Hum. Mol. Genet* **10**:1335-1346

Milne DB, Nielsen FH. 2000. The interaction between dietary fructose and magnesium adversely affects macromineral homeostasis in men. *J Am Coll Nutr* **19**: 31-7

Milsom I, Abrams P, Cardozo L, Roberts RG, Thuroff J, Wein AJ. 2001. How widespread are the symptoms of an overactive bladder and how are they managed? A population-based prevalence study. *BJU Int* **87**: 760-766

Mimata H, Wheeler MA, Fukumoto Y, Takigawa H, Nishimoto T, Weiss RM, Latifpour J. 1995. Enhancement of muscarinic receptor-coupled phosphatidylinositol hydrolysis in diabetic bladder. *Mol Cell Biochem* **152**: 71-76

Minassian VA, Drutz HP, Al-Badr A. 2003. Urinary incontinency as a worldwide problem. *Int J Gynecol Obstet* **82**: 327-338

Minke B. 2001. The TRP channel and phospholipase C-mediated signaling. *Cell Mol Neurobiol* **21**: 629-643

Misaka T, Kusakabe Y, Emori Y, Gonoi T, Arai S, Abe K. 1997. Taste buds have a cyclic nucleotide-activated channel, CNGgust. *J Biol Chem* **272**: 22623-22629

Miyazawa T. 1989. Determination of phospholipid hydroperoxides in human blood plasma by a chemiluminescence-HPLC assay. *Free Radic Biol Med* **7**: 209-17

Moller LA, Lose G, Jorgensen T. 2000. Risk factors for lower urinary tract symptoms in women 40–60 years of age. *Obstet Gynecol* **96**: 446-51

Montgomery BS, Fry CH. 1992. The action potential and net membrane currents in isolated human detrusor smooth muscle cells. *J Urol* **147**: 176-184

Montmayeur JP, Liberless SD, Matsunami H, Buck LB. 2001. A candidate taste receptor gene near a sweet taste locus. *Nat Neurosci* **4**: 492-498

Moore KH, Ray FR, Barden JA. 2001. Loss of purinergic P2X(3) and P2X(5) receptor innervation in human detrusor from adults with urge incontinence. *J Neurosci* **21**: RC166

Moore ED, Voigt T, Kobayashi YM, Isenberg G, Fay FS, Gallitelli MF, Franzini-Armstrong C. 2004. Organization of Ca2+ release units in excitable smooth muscle of the guinea-pig urinary bladder. *Biophysical J* **87(3)**: 1836-47

Moorthy P, Lapitan MC, Quek PL, Lim PH. 2004. Prevalence of overactive bladder in Asian men: an epidemiological survey. *BJU Int* **93**: 528-531

Morano I, Chai GX, Baltas LG, Lamounier-Zepter V, Lutsch G, Kott M, Haase H, Bader M. 2000. Smooth-muscle contraction without smooth-muscle myosin. *Nat Cell Biol* **2**: 371-375

Morgan RW, Wong O. 1985. A review of epidemiological studies on artificial sweeteners and bladder cancer. *Food Chem Toxicol* **23(4-5)**: 529-33

Morita T, Dohkita S, Kondo S, Nishimoto T, Hirano S, Tsuchida S. 1990. Cyclic

adenosine monophosphate production and contractile response induced by beta-adrenoceptor subtypes in rabbit urinary bladder smooth muscle. *Urol Int* **45**: 10-15

Morita T, Tsujii T, Dokita S. 1992. Regional difference in functional roles of cAMP and cGMP in lower urinary tract smooth muscle contractility. *Urol Int* **49**: 191-195

Morita T, Ando M, Ihara K, Ohshima H. 1993. Species differences in cAMP production and contractile response induced by beta-adrenoceptor subtypes in urinary bladder smooth muscle. *Neurourol Urodyn* **12**: 185-190

Morrison AS, Buring JE. 1980. Artificial sweeteners and cancer of the lower urinary tract. *N Engl J Med* **302**: 537-541

Morrison JFB, Nimimo AJ, Whitaker EM. 1986. The localization of β-adrenoceptor subtypes in the rat urinary bladder. *J Physiol* **38:** 29

Morrison J, Steers WD, Brading A, Blok B, Fry C, DeGroat WC, Kakizaki H, Levin R, and Thor K. 2002. Neurophysiology and neuropharmacology. In: *Incontinence, Second International Consultation on Incontinence*, edited by Abrams P, Khoury S, and Wein A. Plymouth, MA: Health Publication, pp 85-163

Mostwin JL. 1985. Receptor operated intracellular calcium stores in the smooth muscle of the guinea pig bladder. *J Urol* **133**: 900-905

Mostwin JL. 1986. The action potential of guinea pig bladder smooth muscle. *J Urol* **135**: 1299-1303

Motulsky H and Christopoulos A. 2003. Fitting dose response curves, in Fitting Models to Biological Data using Linear and Nonlinear Regression. Chapter 41, pp 256-258

Moynihan PJ, Holt RD. 1996. The national diet and nutrition survey of 1.5 to 4.5 year old children: summary of the findings of the dental survey. *Br Dent J* **181**: 328-332

Mumtaz FH, Khan MA, Thompson CS, Morgan RJ, Mikhailidis DP. 2000. Nitric oxide in the lower urinary tract: physiological and pathological implications. *BJU Int* **85**: 567-578

Murad F. 1994. Regulation of cytosolic guanylyl cyclase by nitric oxide: the NO-cGMP signal transduction system. *Adv Pharmacol* **26**: 19-33

Nakahira Y, Hashitani H, Fukuta H, Sasaki S, Kohri K, Suzuki H. 2001. Effects of isoproterenol on spontaneous excitations in detrusor smooth muscle cells of the guinea pig. *J Urol* **166**: 335-340

Nakamura T, Kimura J, Yamaguchi O. 2002. Muscarinic M2 receptors inhibit Ca^{2+}-activated K^{+} channels in rat bladder smooth muscle. *Int J Urol* **9(12)**: 689-96

Nakashima K, Ninomiya Y. 1999. Transduction for sweet taste of saccharin may involve both inositol 1,4,5-trisphosphate and cAMP pathways in the fungiform taste buds in C57BL mice. *Cell Physiol Biochem* **9**: 90-98

Nakayama S, Brading AF. 1993. Inactivation of the voltage-dependent Ca^{2+} channel current in smooth muscle cells isolated from the guinea-pig detrusor. *J Physiol* **471**: 107-127

Nakayama S, Brading AF. 1995. Possible contribution of long open state to noninactivating Ca^{2+} current in detrusor cells. *Am J Physiol* **269**: C48-C54

Namasivayam S, Eardley I, Morrison JF. 1999. Purinergic sensory neurotransmission in the urinary bladder: an in vitro study in the rat. *BJU Int* **84(7)**: 854-60

National Toxicology Program. 1997. US Natl Inst Environ Health Sci. Draft RC background document for saccharin

Navia JM. 1994. Carbohydrates and dental health. *Amer J Clin Nutr* **59**: 719-27

Nelson G, Hoon MA, Chandrashekar J, Zhang Y, Ryba NJP, Zukar CS. 2001. Mammalian sweet taste receptors. *Cell* **106**: 381-390

Nelson G, Chandrashekar J, Hoon MA, Feng L, Zhao G, Ryba NJP, Zukar CS. 2002. An amio-acid taste receptor. *Nature (Lond)* **726**: 199-202

Nelson DL, Cox MM. 2005. Lehninger Principles of Biochemistry, 4^{th} edition, Chapter **5.3**: 182-186

Nergardh A, Borés LO, Naglo AS. 1977. Characterization of the adrenergic β-receptors in the urinary bladder of man and cat. *Acta Pharmacol Toxicol* **40**: 14-21

Nergardh A, Kinn AC. 1983. Neurotransmission in activation of the contractile response in the human urinary bladder. *Scand J Urol Nephrol* **17**: 153-157

Neuhaus J, Dorschne W, Mondry J, Stolzenburg JU. 2001. Comparative Anatomy of the Male Guinea-Pig and Human Lower Urinary Tract: Histomorphology and Three-Dimensional Reconstruction. *Anatomia, Histologia, Embryologia* **30(4)**: 185-91

Nicar MJ. Skurla C. Sakhaee K. Pak CY. 1983. Low urinary citrate excretion in nephrolithiasis. *Urology* **21(1)**:8-14

Nichols DH, Milley PS, Randall CL. 1970. Significance of restoration of normal vaginal depth and axis. *Obstet Gynecol* **36**: 251-6

Nilvebrant L, Ekström J, Malmberg L. 1986. Muscarinic receptor density in the rat urinary bladder after denervation, hypertrophy and urinary diversion. *Acta Pharmacol. Toxicol* **59**: 306-314

Nilvebrant L, Andersson KE, Gillberg PG. 1997. Tolteradine – a new bladder-selective antimuscarinic agent. *Eur J Pharmacol* **327**: 195-207

Noda K, Takebe M, Oka M, Ukai HirouchiM, Toda Y. 2002. Functional role of inhibitory and excitatory nerves in the porcine lower urinary tract. *Eur J Pharmacol* **456**: 81-90

Nomiya M, Shishido K, Uchida W, Yamaguchi O. 2002. A quantitative analysis of mRNA expression of α_1- and β -adrenoceptor subtypes and their functional roles in human normal and obstructed bladders. *Neurourol Urodyn* **21**: 299-300

Nomura AM, Kolonel LN, Hankin JH, Yoshizawa CN. 1991. Dietary factors in cancer of the lower urinary tract. *Int J Cancer* **48(2)**: 199-205

North RA. 2002. Molecular physiology of P2X receptors. *Physiol Rev* **82**: 1013-1067

Noto H, Roppolo JR, Steers WD, de Groat WC. 1991. Electrophysiological analysis of the ascending and descending components of the micturition reflex pathway in the rat. *Brain Res* **549**: 95-105

Nour S, Svarer C, Kristensen JK, Paulson OB, Law I. 2000. Cerebral activation during micturition in normal men. *Brain* **123**: 781-9

Nurse DE, Restorick JM, Mundy AR. 1991. The effect of cromakalim on the

normal and hyper-reflexic human detrusor muscle. *Br J Urol* **68** (Suppl 1): 27-31

Nygaard IE, Heit M. 2004. Stress urinary incontinence. *Obstet Gynecol* **104**: 607-20

Oike M, Creed K E, Onoue H, Tanaka H, Ito Y. 1998. Increase in calcium in smooth muscle cells of the rabbit bladder induced by acetylcholine and ATP. *J Auton Nerv Sys* **69**: 141-147

Okamoto-Koizumi T, Takeda M, Komeyama T, Hatano A, Tamaki M, Mizusawa T, Tsutsui T, Obara K, Tomita Y, Arai K, Takahashi K. 1999. Pharmacological and molecular biological evidence for ET_A endothelin receptor subtype mediating mechanical responses in the detrusor smooth muscle of the human urinary bladder. *Clin Sci* **96**: 397-402

Olney JW, Farber NB, Spitznagel E, Robins LN. 1996. Increasing brain cancer rates: is there a link to aspartame? *J Neuropathol Exp Neurol* **55**: 1115-23

O'Reilly BA, Kosaka AH, Chang TK, Ford AP, Popert R, McMahon SB. 2001. A quantitative analysis of purinoceptor expression in the bladders of patients with symptomatic outlet obstruction. *BJU Int* **87**: 617-622

O'Reilly BA, Kosaka AH, Knight GF, Chang TK, Ford AP, Rymer JM, Popert R, Burnstock G, McMahon SB. 2002. P2X receptors and their role in female idiopathic detrusor instability. *J Urol* **167**: 157-164

Osborn M, Caselitz J, Puschel K, Weber K. 1987. Intermediate filament expression in human vascular smooth muscle and in arteriosclerotic plaques. *Virchows Arch A Pathol Anat Histopathol* **411**: 449-458

Ostberg O. 1931. *Skand Arch Physiol* **62**: 81

Osterman A, Arner A. 1995. Effects of inorganic phosphate on cross-bridge kinetics at different activation levels in skinned guinea-pig smooth muscle. *J Physiol* **484**: 369-383

Oswald J, Schwentner C, Lunacek A, Fritsch H, Longato S, Sergi C, Bartsch G, Radmayr C. 2006. Reevaluation of the fetal muscle development of the vesical trigone. *J Urol* **176(3)**: 1166-70

Pak CY, Sakhaee K, Fuller CJ. 1983. Physiological and physiochemical correction and prevention of calcium stone formation by potassium citrate therapy. *Trans Assoc*

Am Physicians **96**: 294-305

Pak CYC, Koenig K, Khan R, Haynes S, Padalino P. 1992. Physicochemical action of potassium-magnesium citrate in nephrolithiasis. *J Bone Miner Res* **7**: 281-285

Pak CY. 1994. Citrate and renal calculi: an update. *Miner Electrolyte Metab* **20(6)**: 371-7

Palea S, Artibani W, Ostardo E, Trist DG, Pietra C. 1993. Evidence for purinergic neurotransmission in human urinary bladder affected by interstitial cystitis. *J Urol* **150**: 2007-2012

Palmer MH, Baumgarten M, Langenberg P, Carson J. 2002. Risk factors for hospital-acquired incontinence in elderly female hip fracture patients. *J Gerontol Ser A: Biol Sci Med Sci 57* **(10)**: M672-M677

Palmer MH, Fogarty L, Somerfield M, Powell L. 2003. Incontinence after prostatectomy: Coping with incontinence after prostate cancer surgery. *Onc Nurs Forum* **30 (2)**: 229-238

Parekh AB. Putney JW Jr. 2005. Store-operated calcium channels. *Physiological Reviews.* **85(2)**: 757-810

Parija SC, Raviprakash V, Mishra SK. 1991. Adenosine- and alpha, beta-methylene ATP-induced differential inhibition of cholinergic and non-cholinergic neurogenic responses in rat urinary bladder. *Br J Pharmacol* **102**: 396-400

Parks JH, Ruml LA, Pak CYC. 1996. Hypocitraturia. In: Kidney Stones: Medical and Surgical Management. Edited by F. L. Coe, M. J. Favus, C. Y. C. Pak, J. H. Parks and G. M. Preminger. Philadelphia: Lippincott-Raven Publishers, part IV. 905-920

Parmentier ML, Joly C, Restituito S, Bockaert J, Grau Y, Pin JP. 1998. The G-protein coupling profile of metabotropic glutamate receptors, as determined with exogenous G-proteins, is independent of their ligand recognition domain. *Mol Pharmacol* **53**: 778-786

Patel AS, O'Leary ML, Stein RJ, Leng WW, Chancellor MB, Patel SG, Borello-France D. 2006. The relationship between overactive bladder and sexual activity in women. *International Braz J Urol* **32(1)**: 77-87

Perlberg S, Caine M. 1982. Adrenergic response of bladder muscle in prostatic obstruction. Its relation to detrusor instability. *Urol* **20**: 524-527

Persson K, Garcia-Pascual A, Holmquist F, Andersson KE. 1992. Endothelin-1-induced contractions of isolated pig detrusor and vesical arterial smooth muscle: calcium dependence and phosphoinositide hydrolysis. *Gen Pharmacol* **23**: 445-453

Persson K, Andersson KE. 1994. Non-adrenergic, non-cholinergic relaxation and levels of cyclic nucleotides in rabbit lower urinary tract. *Eur J Pharmacol* **268**: 159-167

Persson K, Pandita RK, Aszodi A, Ahmad M, Pfeifer A, Fassler R, Andersson KE. 2000. Functional characteristics of urinary tract smooth muscles in mice lacking cGMP protein kinase type I. *Am J Physiol Regul Integr Comp Physiol* **279**: R1112-R1120

Petkov GV, Heppner TJ, Bonev AD, Herrera GM, Nelson MT. 2001. Low levels of K(ATP) channel activation decrease excitability and contractility of urinary bladder. *Am J Physiol Regul Integr Comp Physiol* **280**: R1427-R1433

Petridou E, Karpathios T, Dessypris N, Simou E, Trochopoulos D. 1997. The role of dairy products and non alcoholic beverages in bone fractures among schoolage children. *Scand J Soc Med* **25**: 119-25

Peyrat L, Haillot O, Bruyere F, Boutin JM, Bertrand P, Lanson Y. 2002. Prevalence and risk factors of urinary incontinence in young women. *Progres en Urologie* **12(1)**: 52-9

Pfisterer MH, Griffiths DJ, Schaefer W, Resnick NM. 2006. The effect of age on lower urinary tract function: a study in women. *J Am Geriatr Soc* **54(3)**: 405-12

Pfitzer G, Hofmann F, DiSalvo J, Rüegg JC. 1984. cGMP and cAMP inhibit tension development in skinned coronary arteries. *Pflügers Arch* **401**: 277-280

Phillips CA. 1999. The effect of citric acid, lactic acid, sodium citrate and sodium lactate alone and in combination with nisin, on the growth of Arcobacter butzleri. *J Appl Microbiol* **29**: 424-428

Pin JP, Duvoisin R. 1995. The metabotropic glutamate receptors: structure and function. *Neuropharmacology* **34**: 1-26

Pin JP, Galvez T, Prezeau L. 2003. Evolution, structure, and activation mechanism of family 3/C G-protein-coupled receptors. *Pharmacol Ther* **98**: 325-354

Piper JM, Matanoski GM, Tonascia J. 1986. Bladder cancer in young women. *Am J Epidemiol* **123**: 1033-1042

Poli E, Monica B, Zappia L, Pozzoli C, Bertaccini G. 1992. Antimuscarinic activity of telenzepine on isolated human urinary bladder: no role for M1-muscarinic receptors. *Gen Pharmacol* **23(4)**: 659-64

Pontari MA, Braverman AS, Ruggieri MR Sr. 2004. The M_2 muscarinic receptor mediates in vitro bladder contractions from patients with neurogenic bladder dysfunction. *Am J Physiol Regul Integr Comp Physiol* **386(5)**: 874-80

Powiardowska E, Puglisi L. 1980. The effects of L-ascorbic acid and prostaglandins on isolated tracheal smooth muscle. *Acta Physiologica Polonica* **31(2)**: 187-94

Price KR, Fligner DJ. 1990. Treatment of caffeine toxicity with esmolol. *Ann Emerg Med* **19**: 44-6

Prodolliet J. 1996. L.M.L. Nollet (Ed.), Handbook of Food Analysis (Marcel Dekker, New York), Vol. 2: 1835-1865

Radley SC, Rosario DJ, Chapple CR, Farkas AG. 2001. Conventional and ambulatory urodynamic findings in women with symptoms suggestive of bladder overactivity. *J Urol* **66(6)**: 2253-8

Ralevic V, Burnstock G. 1998. Receptors for purines and pyrimidines. *Pharmacol Rev* **50**: 413-492

Ranney RE, Opperman JA, Maldoon E, McMahon FG. 1976. Comparative metabolism of aspartame in experimental animals and humans. *J Toxicol Environ Health* 2: 441-451

Reed DR, Li S, Li X, Huang L, Tordoff MG, Starling-Roney R, Taniguchi K, West DB, Ohmen JD, Beauchamp GK, Bachmanov AA. 2004. Polymorphisms in the taste receptor gene (*Tas1r3*) region are associated with saccharin preference in 30 mouse strains. *J Neurosci* **28**: 938-946

Renart J, Reiser J, Stark GR. 1979. Transfer of proteins from gels to diazobenzyloxymethyl-paper and detection with antisera: a method for studying antibody specificity and antigen structure. *Proc Natl Acad Sci USA* **76(7)**: 3116-20

Renwick AG. 1986. The metabolism of intense sweeteners. *Xenobiotica* **16(10/11)**: 1057-1071

Restorick JM, Mundy AR. 1989. The density of cholinergic and alpha and beta adrenergic receptors in the normal and hyper-reflexic human detrusor. *Br J Urol* **63**: 32-35

Reubi JC. 2000. In vitro evaluation of VIP/PACAP receptors in healthy and diseased human tissues. Clinical implications. *Ann NY Acad Sci* **921**: 1-25

Reznik VM, Griswold WR, Brams MR, Mendoza SA. 1980. Does high-dose ascorbic acid accelerate renal failure? *N Engl J Med* **302(25)**: 1418-9

Richter C. 1943. The self-selection of diets. In: Essays in biology. In honor of Herbert M. Evans. Written by his friends. Berkeley and Los Angeles: University of California Press, pp. 501-505

Rivers JM. 1989. Safety of high-level vitamin C ingestion. *Int J Vitam Nutr Res Suppl* **30**: 95-102

Robertson D, Frolich JC, Carr RK, Watson JT, Hollifield JW, Shand DG, Oates JA. 1978. Effects of caffeine on plasma renin activity, catecholamines and blood pressure. *New Eng J Med* **298(4)**: 181-6

Rodgers A. 1999. Effect of cola consumption on urinary biochemical and physicochemical risk factors associated with calcium oxalate urolithiasis.*Urol Res* **27(1)**: 77-81

Rodgers A, Allie-Hamdulay S, Jackson G. 2006. Therapeutic action of citrate in urolithiasis explained by chemical speciation: increase in pH is the determinant factor. *Nephrology Dialysis Transplantation* **21(2)**: 361-9

Roe B, Rn F, Doll H. 2000. Prevalence of urinary incontinence and its relationship with health status. *J Clin Nurs* **9(2)**: 178-187

Rogers PJ, Carlyle J, Hill AJ, Blundell JE. 1988. Uncoupling sweet taste and

calories: comparison of the effects of glucose and three intense sweeteners on hunger and food intake. *Physiol Behav* **43**: 547-52

Rogers M, Colquhoun LM, Patrick JW, Dani JA. 1997. Calcium flux through predominantly independent purinergic ATP and nicotinic acetylcholine receptors. *J Neurophysiol* **77**: 1407-1417

Rohner TJ, Hannigan JD, Sanford EJ. 1978. Altered in vitro adrenergic responses of dog detrusor muscle after chronic bladder outlet obstruction. *Urol* **11**: 357-361

Rohr G, Stovring H, Christensen K, Gaist D, Nybo H, Kragstrup J. 2005. Characteristics of middle-aged and elderly women with urinary incontinence. *Scand J Pri Health Care* **23(4)**: 203-8

Rolls BJ. 1991. Effects of intense sweeteners on hunger, food intake, and body weight: a review. *Am J Clin Nutr* **53(4)**: 872-8

Roper SD. 1989. The cell biology of vertebrate taste receptors. *Annu Rev Neurosci* **12**: 329-353

Rortveit G, Daltveit AK, Hannested YS, Hunskaar S. 2003. Urinary incontinence after vaginal delivery or caesarean section. *N Eng J Med* **348**: 900-07

Ross JA. 1998. Brain tumors and artificial sweeteners? A lesson on not getting soured on epidemiology. *Med Pediatr Oncol* **30**: 7-8

Rubenstein LA, Lanzara RG. 1998. Activation of G protein-coupled receptors entails cysteine modulation of agonist binding. *J Mol Structure* **430**: 57-71

Rud T, Andersson KE, Asmussen M. 1980. Factors maintaining the intraurethral pressure in women. *Invest Urol* **17**: 343-7

Rüegg JC. 1992. *Calcium in Muscle Contraction.* Heidelberg, Germany: Springer

Ruggieri Sr MR. 2006. Mechanisms of Disease: Role of purinergic signalling in the pathophysiology of bladder dysfunction. *Nature Clin Prac Urol* **3**: 206-215

Rugg-Gunn AJ. 1996. Diet and dental caries. In: Prevention of oral disease. Murray JJ, editor. pp. 3-31

Runnels LW, Yue L, Clapham DE. 2002. The TRPM7 channel is inactivated by PIP2 hydrolysis. *Nat Cell Biol* **4**: 329-336

Saenz DTI, Mueller JD, de Las MA, Machado M, Moreland RB, Krane RJ, Wolfe HJ, Traish AM. 1992. Endothelin in the urinary bladder. I. Synthesis of endothelin-1 by epithelia, muscle and fibroblasts suggests autocrine and paracrine cellular regulation. *J Urol* **148**: 1290-1298

Sagoo SK, Board R, Roller S. 2002. Chitosan potentiates the antimicrobial action of sodium benzoate on spoilage yeasts. *Soc Appl Microbiol*, Letters in Applied Microbiology **34**: 168-172

Sainz E, Korley JN, Battey JF, Sullivan SL. 2001. Identification of a novel member of the T1R family of putative taste receptors. *J Neurochem* **77**: 896-903

Saito M, Kondo A, Kato T, Miyake K. 1993. Response of the human neurogenic bladder induced by intramural nerve stimulation. *Nippon Hinyokika Gakkai Zasshi* **84**: 507-513

Salmond CV, Kroll RG, Booth IR. 1984. The effect of food preservatives on pH homeostasis in Escherichia coli. *J Gen Microbiol* **130**: 2845-2850

Sampselle CM, Harlow SD, Skurnick J, Brubaker L, Bondarenko I. 2002. Urinary incontinence predictors and life impact in ethnically diverse perimenopausal women. *Obstet Gynecol* **100**: 1230-1238

Sanders KM. 2001. Invited review: mechanisms of calcium handling in smooth muscles. *J Appl Physiol* **91**: 1438-1449

Sauve R, Parent L, Simoneau C, Roy G. 1988. External ATP triggers a biphasic activation process of a calcium- dependent K+ channel in cultured bovine aortic endothelial cells. *Pflugers Arch* **412**: 469-481

Sayegh A, Dini EL, Holt RD, Bedi R. 2002. Food and drink consumption, sociodemographic factors and dental caries in 4–5-year-old children in Amman, Jordan. *Br Dent J* **193**: 37-42

Schneider P, Hopp HH, Isenberg G. 1991. Ca^{2+} influx through ATP-gated channels increments $[Ca^{2+}]_i$ and inactivates I_{Ca} in myocytes from guinea-pig unrinary bladder. *J*

Physiol **440**: 479-496

Schneider T, Fetscher C, Krege S, Michel MC. 2004. Signal transduction underlying carbachol-induced contraction of human urinary bladder. *J Pharmacol Exp Ther* **309(3)**: 1148-53

Schumacher S. 2006. Epidemiology and pathophysiology of overactive bladder. *Urologe (Ausg. A).* **45(7)**: 822-5

Schwartz GR. 1999. Aspartame and breast and other cancers. *West J Med* **171**: 300-301

Sellers JR. 1985. Mechanism of the phosphorylation-dependent regulation of smooth muscle heavy meromyosin. *J Biol Chem* **260**: 15815-15819

Sellers JR. 2000. Myosins: a diverse superfamily. *Biochim Biophys Acta* **1496**: 3-22

Selvam R, Kurien TB. 1992. Restoration of antioxidants in liver by methionine feeding in experimental rat urolithiasis. *Indian J Biochem Biophys* **29(4)**: 364-70

Sen I, Onaran M, Tan MO, Acar C, Camtosun A, Sozen S, Bozkirli I. 2007. Evaluation of sexual function in women with overactive bladder syndrome. *Urologia Internationalis* **78(2)**: 112-5

Sgadari A. Topinkova E. Bjornson J. Bernabei R. 1997. Urinary incontinence in nursing home residents: a cross-national comparison. *Age Ageing* **26 (Suppl 2)**: 49-54

Shefchyk SJ. 2001. Sacral spinal interneurones and the control of urinary bladder and urethral striated sphincter muscle function. *J Physiol* **533(Pt 1)**: 57-63

Shenfeld OZ, Morgan CW, Ratz PH. 1998. Bethanecol activates a post-receptor negative feedback mechanism in rabbit urinary bladder smooth muscle. *J Urol* **159**: 252-257

Sherman CC, Mendel L B, Smith A H. 1935. The metabolism of orally administered citric acid. *J Biol Chem* **110**: 265-271

Shirazi A, Iizuka K, Fadden P, Mosse C, Somlyo AP, Somlyo AV, Haystead TA. 1994. Purification and characterization of the mammalian myosin light chain

phosphatase holoenzyme. The differential effects of the holoenzyme and its subunits on smooth muscle. *J Biol Chem* **269**: 31598-31606

Sibley GN. 1984. A comparison of spontaneous and nerve-mediated activity in bladder muscle from man, pig and rabbit. *J Physiol* **354**: 431-443

Sibley GN. 1985. An experimental model of detrusor instability in the obstructed pig. *Br J Urol* **57**: 292-298

Siemankowski RF, Wiseman MO, White HD. 1985. ADP dissociation from actomyosin subfragment 1 is sufficiently slow to limit the unloaded shortening velocity in vertebrate muscle. *Proc Natl Acad Sci USA* **82**: 658-662

Sigala S, Mirabella G, Peroni A, Pezzotti G, Simeone C, Spano P, Cunico SC. 2002. Differential gene expression of cholinergic muscarinic receptor subtypes in male and female normal human urinary bladder. *Urol* **60**: 719-725

Silva C, Ribeiro MJ, Cruz, F. 2002. The effect of intravesical resiniferatoxin in patients with idiopathic detrusor instability suggests that involuntary detrusor contractions are triggered by C-fiber input. *J Urol* **168(2):** 575-9

Simon D, Yen S, Cole P. 1975. Coffee drinking and cancer of the lower urinary tract. *J Natl Cancer Inst* **54**: 587-591

Singer D, Biel M, Lotan I, Fockerzi V, Hofmann F, Dascal N. 1991. The roles of the subunits in the function of the calcium channel. *Science* **253**: 1553-7

Sjogren C, Andersson KE, Husted S, Mattiasson A, Moller-Madsen B. 1982. Atropine resistance of transmurally stimulated isolated human bladder muscle. *J Urol* **128**: 1368-1371

Sjogren C, Andersson KE, Mattiasson A. 1985. Effects of vasoactive intestinal polypeptide on isolated urethral and urinary bladder smooth muscle from rabbit and man. *J Urol* **133**: 136-140

Sjuve R, Arner A, Li Z, Mies B, Paulin D, Schmittner M, Small JV. 1998. Mechanical alterations in smooth muscle from mice lacking desmin. *J Muscle Res Cell Motil* 19: 415-429

Sjuve R, Haase H, Ekblad E, Malmqvist U, Morano I, Arner A. 2001. Increased expression of non-muscle myosin heavy chain-B in connective tissue cells of hypertrophic rat urinary bladder. *Cell Tissue Res* **304(2)**: 271-8

Slanina P, Frech W, Ekstrom LG, Loof L, Slorach S, Cedergren A. 1986. Dietary citric acid enhances absorption of aluminum in antacids. *Clin Chem* **32(3)**: 539-41

Slobodan J M, Dragen M, Milovan M, Violeta I-C. 1999. The effects of excitatory amino acids on isolated gut segments of the rats. *Pharmacol Res* **39**: 143-148

Small JV, Gimona M. 1998. The cytoskeleton of the vertebrate smooth muscle cell. *Acta Physiol Scand* **164**: 341-348

Smet PJ, Jonavicius J, Marshall VR, de Vente J. 1996. Distribution of nitric oxide synthase-immunoreactive nerves and identification of the cellular targets of nitric oxide in guinea-pig and human urinary bladder by cGMP immunohistochemistry. *Neuroscience* **71**: 337-348

Smeulders N, Woolf AS, Wilcox DT. 2002. Smooth muscle differentiation and cell turnover in mouse detrusor development. *J Urol* **167(1)**: 385-90

Smith S, Swain J, Brown EM, Wyshak G, Albright T, Ravnikar VA, Schiff I. 1989. A preliminary report of the short-term effect of carbonated beverage consumption on calcium metabolism in normal women. *Arch Intern Med* **149**: 2517-9

Smith DJ, Chapple CR. 1994. In vitro response of human bladder smooth muscle in unstable obstructed male bladders: a study of pathophysiological causes? *Neurourol Urodyn* **34**: 14-15

Smith DV, Margolskee RF. 2001. Making sense of taste. *Sci Am* **284**: 32-39

Smith PP, McCrery RJ, Appell RA. 2006. Current trends in the evaluation and management of female urinary incontinence. *Canadian Med Asso J* **175(10)**: 1233-40

Snell RD. 2006. Clinical Neuroanatomy, 6th edition, Chapter **14:** 396-397

Snooks SJ, Barnes PR, Swash M, Henry MM. 1985. Damage to the innervation of the pelvic floor musculature in chronic constipation. *Gastroenterology* **89**: 977-81

Snooks SJ, Swash M, Henry MM, Setchell M. 1986. Risk factors in childbirth

causing damage to the pelvic floor innervation. *Int J Colorectal Dis* **1(1)**: 20-4

Somlyo AP, Somlyo AV. 1970. Vascular smooth muscle. II. Pharmacology of normal and hypotensive vessels. *Pharmacol Rev* **22**: 249-353

Somlyo AV. 1980. Ultrastructure of vascular smooth muscle. In: *Vascular Smooth Muscle*, edited by Bohr DF, Somlyo AP, and Sparks HV. Bethesda, MD: Am. Physiol. Soc.

Somlyo AP, Walker JW, Goldman YE, Trentham DR, Kobayashi S, Kitazawa ST, Somlyo AV. 1988. Inositol trisphosphate, calcium and muscle contraction. *Phil Trans Roy Soc London B.* **320**: 399-414

Somlyo AP. 1993. Myosin isoforms in smooth muscle: how may they affect function and structure? *J Muscle Res Cell Motil* **14**: 557-563

Somlyo AP, Somlyo AV. 1994. Signal transduction and regulation in smooth muscle. *Nature* **372**: 231-36

Somlyo AP, Wu X, Walker LA, Somlyo AV. 1999. Pharmacomechanical coupling: the role of calcium, G-proteins, kinases and phosphatases. *Rev Physiol Biochem Pharmacol* **134**: 201-234

Somlyo AP, Somlyo AV. 2000. Signal transduction by G-proteins, rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. *J Physiol* **522**: 177-185

Somlyo AP, Somlyo AV. 2003. Ca^{2+} sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiol Rev* **83**: 1325-1358

Somogyi GT, de Groat WC. 1990. Modulation of the release of [3H] norepinephrine from the base and body of the rat urinary bladder by endogenous adrenergic and cholinergic mechanisms. *J Pharmacol Exp Ther* **255(1)**: 204-10

Somogyi GT, de Groat WC. 1992. Evidence for inhibitory nicotinic and facilitatory muscarinic receptors in cholinergic nerve terminals of the rat urinary bladder. *J Auton Nerv Syst* **37:** 89-97.

Spencer H, Menczel J, Lewin I, Samachson J. 1965. Effect of high phosphorus

intake on calcium and phosphorus metabolism in man. *J Nutr* **86**: 125-32

Spencer H, Kramer L, Osis D, Norris C. 1978. Effect of phosphorus on the absorption of calcium and on the calcium balance in man. *J Nutr* **108**: 447-57

Spielman AL, Huque T, Whitney G, Brand JG. 1992. The diversity of bitter taste signal transduction mechanisms. *Soc Gen Physiol Series* **47**: 307-324

Squire RA. 1985. Histopathological evaluation of rat urinary bladders from the IRDC two-generation bioassay of sodium saccharin. *Food Chem Toxicol* **23**: 491-497

Steers WD. 1994. Rat: overview and innervation. *Neurourol Urodyn* **13**: 97-118

Steers WD. 2002. Pathophysiology of Overactive Bladder and Urge Urinary Incontinence. *Rev Urol* **4(Suppl 4)**: S7-S18

Stewart WF, Van Rooyen JB, Cundiff GW, Abrams P, Herzog AR, Corey R, Hunt TL, Wein AJ. 2003. Prevalence and burden of overactive bladder in the United States. *World J Urol* **20**: 327-336

Striem BJ, Pace U, Zehavi U, Naim M, Lancet D. 1989. Sweet tastants stimulate adenylate cyclase coupled to GTP-binding protein in rat tongue membranes. *Biochem J* **260:** 121-126

Strubelt O, Diederich KW. 1999. Experimental treatment of the acute cardiovascular toxicity of caffeine. *J Toxicol Clin Toxicol* **37**: 29-33

Stull JT, Kamm KE, Taylor DA. 1988. Calcium control of smooth muscle contraction. *Am J Med Sci* **31**: 241-5

Stull JT, Hsu LC, Tansey MG, Kamm KE. 1990. Myosin light chain kinase phosphorylation in tracheal smooth muscle. *J Biol Chem* **265**: 16683-16690

Stull JT, Krueger JK, Kamm KE, Gao ZH, Zhi G, Padre R. 1996. Myosin light chain kinase. In: *Biochemistry of Smooth Muscle*, edited by Bárány M. New York: Academic, p. 119-130

Sturgeon SR, Hartge P, Silverman DT, Kantor AF, Linehan WM, Lynch C, Hoover RN. 1994. Associations between bladder cancer risk factors and tumor stage and grade at diagnosis. *Epidemiology* **5(2)**: 218-25

Sugaya K, Nishijima S, Miyazato M, Ogawa Y. 2005. Central nervous control of micturition and urine storage. *J Smooth Mus Res* **41(3)**: 117-32

Sugita M, Tokutomi N, Tokutomi Y, Terasaki H, Nishi K. 1998. The properties of caffeine- and carbachol-induced intracellular Ca2+ release in mouse bladder smooth muscle cells. *Eue J Pharmacol* **348(1)**: 61-70

Sundin T, Dahlstrom A. 1973. The sympathetic innervation of the urinary bladder and urethra in the normal state and after parasympathetic denervation at the spinal root level. An experimental study in cats. *Scand J Urol Nephrol* **7**: 131-149

Suzuki S, Platz EA, Kawachi I, Willett WC, Giovannucci E. 2002. Intakes of energy and macronutrients and the risk of benign prostatic hyperplasia. *Am J Clin Nutr* **75**: 689-97

Swartz RD, Wesley JR, Somermeyer MG, Lau K. 1984. Hyperoxaluria and renal insufficiency due to ascorbic acid administration during total parenteral nutrition. *Ann Intern Med* **100(4)**: 530-1

Szell EA, Yamamoto T, de Groat WC, Somogyi GT. 2000. Smooth muscle and parasympathetic nerve terminals in the rat urinary bladder have different subtypes of alpha(1) adrenoceptors. *Br J Pharmacol* **130**: 1685-91

Tagliani M, Candura SM, Di Nucci A, Franceschetti GP, D'Agostino G, Ricotti P, Fiori E, Tonini M. 1997. A re-appraisal of the nature of the atropine-resistant contraction to electrical field stimulation in the human isolated detrusor muscle. *Naunyn-Schmiedebergs Arch Pharmacol* **356**: 750-755

Taira N. 1972. The autonomic pharmacology of the bladder. *Annu Rev Pharmacol* **12**: 197-208

Takahashi K, Tsuchida K, Tanabe Y, Masu M, Nakanishi S. 1993. Role of the large extracellular domain of metabotropic glutamate receptors in agonist selectivity determination. *J Biol Chem* **268**: 19341-19345

Takayama S, Sieber SM, Adamson RH, Thorgeirsson UP, Dalgard DW, Arnold LL, Cano M, Eklund S, Cohen SM. 1998. Long-term feeding of sodium saccharin to nonhuman primates: implications for urinary tract cancer. *J Natl Cancer Inst* **90**: 19-25

Takayama S, Renwick AG, Johansson SL, Thorgeirsson UP, Tsutsumi M, Dalgard DW, Sieber SM. 2000. Long-term toxicity and carcinogenicity study of cyclamate in nonhuman primates. *Toxicol Sci* **53**: 33-39

Takeda M, Obara K, Mizusawa T, Tomita Y, Arai K, Tsutsui T, Hatano A, Takahashi K, Nomura S. 1999. Evidence for beta3-adrenoceptor subtypes in relaxation of the human urinary bladder detrusor: analysis by molecular biological and pharmacological methods. *J Pharmacol Exp Ther* **288**: 1367-1373

Tampakoudis P, Tantanassis T, Grimbizis G, Papaletsos M, Mantalenakis S. 1995. Cigarette smoking and urinary incontinence in women--a new calculative method of estimating the exposure to smoke. *Eur J Obstet Gynecol Rep Biol* **63(1)**: 27-30

Tang J, Taylor DW, Taylor KA. 2001. The three-dimensional structure of alpha-actinin obtained by cryoelectron microscopy suggests a model for Ca^{2+}-dependent actin binding. *J Mol Biol* **310**: 845-858

Tanabe Y, Masu M, Ishii T, Shigemoto R, Nakanishi S. 1992. A family of metabotropic glutamate receptors. *Neuron* **8(1)**: 169-179

Tanagho EA. 1992. Surgical anatomy of the genitourinary tract: anatomy of the lower urinary tract. In: Walsh P.C., Retik, A.B., Stamey, T.A. Campbell's Urology, 6^{th} ed. Philadelphia: Saunders.

Tanagho EA, Smith DR. 1966. The anatomy and function of the bladder neck. *Br J Urol* **38**: 54-57

Tanagho EA, Miller ER. 1970. Initiation of voiding. *Br J Urol* **42**: 175- 83

Taniguchi K. 2004. Expression of sweet receptor protein, T1R3, in human liver and pancreas. *J Vet Med Sci* **66(11)**: 1311-1314

Taylor HC, Watt CH. 1917. Incontinence of urine in women. *Surg Gynecol Obstet* **24**: 296

Taylor JM, WeinbergerMA, Friedman L. 1980. Chronic toxicity and carcinogenicity to the urinary bladder of sodium saccharin in the in utero-exposed rat. *Toxicol Appl Pharmacol* **54**: 57-75

Teloken C, Caraver F, Weber FA, Teloken PE, Moraes JF. 2006. Overactive bladder: Prevalence and implications in Brazil. *Eur Urol* **49(6)**: 1087-1092

Temml C, Heidler S, Ponholzer A, Madersbacher S. 2005. Prevalence of the overactive bladder syndrome by applying the International Continence Society definition. *Eur Urol* **48(4)**: 622-627

Teo JS, Briffa NK, Devine A, Dhaliwal SS, Prince RL. 2006. Do sleep problems or urinary incontinence predict falls in elderly women? *Aust J Physiother* **52(1)**: 19-24

Terada M, Hayashi S, Hayashi E. 1980. Spasmogenic effects of L-ascorbic acid on the guinea pig isolated ileum preparation. *Jpn J Pharmacol* **30(1)**: 21-7

Tham DM, Gardner CD, Haskell WL. 1988. Potential health benefits of dietary phytoestrogens: a review of the clinical, epidemiological, and mechanistic evidence. *J Clin Endocrinol Metab* **83**: 2223-35

Theobald R. 1982. Arylazido aminopropionyl-ATP (ANAPP3) antagonism of cat urinary bladder contractions. *J Auton Pharmacol* **3**: 175-179

Theobald RJ Jr. 1995. Purinergic and cholinergic components of bladder contractility and flow. *Life Sciences* **56(6)**: 445-54

Theobald RJ Jr. 1996. The effect of N^G-monomethyl-L-arginine on bladder function. *Eur J Pharmacol* **311**: 73-78

Thomas JC, Demarco RT, Pope JC. 2005. Molecular biology of ureteral bud and trigonal development. *Curr Urol Rep* **6(2)**: 146-51

Thor KB, Katofiasc MA. 1995. Effects of duloxetine, a combined serotonin and norepinephrine reuptake inhibitor, on central neural control of lower urinary tract function in the chloralose-anesthetized female cat. *J Pharmacol Exp Ther* **274(2)**: 1014-24

Thor KB, Katofiasc MA, Danuser H, Springer J, Schaus JM. 2002. The role of 5-HT(1A) receptors in control of lower urinary tract function in cats. *Brain Res* **946(2)**: 290-7

Thor KB, Donatucci C. 2004. Central nervous system control of the lower urinary tract: new pharmacological approaches to stress urinary incontinence in women. *J Urol* **172(1)**: 27-33

Thorneloe KS, Nelson MT. 2003. Properties and molecular basis of the mouse urinary bladder voltage-gated K^+ current. *J Physiol* **549**: 65-74

Thyssen HH, Clevin L, Olesen S, Lose G. 2002. Urinary incontinence in elite female athletes and dancers. *Int Urogynecol J* **13(1)**: 15-7

Tikkinen KA, Tammela TL, Rissanen AM, Valpas A, Huhtala H. Auvinen A. 2007. Is the Prevalence of Overactive Bladder Overestimated? A Population-Based Study in Finland. *PLoS ONE* **2(2)**: 195

Tobin G, Sjogren C. 1995. In vivo and in vitro effects of muscarinic receptor antagonists on contractions and release of [^{3}H] acetylcholine in the rabbit urinary bladder. *Eur J Pharmacol* **281**: 1-8

Tomlinson BU, Dougherty MC, Pendergast JF, Boyington AR, Coffman MA, Pickens SM. 1999. Dietary caffeine, fluid intake and urinary incontinence in older rural women. *Int Urogynecol J* **10(1)**: 22-8

Tones MA, Bendali H, Flor PJ, Knopfel T, Kuhn R. 1995. The agonist selectivity of a class III metabotropic glutamate receptor, human mGluR4a, is determined by the N-terminal extracellular domain. *Neuroreport* **7**: 117-120

Tong YC, Hung YC, Lin SN, Cheng JT. 1997a. Pharmacological characterization of the muscarinic receptor subtypes responsible for the contractile response in the rat urinary bladder. *J Auton Pharmacol* **17:** 21-25

Tong YC, Hung YC, Shinozuka K, Kunitomo M, Cheng JT. 1997b. Evidence of adenosine 5'-triphosphate release from nerve and P2x-purinoceptor mediated contraction during electrical stimulation of rat urinary bladder smooth muscle. *J Urol* **158(5)**: 1973-7

Tong YC, Cheng JT. 2002. Alterations of M3 subtype muscarinic receptors in the diabetic rat urinary bladder. *Pharmacology* **64**: 148-151

Tonosaki K, Funakoshi M. 1988. Cyclic nucleotides may mediate taste transduction.

Nature **331:** 354-356

Towbin H, Staehelin T, Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* **76(9)**: 4350-4

Traish A, Moran E, Krane RJ, Saenz DTI. 1992. Endothelin in the urinary bladder. II. Characterization of endothelin receptor subtypes. *J Urol* **148**: 1299-1306

Travo P, Weber K, Osborn M. 1982. Co-existence of vimentin and desmin type intermediate filaments in a subpopulation of adult rat vascular smooth muscle cells growing in primary culture. *Exp Cell Res* **139**: 87-94

Truss MC, Uckert S, Stief CG, Forssmann WG, Jonas U. 1996. Cyclic nucleotide phosphodiesterase (PDE) isoenzymes in the human detrusor smooth muscle. II Effect of various PDE inhibitors on smooth muscle tone and cyclic nucleotide levels in vitro. *Urol Res* **24**: 129-134

Tsao CS. 1984. Equilibrium constant for calcium ion and ascorbate ion. *Experientia.* **40**: 168-170

Tubaro A, Palleschi G. 2005. Overactive bladder: epidemiology and social impact. *Curr Opin Obstet Gynecol* **17**: 507-511

Tucker KL, Morita K, Qiao N, Hannan MT, Cupples LA, Kiel DP. 2006. Colas, but not other carbonated beverages, are associated with low bone mineral density in older women: The Framingham Osteoporosis Study. *Am J Clin Nutr* **84(4)**: 936-42

Turner WH, Brading AF. 1997. Smooth muscle of the bladder in the normal and the diseased state: pathophysiology, diagnosis and treatment. *Pharmacol Ther* **75**: 77-110

Uchida Y, Sato T. 1997. Changes in outward K+ currents in response to two types of sweeteners in sweet taste transduction of gerbil taste cells. *Chem Senses* **22:** 163-169

Uchiyama T, Chess-Williams R. 2004. Muscarinic receptor subtypes of the bladder and gastrointestinal tract. *J Smooth Mus Res* **40(6)**: 237-47

Uckert S, Stief CG, Lietz B, Burmester M, Jonas U, Machtens SA. 2002. Possible role of bioactive peptides in the regulation of human detrusor smooth muscle. *World J*

Urol **20**: 244-249

Ursillo RC. 1961. Electrical activity of the isolated nerve-urinary bladder strip preparation of the rabbit. *Am J Physiol* **201**: 408-412

Uvelius, B. 1976. Isometric and isotonic length-tension relations and variations in cell length in longitudinal smooth muscle from rabbit urinary bladder. *Acta Physiol Scand* **97**: 1-12

Uvelius B, Gabella G. 1980. Relation between cell length and force production in urinary bladder smooth muscle. *Acta Physiol Scand* **110**: 357-365

Uvelius B. 2001. Length-tension relations of in vitro urinary bladder smooth muscle strips. *J Pharmacol Toxicol Methods* **45**: 87-90

Valera S, Hussy N, Evans RJ, Adami N, North RA, Surprenant A, Buell G. 1994. A new class of ligand-gated ion channel defined by P2X receptor for extracellular ATP. *Nature (Lond)* **371**: 516-519

Van Soeren MH, Graham TE. 1998. Effect of caffeine on metabolism, exercise endurance, and catecholamine responses after withdrawal. *J Appl Physiol* **85**: 1493-1501

Vandekerckhove J, Weber K. 1979. The complete amino acid sequence of actins from bovine aorta, bovine heart, bovine fast skeletal muscle, and rabbit slow skeletal muscle. A protein-chemical analysis of muscle actin differentiation. *Differentiation* **14**: 123–133

Vanderhorst VG, Mouton LJ, Blok BF, Holstege G. 1996. Distinct cell groups in the lumbosacral cord in the cat project to different areas in the periaqueductal gray. *J Comp Neurol* **376**: 361-85

van der Vaart CH, de Leeuw JR, Roovers JP, Heintz AP. 2002. The effect of urinary incontinence and overactive bladder symptoms on quality of life in young women. *BJU Int* **90**: 544-549

Varkevisser B, Kinnamon SC. 2000. Sweet taste transduction in hamster: role of protein kinases. *J Neurophysiol.* **83**: 2526-2532

Vaughan CW, Satchell PM. 2003. Urine storage mechanisms. *Prog Neurobiol* **46**: 215-37

Vecchia LC, Altieri A, Tavani A. 2001. Vegetables, fruit, antioxidants and cancer: a review of Italian studies. *Eur J Nutr* **40**: 261-7

Vecchia LA, Chatenoud LA, Altieri A, Tavani A. 2002. Nutrition and health: epidemiology of diet, cancer and cardiovascular disease in Italy. *Nutr Metab Cardiovasc Dis* **11(4 Suppl)**: 10-5

Venazi TJ, Venazi CA. 1988. A molecular electrostatic-potential study of acesulfame. *Anal Chim Acta* **210**: 213-218

Verly W. 1948. *Arch internat physiol* **56**: 1

Vial C, Evans RJ. 2000. P2X receptor expression in mouse urinary bladder and the requirement of P2X(1) receptors for functional P2X receptor responses in the mouse urinary bladder smooth muscle. *Br J Pharmacol* **131**: 1489-1495

Vigod SN, Stewart DE. 2006. Major depression in female urinary incontinence. *Psychosomatics* **47(2)**: 147-51

Viktrup L, Lose G, Rolff M, Barfoed K. 1992. The symptom of stress incontinence caused by pregnancy or delivery in primiparas. *Obstet Gynecol* **79**: 945-949

Visser AJ, van Mastrigt R. 2000a. The role of intracellular and extra cellular calcium in mechanical and intracellular electrical activity of human urinary bladder smooth muscle. *Urol Res* **28**: 260-268

Visser AJ, van Mastrigt R. 2000b. Simultaneous recording of mechanical and intracellular electrical activity in human urinary bladder smooth muscle. *BJU Int* **86**: 113-120

Visser AJ, van Mastrigt R. 2001. Intracellular electrical activity in human urinary bladder smooth muscle: the effect of high sucrose medium. *Urol Int* **66**: 205-211

Wagg A, Majumdar A, Toozs-Hobson P, Patel AK, Chapple CR, Hill S. 2007. Current and future trends in the management of overactive bladder. *Intl Urogynecol J* **18(1)**: 81-94

Wakabayashi Y, Tomoyoshi T, Fujimiya M, Arai R, Maeda T. 1993. Substance P-containing axon terminals in the mucosa of the human urinary bladder: pre-embedding immunohistochemistry using cryostat sections for electron microscopy. *Histochemistry* **100**: 401-7

Wakabayashi Y. Makiura Y. Tomoyoshi T. Kitahama K. Geffard M. Maeda T. 1994. Adrenergic innervation of the urinary bladder body in the cat with special reference to structure of the detrusor muscle: an immunohistochemical study of noradrenaline and its synthesizing enzymes. *Arch Histol Cytol* **57(3)**: 277-89

Walden PD, Durkin MM, Lepor H, Wetzel JM, Gluchowski C, Gustafson EL. 1997. Localization of mRNA and receptor binding sites for the alpha 1a-adrenoceptor subtype in the rat, monkey and human urinary bladder and prostate. *J Urol* **157**: 1032-1038

Wang P, Luthin GR, Ruggieri MR. 1995. Muscarinic acetylcholine receptor subtypes mediating urinary bladder contractility and coupling to GTP binding proteins. *J Pharmacol Exp Ther* **273**: 959-966

Wang EC, Lee JM, Ruiz WG, Balestreire EM, von Bodungen M, Barrick S, Cockayne DA, Birder LA, Apodaca G. 2005. ATP and purinergic receptor-dependent membrane traffic in bladder umbrella cells. *J Clin Investigation* **115(9)**: 2412-22

Wede OK, Lofgren M, Li Z, Paulin D, Arner A. 2002. Mechanical function of intermediate filaments in arteries of different size examined using desmin deficient mice. *J Physiol* **540**: 941-949

Weihrauch MR, Diehl V. 2004. Artificial sweeteners--do they bear a carcinogenic risk? *Ann Oncol* **15(10)**: 1460-5

Wein AJ, Rackley RR. 2006. Overactive bladder: a better understanding of pathophysiology, diagnosis and management. *J Urol* **175(3 Pt 2)**: S5-10

Wellner MC, Isenberg G. 1993. Properties of stretch-activated channels in myocytes from the guinea-pig urinary bladder. *J Physiol* **466**: 213-227

Westby M, Asmussen M, Ulmsten U. 1982. Location of maximum intraurethral

pressure related to urogenital diaphragm in the female subject as studied by simultaneous urethra-cystometry and voiding urethrocystography. *Am J Obstet Gynecol* **144**: 408-12

Wetle T, Scherr P, Branch LG, Resnick NM, Harris T, Evans D, Taylor JO. 1995. Difficulty with holding urine among older persons in a geographically defined community: prevalence and correlates. *J Am Geriatr Soc* **43(4)**: 349-55

Whalley NA, Meyers AM, Martins M, Margolius LP. 1996. Long-term effects of potassium citrate therapy on the formation of new stones in groups of recurrent stone formers with hypocitraturia. *Br J Urol* **78(1)**: 10-4

Whysner J, Williams GM. 1996. Saccharin mechanistic data and risk assessment: urine composition, enhanced cell proliferation, and tumor promotion. *Pharmacol Ther* **71**: 225-252

Wibberley A, Chen Z, Hu E, Hieble JP, Westfall TD. 2003. Expression and functional role of Rho-kinase in rat urinary bladder smooth muscle. *Br J Pharmacol* **138**: 757-766

Wickenden A. 2002. K^+ channels as therapeutic drug targets. *Pharmacol Ther* **94**: 157-182

Wilson LA, Wilkinson K, Crews HM, Davies AM, Dick CS, Dumsday VL. 1999. Urinary monitoring of saccharin and acesulfame K as biomarkers of exposure to these additives. *Food Additives Contam* **16**: 227-238

Wilson L, Brown JS, Shin G, Luc KO, Subak LL. 2001. Annual Direct Cost of Urinary Incontinence. *Am J Obstet Gynecol* **98(3)**: 398-406

Winkelmayer WC, Stampfer MJ, Willett WC, Curhan GC. 2005. Habitual caffeine intake and the risk of hypertension in men. *JAMA* **294(18)**: 2330-2335

Wise KJ, Bergman EA, Sherrard DJ, Massey LK. 1996. Interactions between dietary calcium and caffeine consumption on calcium metabolism in hypertensive humans. *Am J Hypertens* **9**: 223-9

Wojdan A, Freeden C, Woods M, Oshiro G, Spinelli W, Colatsky TJ, Sheldon JH, Norton NW, Warga D, Antane MM, Antane SA, Butera JA, Argentieri TM.

1999. Comparison of the potassium channel openers, WAY-133537, ZD6169, and celikalim on isolated bladder tissue and In vivo bladder instability in rat. *J Pharmacol Exp Ther* **289 (3)**: 1410-8

Wolfe F, Russell IJ, Vipraio G, Ross K. Anderson J. 1997. Serotonin levels, pain threshold, and fibromyalgia, symptoms in the general population. *J Rheumatol* **24**: 555-559

Wolraich ML, Lindgren SD, Stumbo PJ, Stegink LD, Appelbaum MI, Kiritsy MC. 1994. Effects of diets high in sucrose or aspartame on the behaviour and cognitive performance of children. *N Eng J Med* **330**: 301-7

Wong SY. Hong A. Leung J. Kwok T. Leung PC. Woo J. 2006. Lower urinary tract symptoms and depressive symptoms in elderly men. *J Affect Disord* **96(1-2)**: 83-8

World Cancer Resarch Fund/American Institute for Cancer Research. 1997. Food research and the prevention of cancer: a global perspective. Washington, DC: American Institute for Cancer Research

Wu C, Fry CH. 1997a. The role of the L-type Ca^{2+} channel in cholinergic pathways in guinea-pig detrusor smooth muscle. *J Physiol* **499**: 7-8

Wu C, Sui G, Fry CH. 1997b. Spontaneous action potential and intracellular Ca^{2+} transients in human detrusor smooth muscle cells isolated from stable and unstable bladders. *Br J Urol* **80**: 164

Wu C, Bayliss M, Newgreen D, Mundy AR, Fry CH. 1999. A comparison of the mode of action of ATP and carbachol on isolated human detrusor smooth muscle. *J Urol* **162**: 1840-1847

Wu C, Sui G, Fry CH. 2002. The role of the L-type Ca^{2+} channel in refilling functional intracellular Ca^{2+} stores in guinea-pig detrusor smooth muscle. *J Physiol* **538**: 357-369

Wu Y, Ma J. 2004. Refinement of F-actin model against fiber diffraction data by long-range normal modes. *Biophysical J* **86**:116-24

Wurtman RJ. 1983. Neurochemical changes following high-dose aspartame with

dietary carbohydrates. *N Engl J Med* **309**: 429-430

Wyndaele JJ. 2001. The overactive bladder. *BJU Int* **88(2)**: 135-40

Wynder EL, Goldsmith R. 1977. The epidemiology of bladder cancer: a second look. *Cancer* **40**: 1246-1268

Wynder EL, Stellman SD. 1980. Artificial sweetener use and bladder cancer: a case-control study. *Science* **207**: 1214-1216

Wyshak G, Frisch RE, Albright TE, Albright NL, Schiff I, Witschi J. 1989. Nonalcoholic carbonated beverage consumption and bone fractures among women former college athletes. *J Orthop Res* **7**: 91-9

Wyshak G, Frisch RE. 1994. Carbonated beverages, dietary calcium, the dietary calcium/phosphorus ratio, and bone fractures in girls and boys. *J Adolesc Health* **15**: 210-5

Xu L, Lai FA, Cohn A, Etter E, Guerrero A, Fay FS, Meissner G. 1994. Evidence for a Ca^{2+}-gated ryanodine-sensitive Ca^{2+} release channel in visceral smooth muscle. *Proc Natl Acad Sc. USA* **91**: 3294-3298

Xu JQ, Harder BA, Uman P, Craig R. 1996. Myosin filament structure in vertebrate smooth muscle. *J Cell Biol* **134**: 53-66

Xu H, Staszewski L, Tang H, Adler E, Zoller M, Li X. 2004. Different functional roles of T1R subunits in the heteromeric taste receptors. *Proc. Natl Acad Sci USA* **101**: 14258-14263

Yamaguchi O, Shishido K, Tamura K. 1996. Evaluation of mRNAs encoding muscarinic receptor subtypes in human detrusor muscle. *J Urol* **156**: 1208-13

Yamaguchi O. 2002. Beta3-adrenoceptors in human detrusor muscle. *Urology* **59**: 25-29

Yamanishi T, Chapple CR, Chess-Williams R. 2001. Which muscarinic receptor is important in the bladder? *World J Urol* **19(5)**: 299-306

Yono M, Yoshida M, Takahashi W, Inadome A, Ueda S. 2000. Comparison of the effects of novel antimuscarinic drugs on human detrusor smooth muscle. *BJU Int* **86**: 719-725

Yong LC, Brown CC, Schatzkin A, Dresser CM, Slesinski MJ, Cox CS, Taylor PR. 1997. Intake of vitamins E, C, and A and risk of lung cancer. *Am J Epidemiol* **146**: 231-43

Yoshida M, Homma Y, Inadome A, Yono M, Seshita H, Miyamoto Y, Murakami S, Kawabe K, Ueda S. 2001. Age-related changes in cholinergic and purinergic neurotransmission in human isolated bladder smooth muscles. *Exp Gerontol* **36**: 99-109

Yoshida M, Inadome A, Maeda Y, Satoji Y, Masunaga K, Sugiyama Y, Murakami S. 2006. Non-neuronal cholinergic system in human bladder urothelium. *Urology* **67(2)**: 425-30

Yoshimura N, Seki S, Chancellor MB, de Groat WC, Ueda T. 2002. Targeting afferent hyperexcitability for therapy of the painful bladder syndrome. *Urology* **59(5 Suppl 1)**: 61-7

Yoshino M, Yabu H. 1995. Muscarinic suppression of Ca^{2+} current in smooth muscle cells of the guinea-pig urinary bladder. *Exp Physiol* **80**: 575-587

Yoshino M, Matsufuji Y, Yabu H. 1996. Voltage-dependent suppression of calcium current by caffeine in single smooth muscle cells of the guinea-pig urinary bladder. *Naunyn-Schmiedebergs Archives of Pharmacology* **353(3)**: 334-41

Zamburlini A, Maiorino M, Barbera P, Roveri A, Ursini F. 1995. Direct measurement by single photon counting of lipid hydroperoxides in human plasma and lipoproteins. *Anal Biochem* **232**:107–13

Zhao GQ, Zhang Y, Hoon MA, Chandrashekar J, Erlenbach I, Ryba NJ, Zuker CS. 2003. The receptors for mammalian sweet and umami taste. *Cell* **115**: 255-266

Zhang Y, Hoon MA, Chandrashekar J, Mueller KL, Cook B, Wu D, Zuker CS, Ryba NJP. 2003. Coding of Sweet, Bitter, and Umami Tastes: Different Receptor Cells Sharing Similar Signaling Pathways. *Cell* **112**: 293-301

Zhou X, Galligan JJ. 1996. P2X purinoceptors in cultured myenteric neurons of guinea-pig small intestine. *J Physiol (Lond)* **496:** 719-729

Zhu Y, Guo Y, Ye M, James FS. 2005. Separation and simultaneous determination of four artificial sweeteners in food and beverages by ion chromatography. *J Chromatogr A* **1085**: 143-146

Zimmerman PM, Pulliam J, Schwengels J, MacDonald SE. 1985. Caffeine intoxication: a near fatality. *Ann Emerg Med* **14**: 1227–9

Zorn BH, Montgomery H, Pieper K, Gray M, Steers WD. 1999. Urinary incontinence and depression. *J Urol* **162**: 82-84

Weblinks

URL A:
http://academic.kellogg.cc.mi.us/herbrandsonc/bio201_McKinley/f27-9a_urinary_bladder_c.jpg (updated 14.06.2007)

URL 1:
http://www.canmakers.co.uk/industry/can_makers_report_2002_print.asp (updated 14.11.2007)

URL2:
http://www.bbc.co.uk/health/healthy_living/nutrition/drinks_soft2.shtml#carbonated_soft_drinks (updated 28.05.2007)

URL3:
http://www.cokecce.co.uk/cce/aboutus/brands.html (updated 28.05.2007)

URL4:
http://en.wikipedia.org/wiki/Sodium_saccharin (updated 10.06.2007)

URL5:
http://en.wikipedia.org/wiki/Acesulfame_potassium (updated 10.06.2007)

URL6:
http://en.wikipedia.org/wiki/Aspartame (updated 10.06.2007)

URL7:
http://www.nealkonapoje.sk/add/2005TheRoleOfSoftDrinksInHealthAndNutrition.doc (updated: 29.05.2007)

URL8:
http://en.wikipedia.org/wiki/Ascorbic_acid (updated 10.06.2007)

URL9:
http://www.food.gov.uk/multimedia/pdfs/evm_c.pdf (updated 29.05.2007)

URL10:
http://en.wikipedia.org/wiki/Citric_acid (updated 10.06.2007)

URL11:
http://www.expasy.org/cgi-bin/niceprot.pl?Q8TE23 (updated 14.06.2007)

URL12:
http://www.sanger.ac.uk/cgi-bin/Pfam/swisspfamget.pl?name=Q8TE23(updated 14.06.2007)

APPENDIX

ABSTRACTS AND PUBLISHED PAPERS

Abstract for IUGA Buenos Aires, Argentina, October 2003

THE EFFECT OF FIZZY DRINK COMPONENTS ON THE CONTRACTILE RESPONSE OF ISOLATED DETRUSOR SMOOTH MUSCLE.

J Dasgupta[1], RA Elliott[2], DG Tincello[1].
[1]Obstetrics and Gynaecology Department & [2]Division of Medicine for the Elderly, University of Leicester, Leicester General Hospital, Gwendolen Road, Leicester. LE5 4PW

Objective

The Leicestershire Incontinence Study collected epidemiological data on 250,000 community dwelling women. Multivariate analysis revealed daily consumption of fizzy drinks to be independently associated with onset of OAB symptoms within the next twelve months (OR 1.62, 95% CI 1.18, 2.22) (Dallosso et al 2003)
Typical components of fizzy drinks are phenylalanine, citric acid, ascorbic acid, sweeteners, preservatives, antioxidants, stabilisers, flavourings and colourings. We investigated the hypothesis that carbonated soft drinks modulate detrusor muscle function by studying the effects of individual ingredients on the contractile response of rat detrusor muscle strips and determining their mechanism of action.

Methods

Bladders were removed from Wistar rats (200-300kg) and placed in Kreb's solution. Strips of bladder muscle (4 x 0.5 x 1 mm) were suspended in an organ bath chamber (0.2 ml) perfused with Kreb's solution heated to 37°C & aerated with 95% O_2 and 5% CO_2. Tissues were allowed to equilibrate for 1 hour under tension of 10 g. The effect of increasing concentrations (10^{-6}M- 10^{-2}M) of each component of fizzy drinks on the contractile response to a single frequency (10 Hz) of electrical field stimulation (EFS) was determined. Those components found to have a significant effect on detrusor muscle contraction were investigated further. Frequency response curves were obtained in the absence and presence of atropine 10^{-6} M by stimulating the samples with 0.5,1,5,10,20,40 and 60 Hz. Dose response curves were obtained by the addition of 20 second exposures to increasing concentrations of carbachol (10^{-8} M – 10^{-4} M).

Results

Benzoic acid and potassium sorbate had no effect on the contractile response of detrusor muscle. Aspartame 10^{-8} M ($p<0.01$) and phenylalanine (10^{-8} M ($p<0.05$) and 10^{-3} M ($p<0.05$)) enhanced the contractile response to 10 Hz EFS. Ascorbic acid 10^{-8} M – 10^{-3} M ($p<0.05$ – $p<0.001$), acesulfame k 10^{-8} M – 10^{-2}M ($p<0.01$ – $p<0.001$) and citric acid 10^{-7} M – 10^{-3} M ($p<0.05$ – $p<0.001$) all enhanced the contractile response of isolated detrusor muscle to 10 Hz EFS. However, citric acid 10^{-2} M completely abolished the response. Ascorbic acid had a concentration dependent enhancing effect on the frequency response curve in the absence and presence of atropine 10^{-6} M. These effects reached significance with ascorbic acid 10^{-4}M but had a slightly inhibitory effect on the carbachol dose response curve.

Conclusions

We have demonstrated here that some components of fizzy drinks enhance the contraction of bladder smooth muscle and that ascorbic acid 10^{-4}M significantly enhances the contractile response of bladder smooth muscle to the atropine resistant component of electrical field stimulation. This component is adenosine triphosphate (ATP) and ascorbic acid may be acting by either enhancing ATP release from the nerves, modulating ectoATPases, or modulating Ca^{2+} influx via L-type calcium channels. Further investigations are in progress to elucidate these mechanisms.

ICS/IUGA Annual Meeting Annual Meeting
Paris, France. 25th – 27th August 2004

Abstract Title:

Do artificial sweeteners affect bladder contraction?

Abstract Text:

Hypothesis / aims of study

An epidemiological study by the Leicestershire MRC Incontinence Programme (1) found daily consumption of carbonated soft drinks to be independently associated with the onset of OAB symptoms in the next twelve months (OR 1.62, 95% CI 1.18, 2.22). We therefore investigated the hypothesis that artificial sweeteners, which are important constituents of carbonated soft drinks, modulate detrusor muscle function.

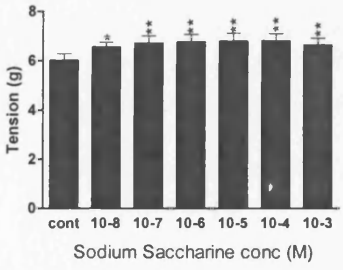
Study design, materials and methods

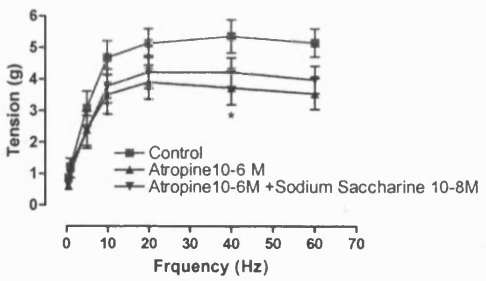
Bladders were removed from male and female Wistar rats (150g-300g) which have been culled in accordance with schedule 1 procedure of the Animal (Scientific Procedures) Act 1986. Bladder muscle strips were suspended in an organ bath perfused with Kreb's solution at 37°C aerated with 95% oxygen and 5% carbon dioxide. The apex of the muscle strip was attached to an isometric transducer connected to a four-channel oscillograph. Electrical field stimulation (EFS) was delivered by platinum electrodes recessed within the organ bath chamber and connected to a Harvard Dual Impedance Research Stimulator. The effect of increasing concentrations (10^{-8} M – 10^{-2} M) of aspartame, sodium saccharine and acesulfame K on the contractile response to a single frequency (10 Hz) of electrical field stimulation was determined. Those concentrations of sweetener found to significantly affect the contraction of rat bladder muscle to 10 Hz were further investigated for their effect on the frequency response curves to electrical field stimulation (0.5 Hz to 60 Hz), in the presence and absence of atropine 10^{-6} M. The effects of these sweeteners on responses to carbachol (10^{-8} M – 10^{-4} M) were also determined.

Statistical analysis was determined by using ANOVA repeated measures; Wilcoxon matched paris and Students t test where appropriate. A value of $p<0.05$ was considered significant.

Results

Sodium saccharine and acesulfame k (10^{-8} M - 10^{-3} M) both significantly enhanced the contractile response of rat detrusor muscle to 10 Hz EFS by 9%, 11.6%, 12.4%, 13%, 13.4%, 10.5%($p<0.01$) and 7%, 8.6%, 8.1%, 8.83%, 10%, 10.4%, 11.4% ($p<0.01$) respectively, compared to control (Fig.1). Sodium saccharine 10^{-8} M increased the maximum response of rat bladder muscle to 40Hz EFS by 10% compared to control and also the atropine resistance response to EFS was increased by 14% (Fig. 2). Sodium saccharine 10^{-7} M had little effect on the carbachol dose response curve although the maximum response was enhanced by 5%. Acesulfame K 10^{-8} M enhanced the maximum contractile response to EFS by 9% and also the atropine resistant response to EFS by 7.5%. The carbachol evoked response was also enhanced by 7.3% in the presence of acesulfame K 10^{-7} M. Aspartame 10^{-7} M had little effect on the atropine resistant response to EFS although the maximum response to EFS in the presence of atropine was enhanced by 5.6%. Aspartame 10^{-7} M also slightly enhanced the maximum response to the frequency response curve by 4.5%. The maximum contractile response to carbachol was however significantly increased by 11% ($p<0.05$) in the presence of aspartame 10^{-8} M.





Interpretation of results

Sodium saccharine, acesulfame K, and to a lesser degree aspartame, significantly

enhanced the amplitude of the contractile response of rat bladder muscle.
The mechanism for this appears to be via an effect on the atropine resistant response to EFS, now known as the purinergic component, which is activated by ATP released from the nerves. Aspartame appears to have more of an enhancing effect on the contractile response to carbachol indicating either a receptor effect or an effect on intracellular calcium movement.

Concluding message
These sweeteners are found in abundance in many foods and soft drinks. It is therefore possible that sweeteners, and other components of soft drinks, may have direct effects on bladder function as demonstrated so far in our study. The likely concentration of these substances in the human bladder and their effect in vivo remains to be determined.

References

1. Dallosso H M, McGrother C W, Matthews R J, Donaldson and the Leicestershire MRC Incontinence Study Group. The association of diet and other lifestyle factors with overactive bladder and stress incontinence: a longitudinal study in women. BJU International. 92(1): 69-77, 2003 Jul.

Abstract for **Blair Bell Research Society Meeting**, Southampton, September 2004

Modification of rat detrusor muscle contraction by artificial sweeteners

Dr Jaydip Dasgupta, Dr Ruth Elliott, Dr Doug Tincello

Dept. of Obstetrics & Gynaecology
University Hospitals of Leicester NHS Trust
Leicester General Hospital
Gwendolen Road
Leicester
LE5 4PW

Soft drinks increase the risk of developing over active bladder symptoms. We established if artificial sweeteners modulate rat detrusor muscle function.

Organ bath experiments were performed to determine the effect of aspartame, sodium saccharine and acesulfame K (10^{-8} M – 10^{-2} M) on the contractile response to electrical field stimulation (EFS) in the presence and absence of atropine 10^{-6} M, carbachol (10^{-8} M – 10^{-4} M), KCl (10 mM- 80 mM) and $\alpha\beta$ methylene ATP (10^{-7} –10^{-3}).

Sodium saccharine, aspartame and acesulfame K significantly enhanced contractile responses to 10 Hz EFS, maximal contractile response to EFS and the atropine resistant response to EFS. Maximal contractile response to α,β, methylene ATP was significantly enhanced by aspartame (10^{-7}M) and the KCl evoked maximum response were slightly enhanced. The maximal response to carbachol was enhanced by acesulfame K only.

Artificial sweeteners directly effect rat bladder function, apparently acting on purinergic component to EFS.

ICS (UK) 2005 Abstract submission form

Name of presenting author	Dr Jaydip Dasgupta	
Institution	University Hospitals of Leicester NHS Trust	
Full Address	Leicester General Hospital, Dept of Obs & Gynae, Gwendolen Road, Leicester, LE5 4PW	
Email address	jd98@le.ac.uk	
Tel number	07834376845	
Abstract Title	Effect of Ascorbic Acid on Rat Bladder Contraction	
Abstract Authors	Dr J Dasgupta, Dr R Elliott and Dr D Tincello	
I would like this abstract to be considered for: a) a poster		
	b) an oral presentation	Yes

Abstract (max 300 words)

An epidemiological study by the Leicestershire MRC Incontinence Programme (1) found an association between daily consumption of carbonated soft drinks and the onset of OAB symptoms. (OR 1.62, 95% CI 1.18, 2.22). We investigated the hypothesis that Ascorbic acid, a constituent of carbonated soft drinks, modulates detrusor muscle function.

Bladder muscle strips were suspended in an organ bath perfused with Kreb's solution at 37°C aerated with 95% oxygen and 5% carbon dioxide. The effect of increasing concentrations (10^{-8} M – 10^{-2} M) of ascorbic acid on the contractile response to EFS 10Hz was determined. Those concentrations found to significantly affect muscle contraction were investigated for their effect on EFS (0.5 Hz to 60 Hz), in the presence and absence of atropine 10^{-6} M. The effect of ascorbic acid on responses to αβ methylene ATP (10^{-3}M – 10^{-7}M), KCl (10mM – 80mM) and carbachol (10^{-8} M – 10^{-2} M) were also determined.

Ascorbic acid (10^{-6} M - 10^{-3} M) significantly enhanced the contractile response of rat detrusor muscle to 10 Hz EFS by 17.2%, 18.57%, 21.18%, and 18.73% ($p<0.01$). The atropine resistance response to EFS (1 Hz – 40 Hz) was enhanced significantly in presence of ascorbic acid 10^{-4} M. The maximal contractile response to αβ methylene ATP (10^{-3} M) and KCl (80mM) was increased by 19.23% and 16.82% ($p<0.01$) respectively. The maximum contractile response to carbachol was however significantly reduced by 8.10% ($p<0.05$).

Ascorbic acid appears to enhance the Ca^{+2} influx through L-type Ca^{+2} channels as demonstrated by its effect on the response to KCl. This may also be its mechanism of action on the atropine resistant component to EFS (ATP), and direct enhancing effect of maximal contractile response to αβ methylene ATP. However, ascorbic acid appears to inhibit carbachol-evoked responses. The mechanism for this will be investigated further.

Reference:

Dallosso H M, McGrother C W, Mathews R J, Donaldson M M K and the Leicestershire MRC Incontinence Study Group. The association of diet and other lifestyle factors with overactive bladder and stress incontinence: a longitudinal study in women. BJU International. 92(1): 69-77, 2003.

ICS Annual Meeting

Montréal, Canada. 29th August – 2nd September 2005

Abstract Title:

Effect of citric acid on rat detrusor muscle contraction.

Abstract Text:

Hypothesis / aims of study

Leicestershire MRC Incontinence Programme (1), an epidemiological study has found daily consumption of carbonated soft drinks to be independently associated with the onset of OAB symptoms in the next twelve months (OR 1.62, 95% CI 1.18, 2.22). We therefore investigated the hypothesis that constituents of carbonated soft drinks modulate detrusor muscle function. Citric acid being one of the components may play an important role.

Study design, materials and methods

Bladders were removed from male and female Wistar rats (150g-300g) which have been culled in accordance with schedule 1 procedure of the Animal (Scientific Procedures) Act 1986. Bladder muscle strips were suspended in an organ bath perfused with Kreb's solution at 37°C aerated with 95% oxygen and 5% carbon dioxide. The apex of the muscle strip was attached to an isometric transducer connected to a four-channel oscillograph. Electrical field stimulation (EFS) was delivered by platinum electrodes recessed within the organ bath chamber and connected to a Harvard Dual Impedance Research Stimulator. The effect of increasing concentrations 10^{-8} M – 10^{-2} M of citric acid on the contractile response to a single frequency (10 Hz) of electrical field stimulation was determined. Those concentrations found to affect muscle contraction were investigated for their effect on EFS (0.5 Hz to 60 Hz), in the presence and absence of atropine 10^{-6} M. The effects of citric acid on responses to α, β-methylene ATP 10^{-3}M – 10^{-7}M, KCl (10mM – 80mM) and carbachol 10^{-8} M – 10^{-2} M were also determined.
Statistical analysis was determined by using Students t test where appropriate. A value of $p<0.05$ was considered significant. Each graph is the mean plus SEM of six different experiments.

Results
Citric acid 10^{-5} M significantly enhanced the contractile response of rat detrusor muscle to 10 Hz EFS by 13.3% (<0.05). The atropine resistance response to 40 Hz EFS was also increased by 10% (<0.01) in presence of citric acid 10^{-5} M compared to control. The maximal contractile response to α, β-methylene ATP 10^{-3} M was increased by 15.8% (<0.001) and the contractile response to KCl (70mM) was enhanced by 38% (<0.01) in presence of citric acid 10^{-5} M compared to control. Citric acid 10^{-5} M however didn't modulate the maximum contractile response to carbachol significantly.

Interpretation of results

Citric acid enhanced the amplitude of the contractile response of rat bladder muscle. It appears to enhance the Ca^{+2} influx through L-type Ca^{+2} channels as demonstrated by its effect on the response to KCl. This may also be its mechanism of action on the atropine resistant component to EFS (ATP), and the direct enhancing effect on the maximal contractile response to αβ methylene ATP.

Concluding message

Citric acid is an important component of many carbonated soft drinks. It is likely that citric acid along with other components of soft drinks, may have direct effects on bladder function as demonstrated so far in our study.

Abstract for Research Urogynaecology Society Meeting, Royal College of Obstetricians & Gynaecologists, November, 2005

Effects of carbonated soft drink components on rat detrusor muscle contraction

Aims

An epidemiological study by the Leicestershire MRC Incontinence Programme found daily consumption of carbonated soft drinks to be independently associated with the onset of OAB symptoms in the next twelve months (OR 1.62, 95% CI 1.18, 2.22). We therefore investigated the hypothesis that the components present in carbonated soft drinks, modulate detrusor muscle function. We determined the effect of important constituents of carbonated soft drinks, like citric acid, artificial sweeteners, antioxidants like ascorbic acid and preservatives like citric acid, sodium benzoate and potassium sorbate, on the contractile properties of isolated bladder muscle strips.

Materials and Methods

Bladders were removed from male and female Wistar rats (150g-300g) which have been culled in accordance with schedule 1 procedure of the Animal (Scientific Procedures) Act 1986. Bladder muscle strips were suspended in an organ bath and perfused with Kreb's solution at 37°C aerated with 95% oxygen and 5% carbon dioxide. Electrical field stimulation (EFS) was delivered by platinum electrodes recessed within the organ bath chamber and connected to a Harvard Dual Impedance Research Stimulator. The pulse width of 0.05 ms was chosen to ensure stimulation of the nerves, not direct stimulation of the muscle .The effect of increasing concentrations (10^{-8} M – 10^{-2} M) of the soft drink components on the contractile response to a single frequency (10 Hz) of EFS was determined. Those concentrations found to significantly affect the contraction of bladder muscle to 10 Hz were further investigated for their action on the frequency response curves to EFS (0.5 Hz to 60 Hz), in the presence and absence of atropine 10^{-6} M. The effects of these chemicals on responses to carbachol (10^{-8} M – 10^{-2} M), $\alpha\,\beta$ methylene ATP (10^{-3} M – 10^{-7} M), KCl (10mM – 80mM) and calcium (0.5mM – 2.5mM) were also determined. Carbachol and α,β methylene ATP are muscarinic and purinergic receptor agonist respectively. KCl and calcium act on the L-type calcium channels causing influx of Ca^{+2}. Statistical analysis was determined by using ANOVA repeated measures and ANOVA followed by multiple comparisons. A value of $p<0.05$ was considered as significant.

Results

Sodium saccharine, aspartame and acesulfame K (10^{-8} M - 10^{-3} M), phenylalanine (10^{-8} M), citric acid (10^{-7} M – 10^{-4} M) and ascorbic acid (10^{-7} M – 10^{-3} M) significantly enhanced the contractile response of rat detrusor muscle to 10 Hz EFS ($p< 0.01$) while sodium benzoate and potassium sorbate had no significant effect. The atropine resistance response to EFS (due to activation of purinergic receptors) was also found to be enhanced by the presence of these components. The maximum contractile response to α β methylene ATP, KCl and calcium was increased significantly by the presence of different concentrations of soft drink components. Most of these chemicals did not affect the carbachol dose response curve. Ascorbic acid, however significantly reduced the maximal contractile response to carbachol, this requires further investigation.

Conclusion

The contractile response to ATP (directly by α β methylene ATP or by EFS in the presence of atropine), KCL and calcium are all initiated by the influx of Ca^{2+} through L-type calcium channels. The contractile response to calcium is a direct result of this mechanism. Carbachol however, initiates smooth muscle contraction via a rise in intracellular Ca^{2+} concentration, resulting from the activation of muscarinic receptors and the secondary messenger inositol trisphosphate (IP_3).

We therefore conclude from our results that the components of soft drinks investigated in this study enhanced the influx of Ca^{+2} through the L-type Ca^{+2} channels, either directly or via purinergic receptor activation pathway thereby increasing the magnitude of bladder muscle contraction which may affect bladder function.

Available online at www.sciencedirect.com
ScienceDirect
Toxicology and Applied Pharmacology 217 (2006) 216–224
Toxicology and Applied Pharmacology
www.elsevier.com/locate/ytaap

Enhancement of rat bladder contraction by artificial sweeteners via increased extracellular Ca^{2+} influx

Jaydip Dasgupta, Ruth A. Elliott*, Angie Doshani, Douglas G. Tincello

University of Leicester, Prolapse and Incontinence Group, Reproductive Sciences Section, Department of Cancer Studies and Molecular Medicine, Robert Kilpatrick Clinical Sciences Building, Leicester Royal Infirmary, Leicester, LE2 7LX, UK

Received 20 June 2006; revised 10 August 2006; accepted 5 September 2006
Available online 12 September 2006

Abstract

Introduction: Consumption of carbonated soft drinks has been shown to be independently associated with the development of overactive bladder symptoms (OR 1.62, 95% CI 1.18, 2.22) [Dallosso, H.M., McGrother, C.W., Matthews, R.J., Donaldson, M.M.K., 2003. The association of diet and other lifestyle factors with overactive bladder and stress incontinence: a longitudinal study in women. BJU Int. 92, 69–77]. We evaluated the effects of three artificial sweeteners, acesulfame K, aspartame and sodium saccharin, on the contractile response of isolated rat detrusor muscle strips.

Methods: Strips of detrusor muscle were placed in an organ bath and stimulated with electrical field stimulation (EFS) in the absence and presence of atropine, and with α,β methylene ATP, potassium, calcium and carbachol.

Results: Sweeteners 10^{-7} M to 10^{-2} M enhanced the contractile response to 10 Hz EFS compared to control ($p<0.01$). The atropine-resistant response to EFS was marginally increased by acesulfame K 10^{-6} M, aspartame 10^{-7} M and sodium saccharin 10^{-7} M. Acesulfame K 10^{-6} M increased the maximum contractile response to α,β methylene ATP by 35% (±9.6%) ($p<0.05$) and to KCl by 12% (±3.1%) ($p<0.01$). Sodium saccharin also increased the response to KCl by 37% (±15.2%) ($p<0.05$). These sweeteners shifted the calcium concentration–response curves to the left. Acesulfame K 10^{-6} M increased the log EC_{50} from −2.79 (±0.037) to −3.03 (±0.048, $p<0.01$) and sodium saccharin 10^{-7} M from −2.74 (±0.03) to 2.86 (±0.031, $p<0.05$). The sweeteners had no significant effect on the contractile response to carbachol but they did increase the amplitude of spontaneous bladder contractions.

Discussion: These results suggest that low concentrations of artificial sweeteners enhanced detrusor muscle contraction via modulation of L-type Ca^{+2} channels.

Keywords: Aspartame; Sodium saccharin; Acesulfame K; Bladder; Smooth muscle; Calcium channels

Introduction

Detrusor muscle overactivity is a common cause of urinary incontinence with a socioeconomic impact comparable to diabetes mellitus (Hampel et al., 2003). The disease is characterised by spontaneous muscle contraction within the bladder wall, causing symptoms of urinary urgency, frequency and incontinence. The International Continence Society also recognises a symptomatic diagnosis of overactive bladder (OAB) that includes urgency, with or without urge incontinence, and usually with frequency and nocturia (Abrams, 2003). A large proportion of patients with OAB will have detrusor muscle overactivity on formal testing. The Leicestershire MRC Incontinence Study of 20,244 community dwelling women aged 40 years and over investigated dietary and lifestyle factors associated with urinary incontinence and demonstrated that daily consumption of carbonated soft drinks was one factor independently associated with the onset of OAB symptoms within the next twelve months (OR 1.62, 95% CI 1.18, 2.22) (Dallosso et al., 2003).

Most of these soft drinks contain high levels of artificial sweeteners, colorants and preservatives that may have diverse effects on tissue integrity or signalling. Sodium saccharin, aspartame and acesulfame K are three important artificial sweeteners found in soft drinks. The carcinogenic potential of

* Corresponding author. Fax: +44 116 2525846.
E-mail address: rac5@leicester.ac.uk (R.A. Elliott).

0041-008X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.taap.2006.09.004

these chemicals has been studied by several groups. Some epidemiological and laboratory studies demonstrated that saccharin is not carcinogenic in the bladder, is not mutagenic and does not bind to DNA (Christ et al., 2004; Takayama et al., 1998; Armstrong and Doll, 1975; Armstrong and Doll, 1974; Auerbach and Garfinkel, 1989). However, other groups have found that male rats developed bladder tumours in up to 30% of all animals after the addition of saccharin to their diet (Taylor et al., 1980; Squire, 1985).

Aspartame is the most extensively used artificial sweetener and some animal studies have also shown that aspartame does not have any cancer inducing properties (Hagiwara et al., 1984; Ishii, 1981), although a more recent study describes an increase in cancers in rats fed aspartame, 20 to 500 mg kg^{-1}, from the age of eight weeks until the natural end of their lives (Soffritti et al., 2006), rather than culling the animals at two years of age, as other groups have done. In both males and females, an increase in lymphomas and leukaemias were found. In female rats there was also an increase in the number of transitional cell carcinomas of the renal pelvis and ureter, and some in the bladder. These results show that the sweetener aspartame, and its metabolites, have access to and are able to alter various tissue systems within the rat, including the urinary tract.

Aspartame has also been found to significantly increase calciuria (Nguyen et al., 1998) and in higher doses to increase striatal levels of serotonin in the brain of rats. This increase resulted in a reduction of aggressive attacks, an action mediated via a mechanism interfering with serotonin synthesis (Chappel, 1992).

The effects of these sweeteners on bladder muscle contraction are yet to be evaluated. The aim of this study was to investigate the previously identified epidemiological association by studying the effects of the artificial sweeteners aspartame, sodium saccharin and acesulfame K on rat detrusor muscle contraction and to elucidate the likely mode of action upon the different pathways of smooth muscle contraction.

Methods

Bladders were removed from male and female Wistar rats (150–300 g), which had been culled in accordance with schedule 1 procedure of the Animal (Scientific Procedures) Act 1986 and were placed into Kreb's solution (NaCl 119 mM, KCl 4.4 mM, $NaHCO_3$ 20 mM, NaH_2PO_4 1.2 mM, $MgCl_2$ 1.2 mM, $CaCl_2$ 2.5 mM and Glucose 11 mM in distilled water; pH 7.2). Two longitudinal muscle strips per bladder (6 mm × 3 mm × 1 mm) were cut and suspended in a Perspex organ bath chamber of 0.2 ml volume perfused at the rate of 1 ml/min with Kreb's solution aerated with 95% O_2 and 5% CO_2 at 37 °C (Brading and Sibley, 1983). The bladder muscle strips were suspended using a fine silk suture between the bottom of the chamber and an isometric force transducer connected to a four-channel Harvard Universal Oscillograph. The strips were allowed to equilibrate for 1 h under tension of 1 g before experimentation. All of the methods used in this paper have been published (Elliott et al., 1992).

Electrical field stimulation (EFS). Detrusor muscle strips were stimulated by EFS using recessed platinum electrodes connected to a Harvard Dual Impedance Research Stimulator capable of delivering electrical impulses at different frequencies, voltage and pulse width.

Frequency–response curves were obtained by stimulating the muscle strips with increasing frequencies, using 0.5, 1, 5, 10, 20, 40 and 60 Hz, at 50 V with a pulse width of 0.05 ms in 10 trains at 3-min intervals. The pulse width of 0.05 ms was chosen to ensure stimulation of the nerves, not direct stimulation of the muscle. We have demonstrated previously that tetrodotoxin 1.6×10^{-6} M abolishes the response to EFS at this pulse width but not to pulse widths higher than this setting (Hudman et al., 2000). When consistent responses had been obtained the tissue sample was washed and re-equilibrated. Time course experiments carried out for 231 min demonstrated that the rat detrusor muscle strips were viable and able to contract consistently over the time of these experiments.

Electrical field stimulation 10 Hz. Detrusor muscle strips were equilibrated then stimulated with EFS (10 Hz) to obtain control contractile responses. This frequency was chosen because both acetylcholine and ATP are released from the nerve terminals after stimulation at this frequency. Aspartame (10^{-8} M to 10^{-2} M) was added to the bath for 15 min followed by three stimulations with EFS (10 Hz). The average of the three responses to EFS (10 Hz) was calculated after the addition of each concentration of aspartame.

Contractile responses to EFS after the addition of acesulfame K (10^{-8} M to 10^{-2} M) and sodium saccharin (10^{-8} M to 10^{-3} M) was determined in the same way. Low concentrations of sweeteners to significantly modulate bladder muscle contractile response to EFS (10 Hz) were found to be aspartame (10^{-7} M), acesulfame K (10^{-6} M) and sodium saccharin (10^{-7} M) and these concentrations were subsequently used for further experiments.

Spontaneous contraction. Smooth muscles in the bladder show spontaneous contractile activity during the filling phase (Brading, 2006). Isolated rat detrusor muscle strips also generate spontaneous phasic contractions. The effect of acesulfame 10^{-6} M, aspartame 10^{-7} M and sodium saccharin 10^{-7} M on spontaneous rat detrusor muscle contractions was determined.

Carbachol concentration–response curves. Stock solution of carbachol, 10^{-2} M, in Kreb's solution was prepared and diluted down to obtain concentrations from 10^{-8} M to 10^{-2} M immediately before use. Concentration–response curves were obtained in the presence of carbachol at increasing concentrations 10^{-8} M to 10^{-2} M for 30 s. Perfusion was repeated after addition and equilibration of each sweetener at previously specified concentrations in EFS (10 Hz) experiments.

Electrical field stimulation in the absence and presence of atropine 10^{-6} M. Firstly, experiments had been conducted to define the contractile response of rat bladder muscle to EFS, over the time course of the subsequent experiments with sweeteners. Three frequency–response curves were obtained at 21 min, 126–147 min and 210–231 min.

A control frequency–response curve was obtained. This was repeated in the presence of each sweetener, at the above concentrations established in EFS (10 Hz) experiments, to determine their individual effect on the frequency–response curve to EFS.

Frequency–response curves were obtained in the presence of atropine 10^{-6} M to block the cholinergic component of EFS (Yono et al., 2000). Similar frequency–response curves were obtained in the presence of atropine 10^{-6} M plus either sweetener, at previously determined concentrations, to establish the effect on the atropine-resistant response to EFS.

Concentration–response curves to ABMA. α,β Methylene ATP was made up as stock in Kreb's solution of 10^{-3} M then diluted further to make concentrations 10^{-7} M to 10^{-3} M before use. Concentration–response curves were obtained after perfusing the detrusor muscle with increasing concentrations of α,β methylene ATP from 10^{-7} M to 10^{-3} M for 45 s. The strips were then perfused with Kreb's solution for 15 min to allow the tension to return to base line and to ensure that purinoceptors were not desensitised by prolonged exposure to α,β methylene ATP. The α,β methylene ATP concentration–response curve was repeated following 20 min of perfusion with Kreb's solution containing each sweetener at previously determined concentrations in EFS (10 Hz) experiments.

Concentration–response curves to KCl. KCl was dissolved in potassium-free Kreb's solution to produce concentrations of 10 mM to 80 mM immediately before use. Concentration–response curves to KCl were obtained by perfusing the detrusor muscle for 60 s with each concentration of KCl. Samples were allowed to relax to baseline between exposures. Another concentration–

response curve was obtained in the presence of each sweetener at previously specified concentrations in EFS (10 Hz) experiments.

Concentration–response curves to calcium. The detrusor muscle strips were depolarised by placing them into calcium-free, potassium-rich Kreb's solution containing 127 mM of KCl. This resulted in an initial contraction of the detrusor muscle strips followed by relaxation. Calcium chloride was dissolved in calcium-free Kreb's solution to give the concentrations of 0.5, 1.0, 1.5, 2.0 and 2.5 mM. After an equilibration period of 60 min, each concentration was perfused through the organ bath chamber for 60 s. When the contractile response had returned to the baseline the next concentration was added 5 min later and so on to produce a control concentration–response curve to calcium. This procedure was then repeated after the strips had been perfused with each sweetener for 20 min, at previously established concentrations in EFS (10 Hz) experiments.

Statistical analysis. Sample size calculations using SPlus 6.2, Insightful, determined from data taken from similar experiments indicated that muscle strips from six animals were required to construct each contractile response curve. Data are expressed as means ± standard error and analysed using repeated measures analysis of variance and one-way analysis of variance followed by Bonferroni's or Dunnett's multiple comparison where appropriate. A p value of <0.05 was regarded as significant. Graphpad Prism was used for data analysis.

Results

Electrical field stimulation 10 Hz

The contractile response of rat detrusor muscle to 10 Hz EFS was significantly enhanced compared to control in the presence of acesulfame K 10^{-8} M to 10^{-3} M ($p<0.01$) (Fig. 1A), aspartame 10^{-7} M to 10^{-2} M ($p<0.01$) (Fig. 1B) and sodium saccharin 10^{-8} M to 10^{-3} M ($p<0.01$) (Fig. 1C). The following concentrations of sweeteners used for subsequent experiments were: acesulfame 10^{-6} M, aspartame 10^{-7} M and sodium saccharin 10^{-7} M. These were chosen because they were a lower, but not the lowest, concentrations to produce a significant effect on bladder contraction at $p<0.01$.

Spontaneous contraction

Aspartame had no effect on spontaneous rat bladder contractions, however, at the point when the perfused solution of acesulfame 10^{-6} M reached the bladder smooth strips in the organ bath, the frequency of the spontaneous contractions was reduced for 60 s but the amplitude of the contractions was increased. Following this immediate effect of acesulfame on spontaneous contractions, the subsequent tone of the strips was increased by a maximum of 30.7% and this increase in tone was maintained for 2 min (not shown).

A similar effect was seen after the addition of sodium saccharin 10^{-7} M to the organ bath. The detrusor muscle showed an initial increase in phasic contractions with the frequency of these contractions being slightly reduced. An increase in tone was maintained for 1 1/2 min. The maximum tone obtained was 65.8% above the resting tone (not shown).

Carbachol evoked responses

Carbachol directly activates cholinergic receptors in bladder smooth muscle. The sweeteners had no significant

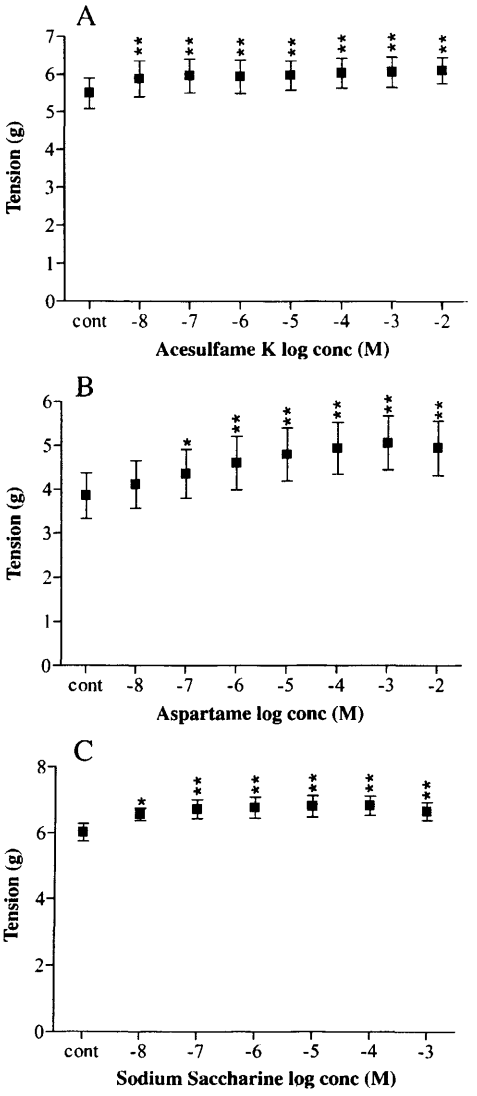


Fig. 1. The effect acesulfame K 10^{-8} M to 10^{-2} M (A), aspartame 10^{-8} M to 10^{-2} M (B) and sodium saccharin 10^{-8} M to 10^{-3} M (C) on rat detrusor muscle contractile response to 10 Hz EFS. $*p<0.05$, $**p<0.01$ (repeated measures ANOVA) $n=6$.

effect on the maximum contractile response to carbachol 10^{-3} M. Acesulfame 10^{-6} M slightly enhanced the maximum response to carbachol by 5.5% (±0.82%) and sodium saccharin 10^{-7} M also slightly enhanced the maximum contractile response to carbachol by 5.5% (±0.78%). Aspartame 10^{-7} M had no affect on the concentration–response curve to carbachol (not shown).

Frequency–response curves

Results of the time course experiments showed no significant difference in the frequency–response curve obtained at 0–21 min, 63–84 min or 210–231 min compared to the control response (data not shown).

Sodium saccharin 10^{-7} M increased the maximum contractile response of bladder muscle to 20 Hz EFS by 12% (±3.84%, NS) compared to control. Aspartame 10^{-7} M and acesulfame K 10^{-6} M enhanced the maximum contractile response to 20 Hz EFS by 10.5% (±2.43%, NS) and 9.0% (±4.3%, NS), respectively (not shown).

Electrical field stimulation in the absence and presence of atropine 10^{-6} M

Atropine blocks the cholinergic component of the nerve-mediated response with the remaining atropine-resistant component being due to the release of the neurotransmitter, ATP (Brading and Williams, 1990). Acesulfame K 10^{-6} M increased the atropine resistance response to 10 Hz EFS by 7.5% (±2.1%) (Fig. 2A) and aspartame 10^{-7} M to 20 Hz EFS by 10.0% (±2.11%) (Fig. 2B). The atropine resistance response to 20 Hz EFS was also marginally increased by 8.3% (±3.2%) (Fig. 2C) after the addition of sodium saccharin 10^{-7} M compared to control. None of these increases were statistically significant.

ABMA responses

α,β Methylene ATP 10^{-4} M directly activates purinergic receptors which depolarizes the cell membrane opening L-type Ca^{+2} channels, resulting in Ca^{+2} influx and muscle contraction (Wu et al., 1999). Acesulfame K 10^{-6} M significantly enhanced the maximum contractile response to α,β methylene ATP, 10^{-3} M by 35% (±9.6%) ($p<0.01$, Fig. 3A) compared to control. Aspartame 10^{-7} M slightly enhanced the contractile response evoked by α,β methylene ATP 10^{-4} M and 10^{-3} M by 34% (±11.24%, NS) and by 25% (±8.46%, NS), respectively, when compared to control (Fig. 3B). Sodium saccharin 10^{-7} M increased the maximum contractile responses to α,β methylene ATP, 10^{-3} M, by 16.3% (±4.3%, NS, Fig. 3C).

KCl-evoked responses

Potassium induces detrusor muscle contraction solely by opening L-type Ca^{+2} channels in the sarcolemma (Maggi et al., 1989). We therefore determined if these sweeteners directly affected Ca^{+2} influx by investigating their effect on contractile responses to KCl. Acesulfame K 10^{-6} M significantly enhanced the contractile response to KCl 60 mM and 70 mM by 21.8% (±3.3%) ($p<0.001$) and 12% (±3.1%) ($p<0.01$, Fig. 4A), respectively. The log EC_{50} −3.28 (±0.009) of the control concentration–response curve to KCl was significantly increased to −3.34 (±0.01, $p<0.01$), shifting the concentration–response curve to the left. Aspartame 10^{-7} M had no significant effect on the contractile response to KCL (Fig. 4B). The contractile responses to KCl, 60 mM, 70 mM and 80 mM, was enhanced in the presence of sodium saccharin 10^{-7} M by 29.4% (±15.8%), 29.17% (±16.0%) and 37% (±15.2%) ($p<0.05$, Fig. 4C), respectively, compared to control. The control log EC_{50} −1.21 (±0.01) was significantly increased to −1.31 (±0.01, $p<0.001$) after the addition of sodium saccharin 10^{-7} M.

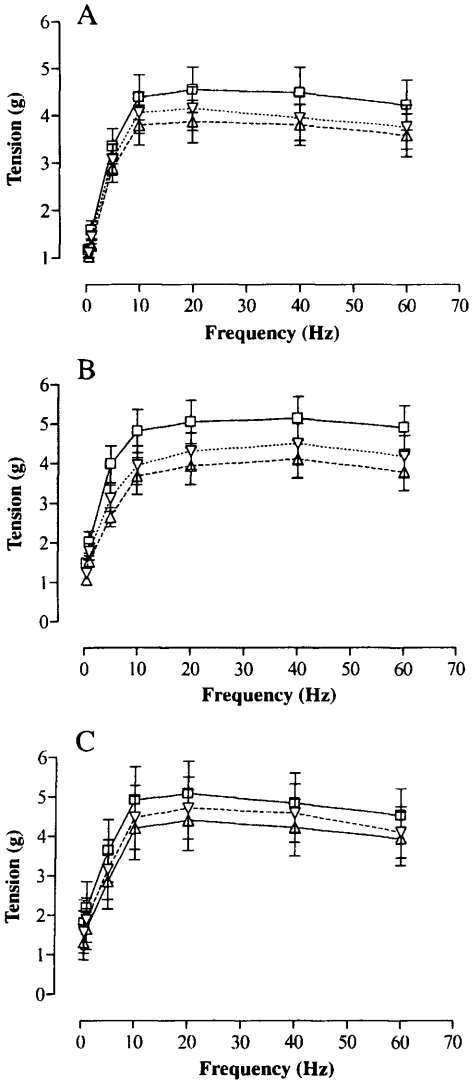


Fig. 2. The effect of acesulfame K 10^{-6} M, aspartame 10^{-7} M and sodium saccharin 10^{-7} M on rat detrusor muscle contractile response to EFS in the presence of atropine 10^{-6} M. □, Control frequency–response curves. △, Frequency–response curves in presence of atropine without sweetener. ▽, Frequency–response curves in presence of atropine and acesulfame K 10^{-6} M (A), aspartame 10^{-7} M (B) and sodium saccharin 10^{-7} M (C) compared to atropine alone. $n=6$.

Concentration–response curves to calcium

In depolarised bladder muscle strips, acesulfame K 10^{-6} M enhanced the contractile response to calcium, 1.0, 2.0 and 2.5 mM by 64%±16.5% ($p<0.05$), 45%±19.0% ($p<0.05$) and 39%±13.7% ($p<0.05$), respectively (Fig. 5A). The concentration–response curve to calcium was significantly shifted to the left after the addition of acesulfame K 10^{-6} M. The log EC_{50} for the control was −2.79±0.03, and after the addition of acesulfame K 10^{-6} M the log EC_{50} was −3.03±0.04 ($p<0.01$).

Aspartame 10^{-7} M enhanced the contractile response to 0.5 mM and 1.0 mM calcium by 73%±19.0% ($p>0.001$) and 35%±3.9% ($p<0.001$), respectively (Fig. 5B). There was no

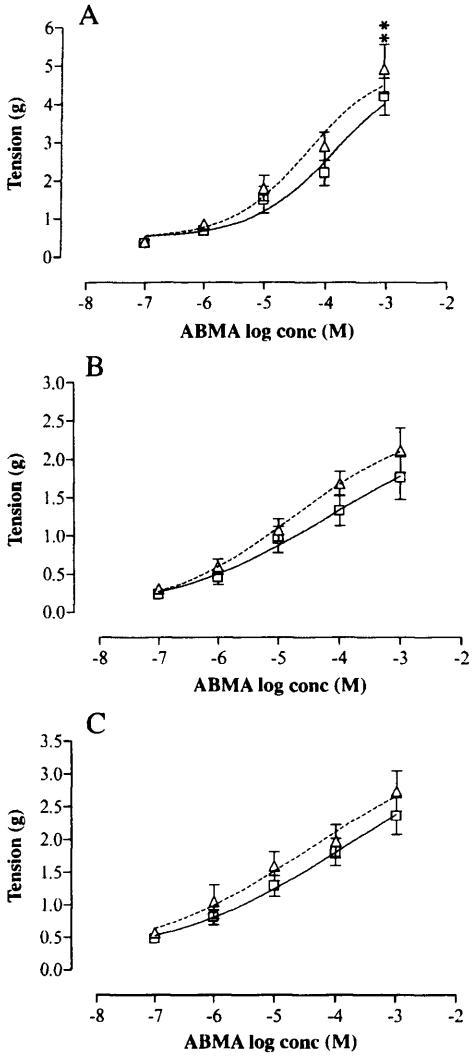


Fig. 3. The effect of acesulfame K 10^{-6} M, aspartame 10^{-7} M and sodium saccharin 10^{-7} M on rat detrusor muscle contractile response to α,β methylene ATP. □, Control concentration–response curves to α,β methylene ATP. ▽, Concentration–response curves to α,β methylene ATP in the presence of acesulfame K 10^{-6} M (A), aspartame 10^{-7} M (B) and sodium saccharin 10^{-7} M (C). $^{**}p<0.01$, $n=6$.

significant difference between the control log EC_{50} −2.74±0.02 and the log EC_{50} after the addition of aspartame 10^{-7} M −2.82± 0.02

Sodium saccharin 10^{-7} M significantly increased the maximum contractile response to calcium by 69%±17.46% ($p<0.01$) and the log EC_{50} for the control concentration–response curve, −2.74±0.03 was significantly shifted to the left after the addition of sodium saccharin 10^{-7} M, −2.86±0.03 ($p<0.05$) (Fig. 5C).

Discussion

The sweeteners sodium saccharin and acesulfame K had a stimulatory effect on the contractile response of rat detrusor muscle to potassium α,β methylene ATP and calcium. Sodium saccharin, acesulfame K and aspartame enhanced the contractile response to 10 Hz EFS significantly throughout the range of concentrations from 10^{-8} M to 10^{-3} M.

Considerable experimental and clinical evidence shows that detrusor overactivity is associated with detectable alterations in the electrical properties of the detrusor smooth muscle cells (Tong et al., 1997). In the stable human bladder detrusor contraction is mediated by ACh release from parasympathetic nerve fibres, which is completely abolished by atropine (Sibley, 1984). Interestingly detrusor muscle contractions from dysfunctional bladders were found to have an atropine-resistant component, believed to be mediated by ATP (Ruggieri, 2006). In contrast most non-primate bladder detrusor muscle contractions involve the muscarinic pathway (via the M_3 receptor

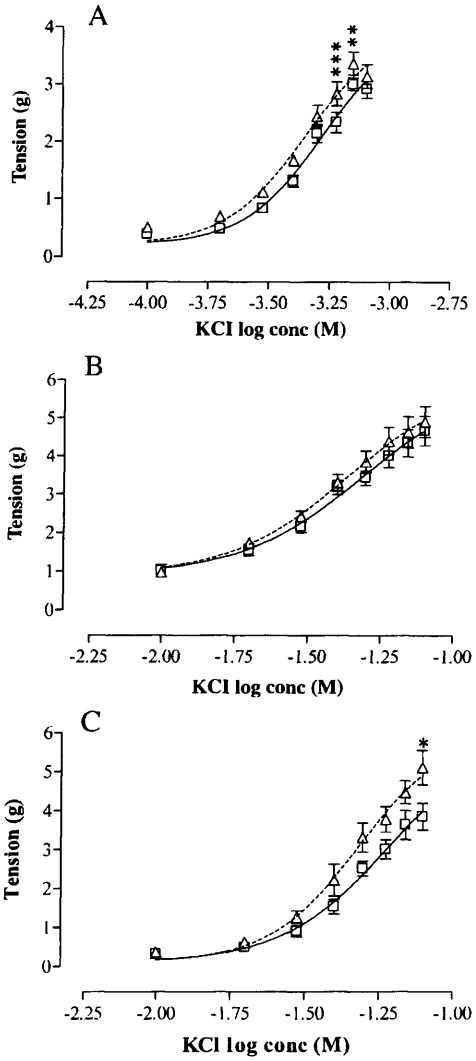


Fig. 4. The effect of acesulfame K 10^{-6} M, aspartame 10^{-7} M and sodium saccharin 10^{-7} M on rat detrusor muscle contractile response to KCl. □ control concentration–response curve. Δ, Concentration–response curve to KCl in presence of acesulfame K 10^{-6} M (A), aspartame 10^{-7} M (B) and sodium saccharin 10^{-7} M (C). $^{*}p<0.05$, $^{**}p<0.01$, $n=6$.

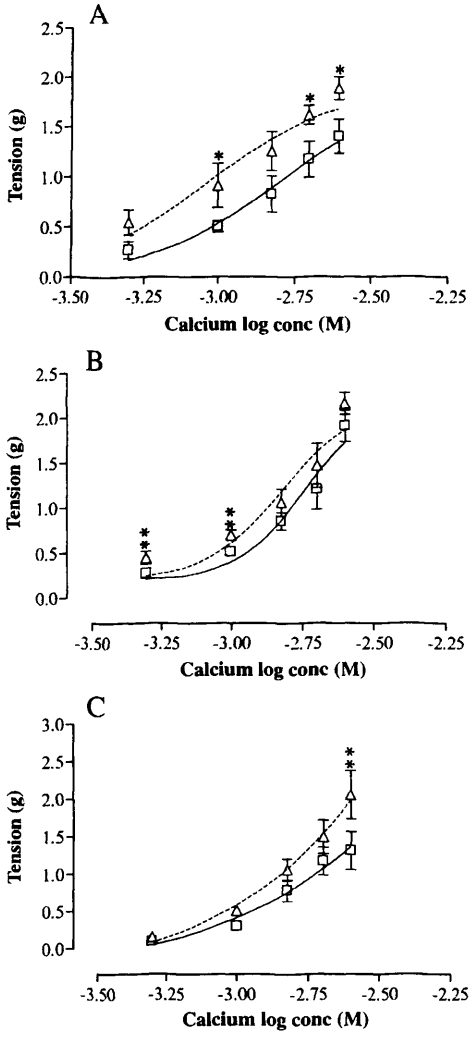


Fig. 5. The effect of acesulfame K 10^{-6} M, aspartame 10^{-7} M and sodium saccharin 10^{-7} M on concentration–response curve to calcium. □, Control calcium concentration–response curve. △, Calcium concentration–response curve in the presence of acesulfame K 10^{-6} M (A), aspartame 10^{-7} M (B) and sodium saccharin 10^{-7} M (C). * $p<0.05$, ** $p<0.01$, $n=6$.

subtype) (Braverman et al., 1998) and purinergic pathway (mediated by ATP) (Namasivayam et al., 1999).

Contraction of rat detrusor muscle to EFS has been shown to be via stimulation of the nerves, as demonstrated by the abolition of this response by the presence of tetrodotoxin (TTX), a sodium channel blocker (Brading and Williams, 1990; Hudman et al., 2000). Nerve stimulation releases the neurotransmitters ACh and ATP, involving the cholinergic and purinergic pathways (Creed et al., 1983). At lower frequencies of EFS, muscle contraction is mainly due to the release of ATP from the nerves with an increasing component due to the release of ACh as the frequency of stimulation increases (Brading and Williams, 1990).

Cholinergic responses in the rat urinary bladder are initiated via activation of muscarinic M_3 receptors (Giglio et al., 2001). Carbachol directly stimulates the muscarinic receptors on cell membranes, activating release of Ca^{+2} from sarcoplasmic reticulum through the second messenger inositol 1,4,5-triphosphate (IP_3) (Oike et al., 1998). The sweeteners had no significant effect on the carbachol concentration–response curve so it appears that their mechanism of action is not via the cholinergic pathway. These sweeteners therefore have no effect on the micturition contraction of the bladder, which is mediated via cholinergic mechanisms particularly in the human detrusor muscle (Sibley, 1984).

When the cholinergic component of the muscle contraction is blocked by atropine, during EFS stimulation, the magnitude of the remaining contractile response was slightly enhanced in the presence of the sweeteners, suggesting that a possible mode of action is through the atropine-resistant component, mediated by ATP.

This hypothesis is supported by the finding that the maximum contractile response to α,β methylene ATP was also enhanced by some of these sweeteners, indicating their direct action on purinoceptor-associated mechanisms. Activation of purinoceptors P2X, which are ligand-gated ion channels, by α,β methylene ATP, a non-hydrolysable stable analogue of ATP, causes depolarisation and opening of L-type Ca^{+2} channels increasing Ca^{+2} influx and thereby initiating contractile response (Wu et al., 1999). α,β Methylene ATP is not only a potent agonist of the P2X purinoceptor, but it causes desensitisation of purinoceptors after prolonged exposure (Brading and Williams, 1990; Yoshida et al., 2001; Noda et al., 2002). Our bladder strips were exposed to α,β methylene ATP for 45 s after which it was washed out with Kreb's solution. This would be considered an inadequate length of time to cause desensitisation of purinoceptors (Bo and Burnstock, 1989).

We investigated the effect of sweeteners on the contractile response of detrusor muscle strips to potassium (KCl). This was to determine the possibility that the sweeteners affect calcium influx through L-type calcium channels. Nifedipine, an L-type Ca^{+2} channel antagonist, completely suppresses the phasic and tonic contractile response to KCl. Exposure to calcium-free medium also rapidly abolishes the response to KCl (Maggi et al., 1989). This suggests that the contractile response to KCl is solely dependent on the influx of calcium through L-type Ca^{+2} channels. As acesulfame 10^{-6} M and sodium saccharin 10^{-7} M enhanced the maximal contractile response to potassium and significantly shifted the concentration–response curves to the left, this suggests that their mode of action involves augmentation of extracellular Ca^{+2} influx through L-type Ca^{+2} channels that is independent of muscarinic or purinergic receptor activity (Maggi et al., 1989; Visser and van Mastrigt, 2000). Further confirmation of this mechanism was obtained by investigating the effect of acesulfame 10^{-6} M, aspartame 10^{-7} M and sodium saccharin 10^{-7} M on the contractile response of rat bladder muscle to calcium $0.5–2.5\times10^{-3}$ M. Rat bladder strips were placed in Ca^{+2}-free, high potassium medium, during which there was no contraction, after an hour's equilibrium in normal Kreb's solution. The high potassium concentration depolarised the smooth muscle cells and opened L-type Ca^{+2} channels. As the medium did not contain Ca^{+2}, contraction of the bladder muscle strips was obtained by the addition of increasing

concentrations of Ca^{+2} to produce a concentration–response curve. Acesulfame and sodium saccharin significantly shifted the calcium concentration–response curve to the left, thereby confirming the mechanism of action of these sweeteners on calcium entry during excitation contraction coupling in bladder muscle.

The chemical structure of acesulfame K resembles that of saccharin in that they both contain the SO_2 and N–C=O functionalities in a rigid framework (Venanzi and Venanzi, 1988). These two sweeteners have had very similar effects on rat bladder contraction whereas aspartame, having a very different chemical structure, has had little effect on rat bladder contractile response. It is therefore likely that acesulfame K and saccharin are interacting with the same structure within the bladder muscle but it is unlikely that this would be 1,4 dihydropyradine calcium channels as the activators of this receptor have a very different chemical structure to acesulfame K and saccharin.

Isolated bladder smooth muscle strips exhibit spontaneous phasic contractions which depend on Ca^{2+} entry through dihydropyridine-sensitive voltage-dependent Ca^{2+} channels (VDCC) (Mostwin, 1986). It would also appear that combined small-conductance Ca^{2+}-activated K^+ (SK) channels, ryanodine receptors (RyRs) and large-conductance Ca^{2+}-activated K^+ (BK) channels function as negative-feedback elements through differential modulation of contractile amplitude, duration and frequency (Herrera et al., 2000). Both acesulfame and sodium saccharin increased the amplitude and decreased the frequency of the rat bladder smooth muscle spontaneous contractions, which may involve modulation of these K^+ channels and activation of RyRs. Further investigation into the effect of these sweeteners on spontaneous contraction of bladder smooth muscle would be required to differentiate the mechanisms involved.

A recent study (Winkelmayer et al., 2005) investigating caffeine intake and the risk of hypertension in women found no association between caffeine consumption and the incidence of hypertension. However, they did find that the consumption of sugared and cola beverages were associated with an increased risk of hypertension. They speculated that some substance other than caffeine was responsible for the increased risk in hypertension. Could compounds, such as the artificial sweeteners, also be affecting vascular smooth muscle function via modulation of Ca^{+2} channels? Further research is required to elucidate this hypothesis.

Although the sweeteners enhance bladder contraction by a small amount compared to the control response, the concentrations of drink components used in the organ bath to examine their effect on the atropine-resistant response; response to α,β methylene ATP, KCl and the response to calcium were also small. A recent study has determined the concentration of four artificial sweeteners in food and beverages by ion chromatography (IC) (Zhu et al., 2005). They determined concentrations in two cola samples as well as in fruit juice and preserved fruit. The average concentrations of sweeteners in the two cola drinks were aspartame 5030 mg/L, acesulfame 1290 mg/L and sodium saccharin 381 mg/L. From these concentrations, we can estimate the concentration of two of the sweeteners in body fluids after the consumption of a 330-ml can of carbonated soft drink. With the use of drug kinetics and calculations to determine the concentration of drugs when distributed in body compartments, we are able to estimate the concentration of these sweeteners in body fluids. We estimate this would be 6.04 mg/L of acesulfame K and 23 mg/L of sodium saccharin, if distribution occurred equally in all body water. The concentrations of these sweeteners used in the organ bath, excluding those used for the EFS 10 Hz responses, were 20.1 ng/L for acesulfame K (10^{-7} M) and 20.5 ng/L for sodium saccharin (10^{-7} M).

The concentration of acesulfame and sodium saccharin has also been determined in urine (Wilson et al., 1999). After the intake of up to 110 mg/L acesulfame and 70 mg/L sodium saccharine, the peak urine concentrations were 118.6 mg/L and 80.2 mg/L, respectively. These levels were far greater that the concentrations we used in the organ bath (excluding EFS 10 Hz responses) and the concentration of these sweeteners in cans of cola have been determined to be even greater than the intake of sweeteners in the Wilson study. We are therefore confident that the concentration of sweeteners used in our organ bath (excluding EFS 10 Hz responses) is below the amount that would be found in body fluids after the consumption of a single can of carbonated soft drink.

Our results showed that aspartame only modified rat detrusor muscle contractile response to EFS 10 Hz and to low concentrations of calcium. This sweetener is different from acesulfame K and sodium saccharin in its affect on bladder contraction. It is possible that it has much weaker effects on calcium influx or it may be affecting a completely different pathway to those described so far. The other possibility is that we have observed non-specific effects. *In vivo* aspartame is not the active compound, as after ingestion it is metabolised to aspartic acid, methanol and phenylalanine (Karikas et al., 1998). Methanol is further metabolized to formaldehyde in both humans and rats (Ranney et al., 1976). Phenylalanine seems to play a role in the transport of precursors of monoamine neurotransmitters into the brain (Pin and Duvoisin, 1995), and aspartic acid (aspartame) has excitatory neurotransmitter functions in the gut mediated by ionotropic receptors (ligand-gated ion channels) and metabotropic receptors, coupled to G-proteins (Slobodan et al., 1999). We have carried out preliminary experiments investigating the effect of aspartic acid and phenylalanine on rat bladder contraction to 10 Hz EFS which showed no significant effect on contractile response (personal observation). It is therefore possible that aspartame itself had non-specific effects on bladder contraction due to direct actions on the muscle rather than through cellular pathways.

We acknowledge that one should employ caution when extrapolating data from epidemiological studies into the laboratory, and vice versa, but nevertheless we consider that the work described is a necessary first step in the hypothesis testing that is required after epidemiology research has generated new hypotheses. These experiments describe the effects of the sweeteners on isolated rat detrusor muscle and we

do not intend to suggest that these can necessarily be extrapolated to human muscle *in vitro*, or indeed to the detrusor muscle in vivo. Testing of these hypotheses requires further work, but we believe that our data are important to raise awareness of the potential for dietary factors to influence bladder and other organ function.

Conclusion

In conclusion, the stimulatory effect of artificial sweeteners on bladder muscle contraction, although small, is clearly evident from the results of our experiments and is greater than any difference found over the time course of our experiments. The probable mechanism of action is by increasing the intracellular Ca^{+2} concentration via extra cellular Ca^{+2} influx through L-type voltage-gated Ca^{+2} channels. A small increase in calcium flux may be all that is required to decrease the threshold for spontaneous, uncontrolled contraction, leading to detrusor muscle over activity, as we have seen in the effect of acesulfame and sodium saccharin on spontaneous bladder smooth muscle contractions. Apart from these sweeteners, the other chemical ingredients present in carbonated beverages, like ascorbic acid, citric acid, phenylalanine and colorants, may also modulate bladder muscle contraction via similar or different mechanism. As carbonated beverages are consumed in vast quantities, these results, and those of an epidemiological study by Dallosso et al. (2003), suggest that many people may be encouraging the future development of OAB. Changes in lifestyle and diet may be required to eliminate these possible predisposing factors for the later development of urinary incontinence.

Acknowledgments

We would like to thank The British Society of Urogynaecology (BSUG) for their generous support of this work. The BSUG had no involvement in the study design, analysis, the preparation of the manuscript or the decision to submit the manuscript for publication.

References

Abrams, P., 2003. Describing bladder storage function: overactive bladder syndrome and detrusor overactivity. Urology 62, 28–37.

Armstrong, B., Doll, R., 1974. Bladder cancer mortality in England and Wales in relation to cigarette smoking and saccharin consumption. Br. J. Prev. Soc. Med. 28, 233–240.

Armstrong, B., Doll, R., 1975. Bladder cancer mortality in diabetes in relation to saccharin consumption and smoking habits. Br. J. Prev. Soc. Med. 29, 73–81.

Auerbach, O., Garfinkel, L., 1989. Histologic changes in the urinary bladder in relation to cigarette smoking and use of artificial sweeteners. Cancer 64, 983–987.

Bo, X.N., Burnstock, G., 1989. [3H]-alpha, beta-methylene ATP, a radioligand labelling P2-purinoceptors. J. Auton. Nerv. Syst. 28, 85–88.

Brading, A.F., 2006. Spontaneous activity of the lower urinary tract smooth muscles: correlation between ion channels and tissue function. J. Physiol. 570, 13–22.

Brading, A.F., Sibley, G.N.A., 1983. A superfusion apparatus to study field stimulation of smooth muscle from mammalian urinary bladder. J. Physiol. (London) 334 (11p).

Brading, A.F., Williams, J.H., 1990. Contractile responses of smooth muscle strips from rat and guinea-pig urinary bladder to transmural stimulation: effects of atropine and alpha, beta-methylene ATP. Br. J. Pharmacol. 99, 493–495.

Braverman, A.S., Kohn, I.J., Luthin, G.R., Ruggieri, M.R., 1998. Prejunctional M1 facilitory and M2 inhibitory muscarinic receptors mediate rat bladder contractility. Am. J. Physiol. 274, R517–R523.

Chappel, Cl., 1992. A review and biological risk assessment of sodium saccharin. Regul. Toxicol. Pharmacol. 15, 253–270.

Creed, K.E., Ishikawa, S., Ito, Y., 1983. Electrical and mechanical activity recorded from rabbit urinary bladder in response to nerve stimulation. J. Physiol. 338, 149–164.

Christ, G.J., Venkateswarlu, K., Day, N.S., Valcic, M., Santizo, C., Zhao, W., 2004. Intercellular communication and bladder function. Adv. Exp. Med. Biol. 539, 239–254.

Dallosso, H.M, McGrother, C.W, Matthews, R.J., Donaldson, M.M.K., 2003. The association of diet and other lifestyle factors with overactive bladder and stress incontinence: a longitudinal study in women. BJU Int. 92, 69–77.

Elliott, R.A., Castleden, C.M., Miodrag, A., Kirwan, P., 1992. The direct effect of diethylstilboestrol and nifedipine on the contractile responses of isolated human and rat detrusor muscles. Eur. J. Pharmacol. 43, 149–155.

Giglio, D., Delbro, D.S., Tobin, G., 2001. On the functional role of muscarinic M2 receptors in cholinergic and purinergic responses in the rat urinary bladder. Eur. J. Pharmacol. 428:, 357–364.

Hagiwara, A., Fukushima, S., Kitaori, M., Shibata, M., Ito, N., 1984. Effects of three sweetners on urinary bladder carcinogenesis initiated by N-butyl-N-(4-hydroxybutyl)-nitrosamine. Gann 75, 763–768.

Hampel, C., Gillitzer, R., Pahernik, S., Hohenfellner, M., Thuroff, J.W., 2003. Epidemiology and aetiology of overactive bladder. Urology 42, 776–786.

Herrera, G.M., Heppner, T.J., Nelson, M.T., 2000. Regulation of urinary bladder smooth muscle contractions by ryanodine receptors and BK and SK channels. Am. J. Physiol.: Regul., Intergr. Comp. Physiol. 279, R60–R68.

Hudman, D., Elliott, R.A., Norman, R.I., 2000. Inhibition of the contractile response of the rat detrusor muscle by the β2 adrenoceptor agonist clenbuterol. Eur. J. Pharmacol. 392, 79–85.

Ishii, H., 1981. Incidence of brain tumours in rats fed aspartame. Toxicol. Lett. 7, 433–437.

Karikas, G.A., Reclos, G., Kokotos, G., 1998. Measurement of molecular interaction of aspartame and its metabolites with DNA. Clin. Biochem. 31, 405–407.

Maggi, C.A., Giuliani, S., Patacchini, R., Turini, D., Barbanti, G., Giachetti, A., Meli, A., 1989. Multiple sources of calcium for contraction of the human urinary bladder muscle. Br. J. Pharmacol. 98, 1021–1031.

Mostwin, J.L., 1986. The action potential of guinea-pig bladder smooth muscle. J. Urol. 135, 1299–1303.

Namasivayam, S., Eardley, I., Morrison, J.F.B., 1999. Purinergic sensory neurotransmission in the urinary bladder: an in vitro study in the rat. BJU Int. 84, 854–860.

Nguyen, U.N., Dumoulin, G., Henriet, M.T., Regnard, J., 1998. Aspartame ingestion increases urinary calcium, but not oxalate excretion, in healthy subjects. J. Clin. Endocrinol. Metab. 83, 165–168.

Noda, K., Takebe, M., Oka, M., Ukai, HirouchiM., Toda, Y., 2002. Functional role of inhibitory and excitatory nerves in the porcine lower urinary tract. Eur. J. Pharmacol. 456, 81–90.

Oike, M., Creed, K.E., Onoue, H., Tanaka, H., Ito, Y., 1998. Increase in calcium in smooth muscle cells of the rabbit bladder induced by acetylcholine and ATP. J. Auton. Nerv. Sys. 69, 141–147.

Pin, J.P., Duvoisin, R., 1995. The metabotropic glutamate receptors: structure and function. Neuropharmacology 34, 1–26.

Ranney, R.E., Opperman, J.A., Maldoon, E., McMahon, F.G., 1976. Comparative metabolism of aspartame in experimental animals and humans. J. Toxicol. Environ. Health 2, 441–451.

Ruggieri Sr., M.R., 2006. Mechanisms of disease: role of purinergic signalling in the pathophysiology of bladder dysfunction. Nat. Clin. Pract. Urol. 3, 206–215.

Sibley, G.N.A., 1984. A comparison of spontaneous and nerve-mediate activity in bladder muscle from man, pig and rabbit. J. Physiol. (London) 354, 431–443.

Slobodan, J.M., Dragen, M., Milovan, M., Violeta, I.-C., 1999. The effects of excitatory amino acids on isolated gut segments of the rats. Pharmacol. Res. 39, 143–148.

Soffritti, M., Belpoggi, F., Degli Esposti, D., Lambertini, L., Tibaldi, E., Rigano, A., 2006. First experimental demonstration of the multipotential carcinogenic effects of aspartame administered in the feed to Sprague–Dawley rats. Environ. Health Perspect. 114, 379–385.

Squire, R.A., 1985. Hispathological evaluation of rat urinary bladders from the IRDC two-generation bioassay of sodium saccharin. Food Chem. Toxicol. 23, 491–497.

Takayama, S., Sieber, S.M., Adamson, R.H., Thorgeirsson, U.P., Dalgard, D.W., Arnold, L.L., Cano, M., Eklund, S., Chen, S.M., 1998. Long-term feeding of sodium saccharin to nonhuman primates: implications for urinary tract cancer. J. Natl. Cancer Inst. 90, 19–25.

Taylor, J.M., Weinberger, M.A., Friedman, L., 1980. Chronic toxicity and carcinogenicity to the urinary bladder of sodium saccharin in the in utero exposed rat. Toxicol. Appl. Pharmacol. 54, 57–75.

Tong, Y.C., Hung, Y.C., Lin, S.N., Cheng, J.T., 1997. Pharmacological characterization of the muscarinic receptor subtypes responsible for the contractile response in the rat urinary bladder. J. Auton. Pharmacol. 17, 21–25.

Venanzi, T.J., Venanzi, C.A., 1988. A molecular electrostatic-potential study of acesulfame. Anal. Chim. Acta 210, 213–218.

Visser, A.J., van Mastrigt, R., 2000. The role of intracellular and extra cellular calcium in mechanical and intracellular electrical activity of human urinary bladder smooth muscle. Urol. Res. 28, 260–268.

Wilson, L.A., Wilkinson, K., Crews, H.M., Davies, A.M., Dick, C.S., Dumsday, V.L., 1999. Urinary monitoring of saccharin and acesulfame-K as biomarkers of exposure to these additives. Food. Addit. Contam. 16, 227–238.

Winkelmayer, W.C., Stampfer, M.J., Willett, W.C., Curham, G.C., 2005. Habitual caffeine intake and the risk of hypertension in women. JAMA 294, 2330–2334.

Wu, C., Bayliss, M., Newgreen, D., Mundy, A.R., Fry, C.H., 1999. A comparison of the mode of action of ATP and carbachol on isolated human detrusor smooth muscle. J. Urol. 162, 1840–1847.

Yono, M., Yoshida, M., Takahashi, W., Inadome, A., Ueda, S., 2000. Comparison of the effects of novel antimuscarinic drugs on human detrusor smooth muscle. BJU Int. 86, 719–725.

Yoshida, M., Homma, Y., Inadome, A., Yono, M., Seshita, H., Miyamoto, Y., Murakami, S., Kawabe, K., Ueda, S., 2001. Age-related changes in cholinergic and purinergic neurotransmission in human isolated bladder smooth muscles. Exp. Gerontol. 36, 99–109.

Zhu, Y., Guo, Y., Ye, M., James, F.S., 2005. Separation and simultaneous determination of four artificial sweeteners in food and beverages by ion chromatography. J. Chromatogr., A 1085, 143–146.