THE EFFECT OF DRUGS ON ISOLATED DETRUSOR MUSCLE CONTRACTION

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By

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The effect of drugs on isolated detrusor muscle contraction. ***Ruth A Elliott-Pearce.***

Detrusor instability is the commonest type of urinary incontinence in the elderly and is present in up to 50% of patients attending continence clinics. Treatment of this condition, aimed at reducing uncontrollable detrusor contractions, is at present unsatisfactory.

For example, calcium antagonists are clinicly disappointing and studies were carried out to investigate why they are ineffective. Rats were treated with nimodipine for 8 days or with a single dose. Treatment for 8 days had no effect on isolated detrusor contraction but a single dose reduced detrusor contractile response. It is propossed that chronic treatment with nimodipine caused an up-regulation of calcium channels as a compensatory mechanism.

Oestrogens have been shown to have an inhibitory effect on detrusor muscle contraction after *in vitro* and *in vivo* treatment. In post-menopausal women with a uterus unopposed oestrogens should not be given, but progesterone has anti-oestrogenic actions. When rats were treated with oestrogen and progesterone for 8 days, there was no effect on rat detrusor contractile response. An anti-oestrogenic effect of progesterone has therefore been demonstrated in rat detrusor smooth muscle.

Caffeine has been shown to increase detrusor pressure on bladder filling in patients with detrusor instability. The effect of low concentrations of caffeine on the contractile response of isolated human and rat detrusor muscle was therefore determined. Caffeine was found to have only a slight potentiating effect on isolated human and rat detruosr muscle contraction.

The results in this thesis have important clinical implications for the treatment of detrusor instability. It may be more effective to administer calcium antagonists in an intermittent manner. Oestrogens are better given alone or with the lowest possible dose of progestogens. Caffeine would not be contraindicated in patients with detrusor instability.

Declaration.

The experimental work presented in this thesis was performed by myself between January 1993 and October 1996, whilst working in the Division of Medicine for the Elderly, University of Leicester. Analysis of data and writing of this thesis was also carried out by myself. Determination of rat serum nimodipine levels was performed by R Paul Whitaker, Toxicology Laboratory, Leicester Royal Infirmary, Leicester.

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CHAPTER 1: INTRODUCTION

Urinary Bladder Anatomy, Structure, Innervation and Mechanisms of Contraction

1.1 Objectives

Urinary incontinence is a major problem in the elderly with considerable medical, psychological, social and economic implications and it also predisposes people to other health problems such as infections and skin breakdown. This frequently led to admission into institutions and increased social isolation (Williams *et al.*, 1982). With an increasing elderly population the total financial and human cost is likely to rise, even though advances have been made in understanding urinary pathophysiology.

Pharmacological therapy and surgical interventions have improved the treatment of urinary incontinence and the introduction of urodynamics has improved diagnosis, but the need for more effective treatment is still of paramount importance. The main objective of this research was to investigate the pharmacology of detrusor muscle contraction and to identify possible beneficial treatments for urinary incontinence, particularly detrusor instability. This required detailed knowledge of the structure, innervation and contractile properties of detrusor smooth muscle with an understanding of the changes that take place resulting in disordered detrusor function. With detrusor instability being of major interest in which the contractility of detrusor muscle is overactive, experiments were designed to investigate the effect of hormones and drugs on detrusor contractile response.

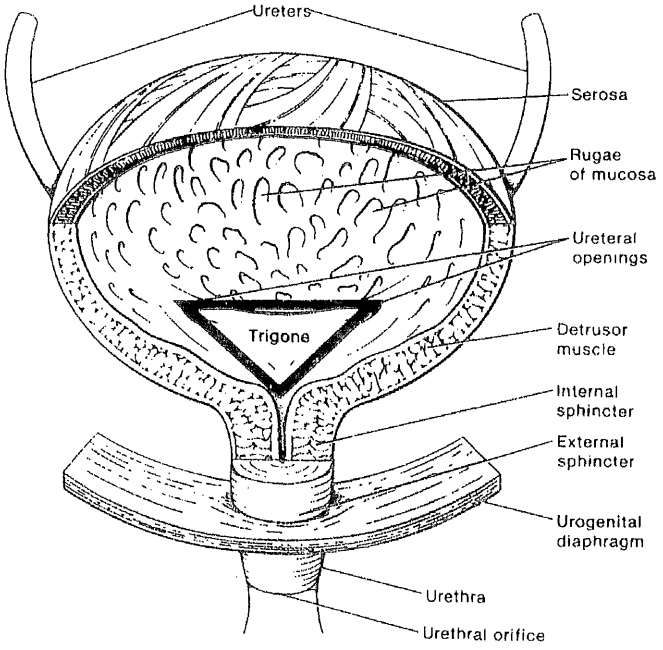


Figure 1. Urinary bladder and female urethra.

(Tortora & Anagnostakos 1996)

1.2 Urinary bladder anatomy.

The urinary bladder is situated in the pelvic cavity and in the male it is directly anterior to the rectum. In the female it is anterior to the vagina and inferior to the uterus (Tortora & Anagnostakos 1996). When the urinary bladder is empty it assumes the shape of a deflated balloon, but becomes pear shaped as the volume of urine inside increases (Figure 1). At the base of the bladder is a small triangular area called the

trigone, and at the apex of this area is found the opening of the urethra. At the top of the trigone is the opening of the ureters into the bladder.

1.3 The structure of the urinary bladder

The urinary bladder is a hollow muscular organ consisting of a serosal layer, a muscular layer called the detrusor muscle and a mucosal layer of transitional epithelium. The detrusor muscle consists of a central region of circularly arranged fibres between longitudinal bundles. There are frequent ionic exchanges between the muscle layers which have no separation between the fibres (Gosling 1979). The arrangement of the muscle layers allows the bladder to contract and expand in all directions.

A property of normal detrusor smooth muscle is the poorly developed electrical coupling between cells which allows continuous electrical activity in the smooth muscle cells during the filling phase without causing a significant rise in intravesical pressure (Brading & Turner 1994). This allows the bladder volume to increase, during storage, whilst keeping the pressure inside the bladder low.

The mechanism by which the urinary bladder contracts and relaxes is related to its nerve supply which arises from the autonomic parasympathetic and sympathetic nervous systems. The nature of this innervation varies between man and the smaller mammals.

1.4 Innervation of the human bladder

The function of the lower urinary tract is related to the localisation of autonomic neuroreceptors. In the lower urinary tract of man, α-adrenergic receptors

predominate in the bladder outlet and along the urethra and contribute to the maintenance of continence by increasing urethral tone. Beta-adrenergic receptors are located in the body and dome of the bladder and have also been shown in the bladder outlet and urethra. The lower urinary tract has only $ß_2$- receptors, stimulation of which aids detrusor relaxation (Eaton & Bates 1982). Parasympathetic nerve stimulation releases acetylcholine which at muscarinic receptor sites initiates contractile responses through the inositol-1,4,5- trisphosphate (IP_3) mediated release of intracellular calcium (Iacovou *et al.*, 1990).

The majority of cholinergic receptors are located in the bladder but some are also found in the bladder outlet and urethra. Stimulation of cholinergic receptors causes detrusor muscle contraction and relaxation of the trigone. It has been claimed that a non-adrenergic, non-cholinergic (NANC) component of parasympathetic nerve stimulation is absent in the human bladder (Sibley 1984), but others have demonstrated atropine-resistant responses, mainly in detrusor muscle from patients with bladder disorders (Hindmarsh *et al.*, 1977, Sjögren *et al.*, 1982, Cowan & Daniel 1983, Nergårdh & Kinn 1983). It has been suggested that this component is purinergic but there seemed to be regional variations in its distribution (Speakman *et al.*, 1989, Hoyle *et al.*, 1989). In the trigone, adenosine triphosphate (ATP) responses are prevalent and purinergic receptors are densely distributed but in the tip of the bladder dome they are low or even absent. The binding characteristics of [^{3}H]α,β-MeATP to washed homogenates and membrane preparations of human bladder were similar to those from rat urinary bladder (Bo & Burnstock, 1995). However, only 38% of human bladder specimens in the binding study and 43% in the localisation study showed specific [^{3}H]α,β-MeATP binding whereas all rat bladder specimens tested showed specific binding (Bo & Burnstock, 1995). Therefore, not all the human detrusor samples tested contained detectable levels of purinoceptors, those which did had lower receptor densities than in the rat detrusor muscle.

Sympathetic impulses aid bladder filling by producing contractions of the urethra and bladder neck, and relaxation of the bladder. The sympathetic nerves supplying the bladder contain neuropeptide Y as well as noradrenaline (Gu *et al.*, 1984, Crowe & Burnstock 1989). In addition, other neuropeptides such as substance P, calcitonin gene-related peptide and vasoactive intestinal polypeptide have been localised in the nerves of the bladder wall (Chapple *et al.*, 1992).

There is some question regarding the role of nitric oxide and relaxation of the detrusor muscle. Previous investigations have shown that isolated, contracted urethral smooth muscles from rabbit, sheep, pig and man respond to transmural stimulation with a relaxant response mediated by a non-adrenergic, non-cholinergic mechanism (Andersson *et al.*, 1983, Klarskov *et al.*, 1983, Andersson *et al.*, 1991, Garcia-Pascual *et al.*, 1991). This electrically evoked relaxation could be completely blocked by N^G-nitro-L-arginine (L-NOARG), which inhibits the synthesis of nitric oxide (NO) from L-arginine (Mülsch & Busse, 1990). To date NANC-nerve mediated relaxation, involving the L-ARG/NO pathway has not been consistently demonstrated in the detrusor smooth muscle (Persson & Andersson, 1992, Persson *et al.*, 1992).

Voiding is mediated predominately by parasympathetic transmission with cholinergic stimuli producing detrusor contraction while simultaneously inhibiting sympathetic activity.

1.5 Innervation of the mammalian urinary bladder

There are important species differences in the nature of the excitatory innervation of the bladder muscle relating to the contributions of cholinergic and non-cholinergic mechanisms (Sibley 1984). It has been known for many years that the

contractile response of mammalian detrusor muscle to pelvic nerve stimulation is only partially blocked by atropine (Langley & Anderson, 1895). They observed that the contractile response of dog, rabbit and cat bladders to nerve stimulation was only slightly reduced by atropine. Later, it was widely accepted that the atropine-resistant response was due to a non-adrenergic, non-cholinergic (NANC) transmitter (Ambache & Zar,1970, Moss & Burnstock,1985). This transmitter was subsequently identified as ATP (Burnstock *et al.,* 1972, 1978, Dean & Downie, 1978, Kasakov & Burnstock, 1983, Levin *et al.,* 1986).

It has been demonstrated recently that the NANC response can be blocked by an ATP receptor antagonist, arylazidoaminopropionyl ATP ($ANAPP_3$), and by α,ß-methylene-ATP, which is an analogue of ATP that desensitises P_2 purinoceptors and abolishes excitatory junction potentials recorded in the smooth muscle of the bladder in response to NANC stimulation (Hoyle & Burnstock 1985, Fujii 1988, Brading & Williams 1990). Indeed, it has been suggested that ACh and ATP are co-transmitters in intrinsic parasympathetic neurones in the bladder (MacKenzie *et al.,* 1982). Recently, the functional importance of these transmitters for micturition contraction in the normal unanaesthetized rat has been demonstrated (Igawa *et al.,* 1993). ATP administered intra-arterially (i.a) close to the bladder produced rapid, phasic, dose dependent increases in bladder pressure with micturition immediately after injection. Pre-treatment with α,ß-methylene ATP blocked the effects of ATP. The administration of carbachol i.a. also produced rapid, sustained, dose-dependent increases in bladder pressure with micturition. However, bladder emptying was not possible after blockade of the micturition reflex with morphine (10μg intrathecally) suggesting that drug induced bladder emptying in the normal, unanaesthetized rat requires an intact micturition reflex. These results also suggests that the two physiologically important transmitters involved in micturition are acetylcholine and ATP (Igawa et al 1993). This dual innervation is found in most animal species and

purinergic activity is probably involved in behavioural activity, such as scent marking, where complete emptying of the bladder is not required (Brading 1992). Others support the hypothesis that ATP may be important in the initiation of micturition since ATP generated pressure is more rapid than cholinergic stimulation alone (Chancellor *et al.*, 1992).

1.6 Mechanism of detrusor smooth muscle contraction

There is much evidence indicating that smooth muscles utilise many sources of calcium ions for contraction (Bolton 1979, Brading & Sneddon 1980, Casteels & Droogmans 1982, Bolton & Kitamura 1983). In the urinary bladder, resting tone, spontaneous activity and contractions induced by agonists and electrical field stimulation are dependent on extracellular calcium (Andersson & Forman 1986). Extracellular calcium enters smooth muscle cells via two pathways, voltage-operated and receptor-operated calcium channels. The properties of bladder calcium channels have been examined in guinea-pigs and humans (Klöckner & Isenberg 1985a, Klöckner & Isenberg 1985b, Montgomery & Fry 1992, Brading 1992). Electrophysiological analysis suggests there is only one type of voltage-operated calcium channel present: L-type which is sensitive to 1,4-dihydropyridine activators and antagonists (Montgomery & Fry 1992, Triggle *et al.*, 1992). Some are also sensitive to ω-conotoxin which has been shown to inhibit EFS-induced contraction in the rabbit detrusor (Zygmunt *et al.*, 1993). Other types of calcium channels, T, N, and P are all insensitive to the organic calcium antagonists and activators (Triggle *et al.*, 1992).

A feature of bladder smooth muscle is its inability to sustain tone in response to prolonged application of agonists and depolarisation with high concentrations of K^+ which may be due to calcium-induced inactivation of the voltage-sensitive channels

(Brading 1992) or reversal of the membrane potential. It is possible that membrane permeability to clacium ions increases transiently with the channels then closing even in the presence of persistent depolarisation (Brading 1992).

It was thought initially that agonists produced contraction by depolarising the cell membrane leading to calcium entry through voltage-sensitive calcium channels (Evans & Schild 1957). It was then observed that depolarised tissue could contract further in response to agonists (Evans *et al.,* 1958) suggesting a voltage-independent mechanism for activating contraction. The contractile response to agonists in calcium free solution is lost only gradually, suggesting an intracellular source of calcium for contraction in addition to that provided by the extracellular medium. Mostwin (1985) investigated receptor-operated intracellular calcium stores in the smooth muscle of the guinea pig bladder. He observed that the bladder muscle retained the ability to contract to muscarinic stimulation in calcium free medium and that the magnitude of the contraction decreased with time. He found that carbachol was capable of producing contraction in a calcium free medium for a longer period of time than K^+ depolarisation (which opens calcium channels in bladder smooth muscle cells) and that once the ability of carbachol to produce contraction was lost it could be restored temporarily by a brief application of calcium containing solution. The loss of response to K^+ depolarisation was more rapid and more profound than that to muscarinic stimulation. In depolarised bladder tissue exposed to the calcium antagonist nifedipine, carbachol could only elicit one large contraction suggesting depletion of an intracellular store. Mostwin (1985), therefore, concluded that the response of the bladder to depolarisation depends primarily on extracellular calcium but that the response to carbachol also involved the release of stored intracellular calcium.

Fovaeus *et al.,* (1987) examined the effects of calcium, calcium channel blockers and the calcium channel agonist Bay K 8644 on muscarinic receptor

stimulation of isolated bladder muscle from rabbit and man. In their experiments they used lanthanum which is a cation considered to compete with calcium for extracellular negative sites in smooth muscle and to bind to negative sites in the calcium channels, thus blocking both potential and receptor operated channels. In contrast, nifedipine, which caused a 40% inhibition of the response to the highest carbachol concentration used, seems to block only one of the activation pathways, probably the potential operated channel (Fovaeus *et al.,* 1987). The effect of nifedipine decreased with increasing carbachol concentrations. In contrast to Mostwin (1985) they concluded that contractions produced by muscarinic receptor stimulation were primarily dependent on calcium bound to the outside of the membrane of the smooth muscle coming from the extracellular medium, and that the release of calcium stored within the cell may not be a major source of activator calcium in the rabbit or human detrusor muscle. Their experimental technique differed to that of Mostwin (1985) with respect to the exposure of bladder muscle to carbachol stimulation. Mostwin subjected his tissue samples to ten second applications of carbachol producing an initial phasic response, dependent on the release of intracellular calcium, followed by a tonic response dependent on the influx of extracellular calcium. Fovaeus *et al.,* (1987) used cumulative additions of carbachol, resulting in prolonged tonic response, which does not represent intrinsic muscarinic receptor stimulation by acetylcholine, as this is rapidly hydrolysed by acetylcholinesterase. The tonic response of detrusor smooth muscle to prolonged muscarinic stimulation may, therefore, explain the discrepancy in their results.

Subsequent studies on the contraction of isolated human bladder muscle found that multiple sources of calcium are mobilised for the contraction of human bladder muscle to different stimulants (Maggi *et al.,* 1989). Carbachol, neurokinin A and endothelin mobilise a calcium pool which is $LaCl_3$-sensitive (lanthanum chloride) but nifedipine-resistant. These agents also mobilise a tightly bound Ca^{2++} pool

independently from membrane depolarisation. This is probably a procaine-sensitive intracellular source of activator Ca^{2++} mobilised by caffeine and carbachol. The failure of procaine to prevent the response to endothelin in high K^{+}, Ca^{2++}-free medium raises the possibility that this peptide mobilises an intracellular source of activator Ca^{2++}, distinct from the caffeine-and carbachol-sensitive pool (Maggi *et al.*, 1989).

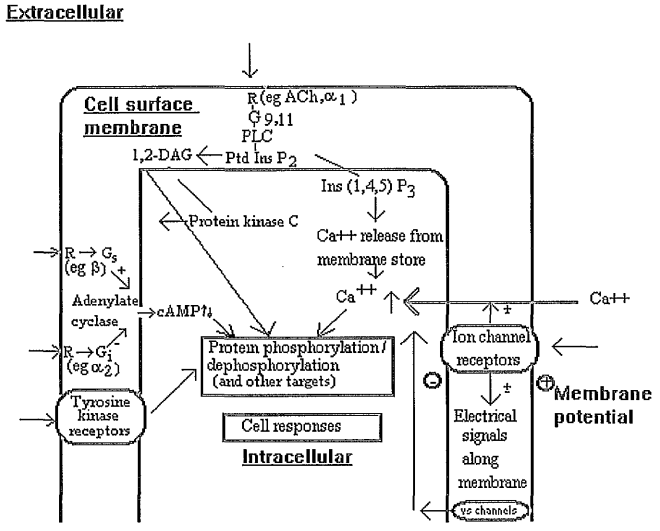


Figure 2. ***Diagram of some of the mechanisms of transmembrane signalling used by cell surface receptors. On the left are receptors possessing intrinsic protein tyrosine kinase activity, alongside the control of adenylate cyclase by stimulatory and inhibitory receptors (R) mediated by G_s and G_i. At the top is receptor stimulated hydrolysis of PtdInsP_2 by phospholipase C leading to intacellular accumulation of 1, 2-DAG, Ins(1,4,5) P_3 and Ca^{2+}. On the right are the actions of receptors possessing intrinsic ion channels. (Michell 1987)***

There is now much evidence that inositol (1,4,5)-trisphosphate (IP_3) is the cytoplasmic second messenger that mobilises intracellular stores of calcium during smooth muscle contraction, including detrusor muscle (Berridge & Irvine, 1989, Iacovou *et al.*, 1990) (Fig 2). IP_3 is one of the messengers that is released when hydrolysis of a membrane phospholipid, phosphatidyl 4,5- bisphosphate (PIP_2), is

stimulated by the action of an agonist at a membrane receptor such as the muscarinic receptors on the surface of smooth muscle cells. The receptor is linked by a guanine-nucleotide binding protein to the enzyme phospholipase C (PLC) (Abdel-Latif 1986, Michell 1987). Stimulation of the receptor activates the enzyme which catalyses the hydrolysis of PIP_2 resulting in the release of two substances, IP_3 and diacylglycerol, both of which are second messengers. Inositol (1,4,5)-trisphosphate rapidly diffuses into the cell where it binds to an IP_3 receptor on the sarcoplasmic reticulum and mediates the phasic release of calcium which is available to activate contraction (Norman 1993) In smooth muscle the IP_3 induced calcium signal acts as a primer to drive a process of calcium-induced calcium release from the IP_3 insensitive pools to produce a spike organised in the form of a wave, spreading the signal throughout the cell (Berridge & Irvine 1989). Waves are not confined to single cells but can travel from cell to cell through various mechanisms (Berridge 1993).

Detrusor smooth muscle contraction is, therefore, initiated by muscarinic receptor activation which stimulates an intracellular pathway of events. These result in the release of bound intracellular calcium which promotes the entry of external calcium and leads to contraction.

Urinary Incontinence: Background, Definition, Prevalence and Classification

1.7 Historical background

Methods of managing urinary incontinence were described by the Egyptians in the Papyrus Ebers (1500 BC). Similar advice is also described in ancient Greek literature. The incontinent person was recommended a special diet and given practical advice on how to live with this handicap (Molander 1992).

From the 18th century there are reports on the occurrence of urinary incontinence after child birth. Apart from these there has been surprisingly little written about incontinence from an historical perspective (Molander 1992). During the Victorian era, in an atmosphere of prudery, the incontinent person would feel embarrassed or ashamed and not tell anyone about his or her condition. In many respects the problem of urinary incontinence is still a symptom which is not discussed in public. Indeed, this problem is hidden from closest relatives and there is also a reluctance to tell the doctor about it (Molander 1992).

1.8 Definition of urinary incontinence

Incontinence is a word derived from the Latin meaning wanting in self control and the involuntary leakage of urine is defined as urinary incontinence (U.I). The International Continence Society (I.C.S) has defined U.I. as a condition where the involuntary loss of urine is a social or hygienic problem and is objectively demonstrable (Bates *et al.*, 1979).

1.9 Prevalence of urinary incontinence

Urinary incontinence is more common in women than men (Brocklehurst 1993) and the reason for this is thought to be due to anatomical differences. The structures contributing to closure of the urethra are more complex in women than men, who have a longer urethra with the middle section supported by a well developed urogenital diaphragm. Incontinence in men is usually associated with outlet obstruction due to prostatic hypertrophy (Williams *et al.,* 1982). The urethra is shorter in women and its attachment is often poor due to weakening of the pubo-urethral ligament. Pregnancy, child birth and increasing age can induce changes in the pelvic floor muscles which may increase urethral motility resulting in stress incontinence. Other causes of urinary incontinence have an association with urogenital symptoms such as vaginal atrophy and lower urinary tract infections (Iosif & Bekassy 1984, Berg *et al.,* 1988).

The prevalence of urinary incontinence in women tends to increase with age (Brocklehurst *et al.,* 1971, Yarnell and St Leger 1979, Williams *et al.,* 1982, McGrother *et al.,* 1987, Molander *et al.,* 1990, Rekers *et al.,* 1992, Brocklehurst 1993) although there are differing opinions on the pattern of this increase (Thomas *et al.,* 1980, Iosif *et al.,* 1981, Ouslander *et al.,* 1982, Diokno *et al.,*1986, Jolleys 1988). These variations are partly due to differing definitions of incontinence and also because of the different methods used to elicit the presence of incontinence. Some use rather liberal definitions of urinary incontinence which include any uncontrolled urine loss in the prior twelve months regardless of severity (Diokno *et al.,* 1986). Others use more strict definitions such as two or more incontinent episodes per month (Thomas *et al .,* 1980). Some studies performed urodynamics to confirm incontinence (McGrother *et al.,* 1987); others relied on symptomology only and made no attempt to gauge the quantity of urine loss (Thomas *et al.,* 1980). The prevalence of urinary

incontinence therefore depends on the populations studied. Some studies are confined to certain age groups, such as over 70 years of age (Vetter *et al.*, 1981, McGrother *et al.*, 1987) others investigated the prevalence rate of urinary incontinence in institutions where the reason for admission is often incontinence (Ouslander *et al.*, 1982). Therefore, the prevalence rate will be high in these populations.

The reported prevalence of urinary incontinence in the community varies from 1.6% to 26% (Issacs and Walkey 1964, Brocklehurst *et al.*, 1968, Feneley *et al.*, 1979, Milne 1976, Yarnell and St Leger 1979, Brocklehurst 1993). Clinically significant urinary incontinence is present in 4% to 10% of elderly people in the community and this prevalence rises to an estimated 15% -16% in men and women aged 85 and over (McGrother *et al.*, 1987). Vetter *et al* (1981) examined the prevalence of urinary incontinence in the elderly at home and found that 14% of the over 70's had any degree of incontinence with 7% in men and 18% in women.

In institutions the prevalence of urinary incontinence varies from 12.9% to 48% and is thus much higher than in the non institutionalised community (Isaacs and Walkey 1964, Milne 1976, Donaldson *et al.*, 1983, Peet *et al.*, 1995). Incontinence in hospitals is not associated with sex or increasing age but is related to physical disability and neurological diseases (Isaacs and Walkey 1964, Milne 1976). The hospital prevalence rate appears to remain stable (Milne 1976), although a recent study in Leicestershire has shown that the decrease in the proportion of highly dependent people in NHS beds has been countered by increases in the proportions of dependent people in other institutions (Stern *et al.*, 1993).

1.10 Classification of urinary incontinence

There are four main types of urinary incontinence (see Table I) as described below.

1.10.1. Stress incontinence.

The diagnosis of stress incontinence is based on a history of urine loss during coughing, sneezing, laughing or straining (Peggs 1992). This is a dysfunction of the bladder outlet leading to transient loss of small volumes of urine when the intra-abdominal pressure is raised above urethral resistance during exertion. Laughing, coughing, straining or bending are activities capable of causing small losses of urine in women with sphincter insufficiency. Fifty percent of young women admitted to occasional minor leakage (Graber 1977, Nemia and Middleton 1954). Normal ageing in women plus multiparity and surgical manipulation can cause incompetence of the pelvic floor muscles resulting in stress incontinence (Green 1975). Local urethral inflammation due to infection or oestrogen deficiency can also cause stress incontinence (Salmon *et al.*, 1941, Wilington 1978). Postmenopausal women with stress incontinence have improved following oestrogen therapy (Hilton and Stanton 1983, Rud 1980, Miodrag *et al.*, 1988).

In men stress incontinence usually occurs after urological surgery and neurological diseases with urinary tract infections, papilloma, chronic inflammation and radiation damage, being very rare causes of stress incontinence (Raz 1978).

1.10.2 Overflow incontinence.

This type of incontinence occurs when the intravesicular pressures exceed urethral closure pressures at high bladder volumes. The elevation of intravesical pressure is associated with bladder distension which may lead to detrusor instability when associated with obstruction due to tumours and prostatic hypertrophy. Surgical operations such as pelvic floor repair can also cause overflow incontinence, which is more common in men than women.

A functional obstruction can be created by dyssynergistic contractions of the detrusor and external sphincter. This condition has been described in patients with severe neurological disease involving the spinal cord (Blaivas *et al.*, 1980).

Detrusor inadequacy is another cause of this type of incontinence and implies insufficient detrusor tone to overcome normal intraurethral resistance. Reasons for detrusor inadequacy include lower motor neurone diseases (McGuire 1980), diabetic autonomic neuropathy, alcoholic neuropathy and medications such as muscle relaxants. Permanent detrusor inadequacy can result from lower spinal cord lesions although most patients develop spontaneous bladder contractions (Perlow and Diokno 1981).

Another cause of overflow incontinence is impaired sensory input from the bladder commonly due to diabetes mellitus or tabes dorsales. These patients are not aware of the need to void but they can control their overflow incontinence so long as they remember to void.

1.10.3 Functional Incontinence.

This type of incontinence occurs when normally continent persons are unable to reach the toilet in time to avoid an accident. Joint abnormalities, arthritic pain, muscle weakness or strokes may prevent an otherwise continent person reaching the toilet in time. An unfamiliar setting, lack of convenient toilet facilities, or other environmental factors can aggravate these conditions (Williams *et al.*, 1982).

Type of Incontinence	Those Affected	Cause
Stress incontinence	Women. All ages.	Inadequate closure of Bladder Outlet. Weak Pelvic floor muscles.
Overflow incontinence	Mainly older men. Neuropathies	High bladder volumes
Functional incontinence	Men & women. All ages.	Environmental
Detrusor instability	Mainly women. Men with BPH.* All Ages	Uninhibited detrusor muscle contractions.

Table 1. Classification of urinary incontinence
(*BPH Benign prostatic hyperplasia)

1.10.4 Detrusor instability.

An unstable bladder is one that is shown objectively to contract, spontaneously or on provocation, during the filling phase while the person is attempting to inhibit micturition (ICS 1988). Detrusor instability is the commonest type of incontinence in

the elderly and is present in up to 50% of patients attending incontinence clinics (Torrens & Griffiths 1974, Abrams *et al.*, 1983, Cardozo 1984). It has been alleged that up to 10% of the population may suffer from detrusor instability (Cardozo 1990). A certain number of patients with unstable bladders have an underlying neurological cause and this type of incontinence is termed detrusor hyper-reflexia. However, for the majority of people with detrusor instability a cause cannot be found and so they are said to have "idiopathic detrusor instability". A diagnosis of the unstable bladder can be obtained by urodynamic investigation. The first International Continence Society Report on the standardisation of terminology (ICS 1976) stated that "the presence of contractions greater than 15cm H_20 clearly indicates an uninhibited detrusor contraction when the patient has been asked to inhibit micturition". It is now generally accepted that any unstable detrusor contraction is significant with respect to the patient's symptoms (Freeman & Malvern 1989). In men this condition is often associated with outflow obstruction due to benign prostatic hypertrophy but in women outflow obstruction is an uncommon association.

1.11 Present knowledge of the mechanism of detrusor instability

Pathophysiological changes in detrusor smooth muscle have been observed in man and animals following bladder outflow obstruction. As there is a similarity between all types of detrusor instability these observations may shed some light on the underlying mechanisms of this condition.

Histological studies have demonstrated denervation associated with bladder outflow obstruction (Gosling *et al.*, 1986) and a re-innervation of the bladder muscle following prostatectomy (Cumming & Chisholm 1992). Speakman *et al* (1987) observed a reduction in the density of acetylcholinesterase positive nerves in all unstable bladders examined in their study. Degenerating nerve profiles were also seen

on electron microscopy. These changes have also been observed in animals with outflow obstruction (Harrison *et al.*, 1990, Kato *et al.*, 1988). Partial denervation in the obstructed bladder of the rabbit has been demonstrated (Harrison *et al.*, 1990, Kato *et al.*, 1988), but denervation was not found in the rat (Gabella & Uvelius 1990). The rat is the only species that does not have intramural ganglia (ganglion neurones in the bladder wall), although the bladder musculature is well innervated having fibres of extrinsic origin (Gabella & Uvelius 1990).

Other studies on detrusor muscle from unstable human bladders have shown a reduction in the density of α-adrenoceptors (Restorick & Mundy 1989) and in pre-synaptic α-adrenoceptor activity (Eaton & Bates 1982). An increase in atropine resistance of transmurally stimulated isolated human bladder muscle has been demonstrated also in unstable detrusor samples (Sjögren *et al.*, 1982).

The contractile properties of isolated smooth muscle strips from unstable bladders differs from normal bladders. Muscle from unstable bladders are less responsive to transmural nerve stimulation and generate less force per unit weight (Brading & Turner 1994). Kinder & Mundy (1987) also reported increased spontaneous activity and fused contractions in smooth muscle from unstable bladders of both neurogenic and idiopathic aetiology. They did not find evidence of decreased effectiveness of intrinsic nerve stimulation and only slight supersensitivity to agonists. Fused contractions observed in isolated strips from unstable bladders reflect an increase in electrical coupling between muscle cells. This phenomenon is rarely seen in muscle from normal bladders. It allows the spread of electrical activity within the bladder wall and therefore increases intravesical pressure (Brading & Turner 1994).

These changes in detrusor muscle are associated with bladder outlet obstruction, it is uncertain whether they are also associated with idiopathic detrusor

instability. As the prevalence of this condition and other lower urinary tract disorders tends to increase with age the physiological changes that take place during the ageing process are considered.

1.12 The effects of ageing on the lower urinary tract.

The ageing process affects bladder function and is an important factor in the response of urinary tract smooth muscle to pharmacological agents (Nishimoto *et al.*, 1995). In the female, ageing is associated with decreased secretion of progesterone and oestradiol with subsequent effects on the female urinary tract. The mucosa of the vagina, urethra and vesical trigone are oestrogen sensitive and show parallel changes under different hormonal climates (Miodrag *et al.*, 1988). Atrophic vaginitis, consequent upon oestrogen deprivation, may be associated with atrophic urethritis, which in turn may cause frequency, dysuria, urgency and incontinence (Miodrag *et al.*, 1988).

Structural changes in the ageing detrusor have been demonstrated by electron microscopy with the dense band pattern (muscle cell membranes) representing structural changes in the normal ageing detrusor (Elbadawi *et al.*, 1993). This heralds a process of muscle cell dedifferentiation in the detrusor accompanying natural ageing, and may affect exchange and storage of ions involved in the excitation-contraction coupling mechanism of muscle cells. In addition widespread degeneration of muscle cells and axons was observed in the ageing detrusor with impaired detrusor contractility (Elbadawi *et al* ., 1993). A possible problem with the study of Elbadawi *et al* (1993) is they included patients who previously had either a hysterectomy (women) or resection of the prostate (men). Both procedures could possibly affect the structure and function of detrusor muscle by alterations in female hormones levels and obstruction due to prostatic hypertrophy, as discussed previously.

Gilpin *et al.*,(1986) examined the density of autonomic innervation in bladders from male and female patients aged 20 to 79 years. In the 60-72 age group a significant reduction in nerve counts was demonstrated compared to the 25-35 age group. A more recent study examined age-dependent alterations in β-adrenergic responsiveness in rat detrusor muscle (Nishimoto *et al.*, 1995). An age related decrease in the responsiveness to β-adrenergic stimulation, density of β-adrenergic receptors and cyclic AMP was demonstrated (Nishimoto *et al.*, 1995). Beta-adrenergic activation by noradrenaline relaxes detrusor smooth muscle and facilitates urine storage (DeGroat & Saum 1972).

In the male the ageing process is associated with prostatic enlargement resulting in bladder outlet obstruction. Benign prostatic hyperplasia (BPH) is associated with irritative bladder symptoms and changes in the pharmacophysiology of detrusor muscle, as described previously.

In conclusion, age-related alterations in detrusor muscle function, structure and innervation have been demonstrated; these affect the relaxant and contractile properties of detrusor smooth muscle.

Pharmacological Treatment of Detrusor Instability: Past, Present and Future

1.13 Past and present treatment of detrusor instability

The pharmacological treatment of detrusor instability has been aimed at reducing the contractility of the detrusor muscle. Because bladder contraction is initiated by the release of acetylcholine from parasympathetic nerves, it is hardly surprising that the majority of pharmacological treatment has centred on the use of various anti-cholinergic drugs.

1.13.1 Anticholinergic agents.

Anticholinergic drugs act by blocking muscarinic receptors competitively at the post-ganglionic parasympathetic receptor sites. Atropine is the classical anticholinergic agent but it is not used to treat detrusor instability because of its generalised antimuscarinic and antinicotinic side effects. As early as 1936 atropine was found to be of benefit in relieving urgency and frequency in patients with spastic paraplegia (Langworthy 1936). Bladder capacity was increased from 150 to 250 ml and urinary frequency was reduced. However atropine sulphate had to be gradually increased to the limits of tolerance which resulted in distressing side effects. This may be partially attributed to low bioavailability making it difficult to achieve sufficient drug concentration in the effector organ.

A commonly used anticholinergic drug is propantheline which can relieve symptoms if given in high doses (Kieswetter & Popper 1972, Beck *et al.*, 1966, Beck *et al.*, 1976, Ostergard 1979). *In vitro* studies have shown that propantheline bromide has a direct anti-muscarinic binding potential similar to atropine (Levin *et al.*, 1982). However anticholinergic side effects are encountered producing a dry mouth, blurred vision, drowsiness, constipation and tachycardia due to the non-bladder specific nature of antimuscarinic drugs. Such side effects make double-blind trials difficult, and

variations in the dosage and route of administration of drugs may affect the interpretation of results. Such problems can be encountered in studies using propantheline which has a low biological availability when given orally and which can vary markedly between individuals (Staskin *et al.*, 1990). The benefit of individual drug titration has been demonstrated in a study (Blaivas *et al.*, 1980) where the propantheline dose, usually 15 to 30 mg four times daily, was varied between 7.5 and 60 mg four times daily in order to obtain a complete response in 25 out of 26 patients. Unfortunately, there are no good clinical trials of this drug.

Emepronium bromide had been used for many years to treat detrusor instability but was withdrawn from the U.K. The recommended maximum dose was 200mg four times daily, but this was minimally effective because only 6% of the dose was absorbed through the gastrointestinal tract (Ritch *et al.*, 1977). In one study no difference between oral emepronium (200 mg three times daily) and placebo was noted, with the overall subjective improvement rate in the drug and placebo groups being 79% (Hansen *et al.*, 1982). These results may be explained by the low dosage of oral emepronium employed in this study combined with its poor absorption. When higher (more than the recommended dose) and more effective doses were administered orally (300-400 mg four times daily) there was a high incidence of oral and oesophageal ulceration. Because of these side effects parenteral preparations were investigated.

Parenteral administration of emepronium bromide abolished detrusor contractions and increased bladder capacity (Cardozo and Stanton 1979), but this preparation is not available for general use. Another preparation, emepronium carrageenate, was developed to overcome the problem of oesophageal ulceration caused by the bromide. The drug was clinically assessed with significant subjective

and objective improvement (Massey and Abrams 1984) but did not reach the U.K. market.

Terodiline is an anticholinergic drug with calcium channel blocking action. It has been used mainly for the treatment of urge incontinence. A study by Husted *et al.*, (1980) on the effect of terodiline on the contractile response of isolated rabbit detrusor muscle, demonstrated mainly anticholinergic effects at low concentrations. At higher concentrations it also had a calcium antagonistic effect which abolished the contractile response to electrical field stimulation. The anticholinergic properties seem to dominate at clinically tolerated doses (Staskin *et al.*, 1990).

Clinically terodiline has been shown to be effective, but it was withdrawn from use due to cardiotoxicity. The clinical efficacy of terodiline was established in a well constructed multi-centre study which used a randomised, double blind, two-period cross over protocol (Peters 1984). The results of this study showed that there was a patient preference for terodiline compared to placebo of 63%. However, 35% of the patients developed side effects on placebo. The frequency of voluntary micturition decreased from 9.6 to 8.9 per 24 hours on placebo, and from 9.9 to 7.3 on terodiline. Involuntary micturitions decreased from 2.3 to 1.7 on placebo and from 2.5 to 1.5 on terodiline. Volume at first desire to void increased on placebo from 159 to 162 and on terodiline from 151 to 198 ml. Bladder capacity increased from 312 to 328 on placebo and from 320 to 374 ml on terodiline.

Although all these differences were statistically significant the clinical significance was not marked. The improvements in voiding frequency and involuntary micturition were statistically significant but the clinical improvement of voiding every 2 hours and 41 minutes (placebo) or every 3 hours and 16 minutes (terodiline), or having 17 incontinent episodes (placebo) against 15 episodes (terodiline) in ten days,

may not actually cause a notable improvement in the patients' lifestyles. Furthermore a single centre study comparing terodiline with bladder retraining, against placebo with bladder retraining, concluded that the possible benefit of terodiline is likely to be small (Wiseman *et al.*, 1991).

Ultimately what matters is how patients perceive their condition, which is reflected in daily bladder diary charts. Urodynamic parameters provide suitable objective measures but correlate poorly with symptoms (Peters 1984).

Further clinical studies demonstrated that 10 out of 12 women improved symptomatically and urodynamically while taking terodiline (Ulmsten *et al.*, 1985). Tapp *et al* (1987) showed that in 70 patients who completed the study, there were significant improvements in frequency, incontinence episodes and volumes voided in the terodiline group compared to the placebo group. Sixty two percent of the treated group considered themselves to be improved while only 42% of the control group improved. A problem with these results, and in most studies, is that total volumes voided per day are rarely recorded. The total urine output would be useful in eliminating the effects of increased fluid intake and output which are often associated with improved continence.

Drugs with anticholinergic activity are, therefore, effective in alleviating the symptoms of detrusor instability. Unfortunately they are all associated with side effects which limit their usefulness in clinical practice.

1.13.2 Musculotropic relaxants

The musculotropic relaxants are direct acting smooth muscle relaxants with anticholinergic activity.

Oxybutinin is a tertiary amine anticholinergic drug. It has less generalised anticholinergic effects and more antispasmodic actions. It is an effective drug for treating detrusor instability (Cardozo *et al.,* 1987, Moisey *et al.,* 1980) although side effects can sometimes be less tolerable than the symptoms of detrusor instability; these can be minimised by starting at low doses (Castleden & Robinson, in press). The difficulty in documenting subjective improvements during the treatment period is illustrated in the study by Moisey *et al.,* (1980). Individual clinical responses to the medication did not correlate with the objective responses to bladder filling established by urodynamics. There are also problems with some of these studies in the low patient numbers employed, no control for non-pharmacological treatment, no placebo control and short treatment periods with little data on follow-up. It is possible that the cross-over design in some studies may be invalid because of the carry-over effect of oxybutinin seen with patients (Castleden & Robinson, in press).

Dicyclomine hydrochloride is usually used to treat gastrointestinal disorders but a study by Awad *et al.,* (1977) showed that the symptoms of 24 out of 27 patients with uninhibited bladder contractions improved when given dicyclomine. Beck *et al* (1976) also showed that this drug is effective for treating detrusor instability but it never gained popularity, maybe because the doses prescribed were inadequate (Wein 1984).

Flavoxate hydrochloride has been used for a number of years to treat detrusor instability but studies demonstrated that it was no more effective than placebo when administered orally or parenterally (Briggs *et al.,* 1980, Cardozo and Stanton 1979).

Oxybutinin would therefore appear to be the most clinically useful of these compounds, but the evidence in the literature for their clinical efficacy is scanty and generally of poor quality.

1.13.3 Tricyclic antidepressants

These drugs have a variety of pharmacological actions including anticholinergic, sympathomimetic and central sedative effects. Imipramine inhibits noradrenaline transport into adrenergic nerve terminals and antagonises muscarinic cholinergic response to neurotransmitters (Beck 1989). As well as an inhibitory action on detrusor muscle it also stimulates urethral smooth muscle contraction. Imipramine hydrochloride has long been prescribed for the treatment of nocturnal enuresis in children but it is also of value in adults. Castleden *et al.*, (1981) demonstrated, in an open study with no placebo controls, that 6 out of 10 elderly incontinent women became dry with imipramine although its effectiveness has been shown to improve when used in conjunction with the anticholinergic propantheline (Raezer *et al.*, 1977). A more recent placebo controlled trial demonstrated that imipramine had no benefit over habit retraining alone (Castleden *et al.*, 1986).

1.13.4 Evaluating experimental data

Problems are encountered when evaluating the effect of drugs on detrusor instability, either in clinical trials or on isolated bladder muscle samples from small mammals or humans.

A major problem in clinical trials is the selection of patients. Urge incontinence is a symptom and a clinical diagnosis which can have different pathological causes. This might explain why the same drug will not benefit all patients

(Staskin *et al.*, 1990). Even a urodynamic diagnosis of detrusor instability does not identify the pathological cause (Wiskind *et al.*, 1994) Compliance, absorption, metabolism and excretion of pharmacological agents also differ between patients which affect the availability and concentration of drugs at receptor sites in detrusor muscle. There is also a major problem in extrapolating experimental results on isolated animal detrusor muscle to humans. The neurotransmitters involved in detrusor muscle contraction differ between humans and smaller mammals and there could also be a difference in the excitation-contraction coupling mechanisms which could account for the discrepancy in experimental results between species (Staskin *et al.*, 1990)). Often drug concentrations used *in vitro* are not comparable to plasma levels which can be obtained in man.

There is clearly a need for improvement in available treatments for detrusor instability. This should include the development of new therapeutic agents which combine good efficacy with a low incidence of side effects. An alternative approach would be the use of pre-existing drugs which are, at present, not indicated for the treatment of detrusor instability.

The latter approach was adopted in this thesis by examining the effect of different types of drugs on detrusor muscle function, oestrogen, progestogens, calcium antagonists and caffeine. All these drugs are known to affect calcium movement within and into smooth muscle cells thereby affecting contractile response.

Female hormones were included because of their known effect on the lower urinary tract and because of the increased prevalence of detrusor instability in women after the menopause, indicating a possible role in the causation of this condition.

Calcium antagonists inhibit calcium influx and smooth muscle contraction. They therefore have a potentially useful role in the treatment of detrusor instability. Experiments have been included to examine the mechanisms of these drugs *in vivo.*

Caffeine is universally consumed in tea and coffee. It is known to have diuretic properties and possibly direct effects on smooth muscle contraction. Experiments to determine the effects of caffeine on detrusor muscle contraction were included to investigate whether caffeine consumption aggravates detrusor instability.

All these compounds, because of their mode of action, have the potential to influence detrusor muscle function. However, their use in the treatment of detrusor instability and other forms of urinary incontinence has not been established. The experiments in this thesis were designed to help clarify this situation.

1.14 Treatment of detrusor instability: the future

1.14.1 Oestrogen and progestogens

The physiology of the female urinary tract is influenced by sex hormones. In order for these hormones to act selectively on urogenital tissue they have to interact with a specific receptor. High affinity oestradiol receptors have been demonstrated in the rabbit urethra and bladder (Batra & Iosif 1983, Urner *et al.,* 1983) and the rat urethra (Lindskog *et al.,* 1980). Oestradiol receptors have also been demonstrated in the human female urethra and bladder, firstly by Iosif *et al* (1981) who showed that the concentration of the receptors in the detrusor and trigone were considerably lower than in the urethra. In two out of four patients undergoing urethrocystectomy, oestradiol receptors could not be detected in either the cytosolic or nuclear fraction of the bladder. Those that were, could only be found in the nuclear fraction which was

due to the transfer of all cytosolic oestradiol receptors to the nucleus as a result of an oestradiol injection given 24 hours before operation. This was administered because these patients had previously had hysterectomies. This study showed inconsistency in demonstrating oestradiol receptors, their sample size was small (n=4) and the tissue samples were not from normal bladders. Three patients had cancer of the bladder and the fourth a neurogenic bladder.

Ingelman-Sundberg *et al* (1981) confirmed the presence of oestrogen receptors in female urogenital tissue taken from women with stress incontinence. They found significant quantities of receptor in the bladder, pubococcygeus, urethra and vaginal epithelium. More recent studies using immunohistochemical techniques demonstrated nuclear oestrogen and progesterone receptors in the smooth muscle of the trigone and the posterior part of the bladder neck (Wolf *et al.*, 1991). Using the same techniques, oestrogen receptors were identified in the trigone but not in the bladder lateral wall whereas progesterone receptors were found in both sites (Pacchioni *et al.*, 1992). Although these results show variations in the location of oestrogen and progesterone receptors in the urinary bladder, the lower urinary tract is clearly a target for such hormonal action.

Animal experiments by the author in collaboration with others in the laboratory (Elliott *et al.*, 1992a, Elliott *et al.*, 1992b), have shown that the physiology and pharmacology of the bladder can be significantly altered by changes in the concentrations of sex hormones (Hodgson & Heesch 1978, Levin *et al.*, 1980, Shapiro 1986, Batra & Andersson 1989, , Ekström *et al.*, 1993). Levin *et al* (1980) showed that the administration of oestrogen to immature female rabbits resulted in both an increased bladder response to carbachol and an increased density of muscarinic receptors. This was in contrast to other studies which report that oestrogen treatment in female rabbits led to a significant decrease in muscarinic

receptor density (Shapiro *et al.,* 1986, Batra & Andersson 1989). This contradiction may be explained by the differences in the maturity of the rabbits and the duration of oestrogen treatment. Levin *et al* (1980) treated immature rabbits with oestrogen for only 4 days, whereas Shapiro (1986) treated mature rabbits continuously for 3 weeks with oestrogen. Batra and Andersson (1989) treated their mature ovariectomized rabbits for up to 8 weeks.

Previous work from this laboratory has shown that the direct application of diethylstilboestrol to the organ bath reduced the contractile response of rat detrusor muscle to stimulation with carbachol, acetylcholine , electrical field stimulation (EFS) and 5-hydroxytryptamine (Elliott *et al.,* 1992a). It was concluded that this inhibitory effect was due to the reduction of calcium influx as the contractile response of depolarised detrusor muscle to calcium was inhibited by bath applied diethylstilboestrol. Whereas when administered *in vivo,* oestrogen would not only have this effect but also act on the metabolic activity of the cell influencing the contractile machinery as a consequence of intracellular oestrogen receptor interactions (Batra 1980, Elliott *et al.,*1992b).

Levin *et al* (1991) investigated the effect of pregnancy on muscarinic receptor density and function in the rabbit urinary bladder. Pregnancy significantly reduced the contractile response of the bladder to bethanechol (chemically related to acetylcholine) and decreased the muscarinic receptor density by 50%. This study demonstrated the influence of physiological levels of sex hormones during pregnancy on the lower urinary tract as opposed to pharmacological levels achieved by hormonal administration.

Urinary incontinence in women has been shown by epidemiological surveys to be more common around the time of the menopause (Feneley *et al.,* 1979, Thomas *et*

al., 1980). Molander *et al* (1990) investigated the prevalence of urinary incontinence in a random sample of women from the 1900-1920 birth cohorts residing in the city of Göteborg, Sweden. The prevalence of urinary incontinence increased from 13.9% in the 1920 birth cohort to 24.6% in the 1900 birth cohort. The mean starting age for urinary incontinence (65+/-13.2 years) occurred 10-15 years after the menopause, indicating a possible connection with the hormonal changes that take place in the peri-menopausal and post-menopausal periods. Another study by Rekers *et al* (1992) found the prevalence of urinary incontinence in post-menopausal women to be 26.4% and their data showed clearly that the menopause had a causal or contributory role in incontinence. As the onset of urinary incontinence in women is associated with the menopause, treatment with sex hormones would be expected to have a beneficial effect on urinary symptoms.

Salmon *et al* (1941) treated 16 post-menopausal women with urinary frequency, urgency and incontinence with oestrogens. In all but 3 patients, relief of symptoms was achieved. After the withdrawal of treatment symptoms gradually recurred along with the re-appearance of signs of oestrogen deficiency. This early study was purely subjective, lacked controls and was performed before the advent of urodynamics. With the introduction of pressure transducers Raz *et al* (1973) reported an increase in maximum urethral pressures together with symptomatic improvement in 26 of 40 women with stress incontinence who underwent treatment with conjugated oral oestrogens. They also treated 10 incontinent patients with medroxyprogesterone acetate which resulted in a definite worsening of the condition in 6 of these patients, 2 of which progressed to severe incontinence. The controls employed in this study had normal urinary function, they were also treated with medroxyprogesterone only and demonstrated no alteration in bladder control. Thus progestogens have been shown in this study to worsen symptoms of urinary incontinence but they had no effect on

normal bladders. This suggests abnormal bladders are more susceptible to the effects of female hormones.

The urodynamic effects of hormones on the lower urinary tract of women with stress incontinence have also been examined by (Rud 1980, Hilton & Stanton 1983). Rud (1980) treated 24 stress incontinent women with a combination of high dose oestradiol and oestriol. He showed a significant increase in transmission of intra-abdominal pressure to the urethra as well as an increase in the maximum urethral pressure and urethral length at rest. Hilton and Stanton (1983) used intravaginal oestradiol cream to treat ten women with urodynamically proven genuine stress incontinence. They demonstrated a significant increase in the stress maximum urethral-closure pressure because of improved pressure transmission in the mid-urethra. Both studies showed significant improvement in the symptoms of stress incontinence, urgency and frequency, although neither were placebo-controlled. Rud (1980) also pointed out that the increased pressure transmission ratio might be due to factors outside the urethra such as the striated musculature of the pelvic floor or in the periurethral vasculature or supporting tissues.

Although the onset of urinary incontinence in women appears to be associated with the menopause it is uncertain whether oestrogen deficiency is a major factor in the pathogenesis of this condition. A study by Benness *et al* (1991a) comprised of a questionnaire administered by medical personnel to two groups of women. One group had received no oestrogen therapy since their menopause and another group had been on continuous hormone replacement therapy (HRT) for ten years or more. They found that oestrogen deficiency did not seem to be an important factor in the pathogenesis of symptoms of incontinence, except for possibly nocturia. It is interesting that they found stress incontinence and voiding difficulty symptoms more

common in those on HRT and therefore they questioned the role of progestogens in the causation of these symptoms.

To date there is considerable experimental support for a possible role of oestrogens in the treatment of urinary incontinence. The converse is true for progestogens, which have been shown to exacerbate symptoms of incontinence. A recent study by Ekström *et al* (1993) investigated the effect of long term treatment with oestrogen or progesterone on the contractile responses of rabbit urinary bladder and urethra. Oestrogen treatment shifted the frequency response curve of the bladder to the right (inhibition of contractile response) and progesterone increased the maximal nerve induced contraction. Progesterone also increased the maximal urethral tension in response to nerve stimulation. The authors concluded that their results provided no objections to the use of progesterone with oestrogen in the treatment of stress incontinence as the effects of progesterone were small and seemed to improve maintenance of urethral closure.

There appear to be conflicting opinions regarding the role of progestogens in the causation and treatment of urinary incontinence. The study by Ekström *et al* (1993) investigated the effect of either oestrogen or progesterone on the contractile response of rabbit detrusor muscle and lacked an oestrogen and progesterone treated group, which would resemble the treatment regime of women on hormone replacement therapy.

Because of these discrepancies and omissions the effect of progesterone and oestrogen treatment, *in vitro* and *in vivo*, on the contractile response of rat detrusor muscle to electrical field stimulation has been included in this thesis.

1.14.2 Calcium Antagonists

To date the treatment of detrusor instability with calcium antagonists has been limited to a drug which combines this action with anticholinergic properties. Terodiline (although now withdrawn) has been shown in animal experiments to be an effective inhibitor of bladder contractions (Husted *et al.,* 1980) and clinical trials have shown it to be particularly helpful for the symptoms of urgency and urge incontinence (Tapp *et al.,* 1989).

The rationale for using calcium antagonists in the treatment of detrusor instability is to limit the influx of calcium through potential-operated calcium channels. The entry of calcium is an important trigger for smooth muscle contraction. Since contraction of detrusor smooth muscle is a contributory factor to urinary incontinence in patients suffering from detrusor hyperactivity it is important to establish the effect of calcium antagonists on the contractile response of detrusor muscle (Castleden *et al.,* 1981).

Shapiro *et al* (1991) compared the binding and functional properties of calcium channel receptors in normal and myelodysplastic bladders. This condition ranges from an atonic poorly emptying bladder to a poorly compliant hyperreflexic bladder. Although they found no differences in calcium channel receptor densities between the two groups, the presence of these receptors in the bladder suggests they have a meaningful role in detrusor function. Bladder activity could, therefore, be modulated by calcium channel antagonists. Regional differences in the density of calcium channel receptors in the lower urinary tract of the rabbit have been identified with the number of receptors in the urethra being three times that in the bladder dome and base (Latifpour *et al.,* 1992).

In vitro studies of isolated human and animal detrusor muscle preparations have shown conclusively that calcium antagonists have a significant inhibitory effect on contractile response (Forman *et al.*, 1978, Hassouna *et al.*, 1986, Bo & Burnstock 1990, Fovaeus *et al.*, 1987, Zar *et al.*, 1990, Scultety 1991, Elliott *et al.*, 1992a). *In vivo* studies in animals were performed after intravenous administration of verapamil, nifedipine and nicardipine, and after a single oral dose of nicardipine and verapamil (Sjögren & Andersson 1979, Angelico *et al.*, 1992, Diederichs *et al.*, 1992). These drugs inhibited bladder contraction in a dose-dependent manner, although verapamil produced inhibition only after toxic oral doses. *In vivo* animal studies have, therefore, been performed after intravenous administration of a calcium antagonist or after a single oral dose. The normal drug regime for treating this condition in man is by daily oral dosage, and there is little information on the effect of these drugs on detrusor contractions using this dosage regime in animals or man.

Clinical investigations, examining the effect of single oral doses of nifedipine (10-40mg) on bladder contraction in women with urge incontinence, demonstrated a reduction in amplitude and frequency of uninhibited detrusor contractions and also a significant increase in residual urine (Forman *et al.*, 1978, Rud *et al.*, 1979). In contrast it has been found that similar oral doses of nifedipine had no significant effect on such bladder contractions in 30 patients (Laval & Lutzeyer 1980). The effect of chronic oral dosing with flunarizine for one week did not demonstrate significant urodynamic improvement in women with proven idiopathic detrusor instability, although their symptoms improved significantly (Palmer *et al.*, 1981). Further experience with calcium antagonists in the treatment of detrusor instability has been disappointing (Levin *et al.*, 1994). However, calcium antagonists have not undergone sufficiently rigorous scientific investigation before being introduced and rejected in clinical practice for the treatment of detrusor instability. Such drugs are known to vary in action between tissues (Fleckenstein *et al.*, 1981) and between compounds of

the same class (Bolton 1979). Furthermore, chronic dosing could produce tachyphylaxis which has been reported with verapamil (Aderka *et al.*, 1986). This would result in a diminished response of the tissue to repetitive exposure to the same concentration of the drug and could explain the disappointing clinical results.

Scientific evidence regarding the effect of chronic oral dosing with calcium antagonists on the contractile response of detrusor muscle is lacking. Because of this, experiments were designed to confirm the sensitivity of human detrusor muscle to nifedipine and verapamil *in vitro* and to establish human detrusor muscle sensitivity to nimodipine. Rat detrusor muscle sensitivity to nimodipine treatment *in vitro* was also established and the contractile response of rat detrusor muscle after chronic oral treatment with nimodipine and after a single oral dose was compared.

1.14.3 Caffeine.

Caffeine is a xanthine derivative which occurs naturally in tea and coffee. It causes a mild diuresis by acting on the renal tubules to increase renal blood flow and decrease sodium and water reabsorption from the distal tubule in a manner similar to that of thiazide diuretics (Maren 1961).

Caffeine also affects the contraction of skeletal and smooth muscles. Observations on caffeine-induced contractions in frog skeletal muscle date back many years (Ransom 1911). These contractions were observed during experiments on the formation of acid in muscles. At low concentrations caffeine also increases contractions induced by direct stimulation of the muscle (Sandow & Brust 1966).

The pharmacological effects of caffeine differ depending on the concentrations used; it has both stimulant and relaxant effects on different smooth muscles and also

on the same smooth muscle under different conditions (Bolton 1979). Caffeine also releases intracellular calcium to induce a transient contraction (Leijten & van Breemen 1984, Karaki *et al.*, 1987). Apart from the contractile effect, caffeine has a potent inhibitory effect on various smooth muscles. This is thought to be associated with a rise in cAMP due to phosphodiesterase inhibition (Bolton 1979) and alterations in membrane permeability resulting in changes in calcium influx (Ito & Kuriyama 1971, Sunano & Miyazaki 1973, Ito *et al.*, 1973).

It is, therefore, possible that caffeine could have a direct effect on detrusor muscle contraction, thereby improving or worsening symptoms of urinary incontinence. Recently, Creighton & Stanton (1990) examined the effect of caffeine on urodynamic studies in asymptomatic women and those with confirmed detrusor instability. The group with detrusor instability showed a statistically significant increase in detrusor pressure on bladder filling following the administration of caffeine. The asymptomatic group showed no abnormality on cystometry. These results indicate that increased urgency and frequency reported after drinking caffeine-containing compounds may not be solely due to the diuretic effect of this agent.

Studies on the effect of caffeine on isolated detrusor muscle preparations are scant. Huddart *et al* (1983) included bladder muscle strips in a study on the effect of methylxanthines on contractile responses and calcium mobilisation in smooth muscles. Caffeine (0.1-5.0 mM) inhibited the K^+-induced tonic contractions of bladder muscle strips without significant effect on phasic responses to K^+. It is of interest that the concentration of caffeine used in this and most other studies is higher than plasma concentrations achieved after the ingestion of 200 mg caffeine (Creighton & Stanton 1990).

A plasma concentration of 50μM is obtained after the ingestion of 250 mg of caffeine, which is equivalent to three cups of coffee (Goodman & Gillman 1985). However, one study did look at the effect of low concentrations of caffeine on the response of *in vitro* whole bladder preparation to field stimulation (Lee *et al.,* 1993). The concentration of caffeine required to increase the rate of pressure generation was inversely proportional to the extracellular calcium concentration. It was concluded that caffeine increased the rate of release of intracellular calcium.

It would be of considerable importance to establish the effect of low concentrations of caffeine (50μM) on the contractile response of isolated detrusor muscle with a view to advising patients with urinary incontinence on caffeine consumption. For this reason an investigation into the effects of low concentrations of caffeine on isolated human and rat detrusor muscle contractile responses was included in this thesis.

1.14.4. Calcium movement: A common theme

The pathophysiological and pharmacological approach to the investigation of detrusor muscle contractile responses in this thesis focuses mainly on the process of intra-cellular and extra-cellular calcium movement.

Oestrogens have been shown previously in this laboratory and by other workers to affect smooth muscle contraction by a reduction in calcium influx when present in the tissue environment or indirectly via muscarinic receptors *in vivo.* Progesterone, *in vitro,* has also been shown to inhibit calcium uptake in uterine smooth muscle but its direct effect on isolated detrusor muscle is not known. The effect of progesterone treatment with oestrogen, *in vivo,* and progesterone alone, *in vitro,* was therefore investigated in this thesis.

Calcium antagonists inhibit calcium entry through voltage-sensitive calcium channels. Their effect on detrusor muscle contraction *in vitro* has concentrated on the use of single doses of drugs. The effect of chronic dosing with calcium antagonists on the contractile response of isolated detrusor muscle in not known. It is important to establish the effect of chronic dosing as this is the normal drug regimen used in humans. Experiments were carried out to establish this effect and to fill an opening in research concerning this method of treatment.

Caffeine has different modes of action depending on the concentrations used. It is known to have an effect on intracellular and extracellular calcium movement and to inhibit cyclic nucleotide phosphodiesterases (Bolton 1979). The direct effect of caffeine on isolated human detrusor muscle, at concentrations likely to be ingested when drinking tea or coffee, has not been investigated previously. It would be important to establish the effect of caffeine on isolated detrusor muscle contraction to advise patients with detrusor instability on caffeine consumption.

This research will increase knowledge of the effects of pharmacological agents on detrusor muscle contractile responses. This will hopefully improve our understanding of the mechanisms involved, and lead to better treatment of urinary incontinence, particularly detrusor instability.

1. 15 Aims and Objectives

The overall aims and objectives of this research were;

(a) To determine the effect of progesterone on isolated detrusor muscle contraction and to investigate the interaction or possible anti-oestrogenic action between progesterone and oestrogen *in vitro.*

(b) To investigate the effect of progesterone and oestrogen treatment for 8 days *in vivo* on isolated rat detrusor muscle contractile response. Oestrogen has been shown previously to have a significant inhibitory effect on rat detrusor muscle contraction and this experiment was designed to determine the effect of the addition of progesterone to treatment regimen. Progesterone is known to have antioestrogenic activity and may therefore affect oestrogen's inhibitory action on rat detrusor muscle contractile response.

(c) To establish the effect of nimodipine on human isolated detrusor muscle contractile response and to investigate the effect of *in vivo* treatment on isolated rat detrusor muscle contractile response. These experiment were designed to determine why some calcium antagonists appear to be ineffective when given to treat detrusor instability.

(d) Caffeine is known to worsen the symptoms of detrusor instability but not that of stable bladders. It is possible caffeine may have a direct effect on detrusor smooth muscle contraction. To investigate this possibility experiments were carried out to determine the effect of caffeine on isolated rat and human detrusor muscle contractile response.

CHAPTER 2: GENERAL METHODS

Experimental procedure and theory

The evaluation of the effect of drugs *in vivo* is complicated by many factors. These include individual variations in drug absorption, distribution, metabolism and alteration of measured target organ action by excretion, homeostatic and compensatory mechanisms of different systems. A way to avoid such complications is to investigate the action of drugs on a simplified system such as a living strip of bladder muscle tissue isolated from the body. The procedure for this is an organ bath which was first introduced by Magnus (Fig 3).

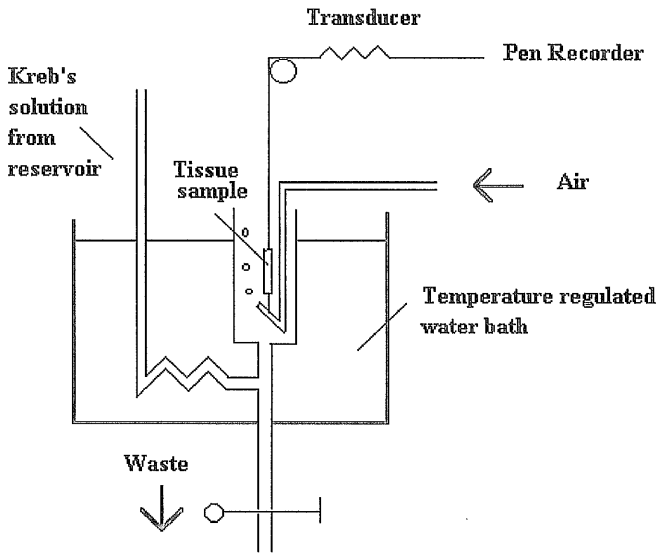


Figure 3. The organ bath

2.1 The organ bath

Strips of bladder muscle were prepared and placed in physiological salt solution (Kreb's solution) having an ionic composition similar to that of blood plasma. The Kreb's solution was contained in a 50ml chamber situated in a temperature regulated water bath maintained at 36-37°C. Oxygen was bubbled through the

solution to keep the tissue sample alive. The muscle sample was set up so that one end was anchored to a fixed hook in the chamber and the other connected to a transducer by silk sutures. The transducer, which was connected to a Washington Oscillograph, converted isometric tension into an electrical signal. When the muscle sample contracted it shortened in length and this was represented as a deflection of the pen recorder. The recorder was calibrated by suspending a known weight from the transducer and recording the size of the pen deflection, from this a relationship between tension and distance of deflection was obtained.

In vivo detrusor muscle contracts in response to activity in neurones which releases neurotransmitters such as acetylcholine (ACh). *In vitro* contraction of isolated detrusor muscle was obtained by the addition of a solution of ACh to the bath chamber. The muscle tissue began to contract within a few seconds and removing the ACh containing Kreb's solution, and replacing it with fresh solution, relaxed the muscle back to its original length. A further application of ACh was applied within a few minutes. This response was also achieved by the exogenous application of other neurotransmitters such as ATP. A solution of ATP added to the organ bath chamber produced a contraction in the muscle strip with an amplitude less than that obtained after the addition of ACh.

The contractile response to nerve stimulation was achieved by passing the muscle tissue through parallel electrodes which were connected to a stimulator capable of delivering electrical impulses at different frequencies, voltage and pulse width. Electrical field stimulation (EFS) not only produces contraction due to the release of endogenous neurotransmitters from nerve terminals, but also due to direct muscle stimulation. This is related to the setting of the pulse width, the higher the setting resulting in more direct muscle stimulation.

2.2 Dose response curves

When the concentration of ACh added to the organ bath chamber was reduced it eventually reached a concentration which did not produce a contraction. When the concentration was increased from this level the degree of contraction increased with dose until a maximum was reached when a further increase in ACh concentration did not produce a greater effect (Burgen & Mitchell 1985). Acetylcholine produced its effect by combining with its receptor to initiate a pathway of events within the cell which resulted in contraction of the muscle sample. The curve relating dose of ACh to contractile response is a dose-response curve (Fig 4).

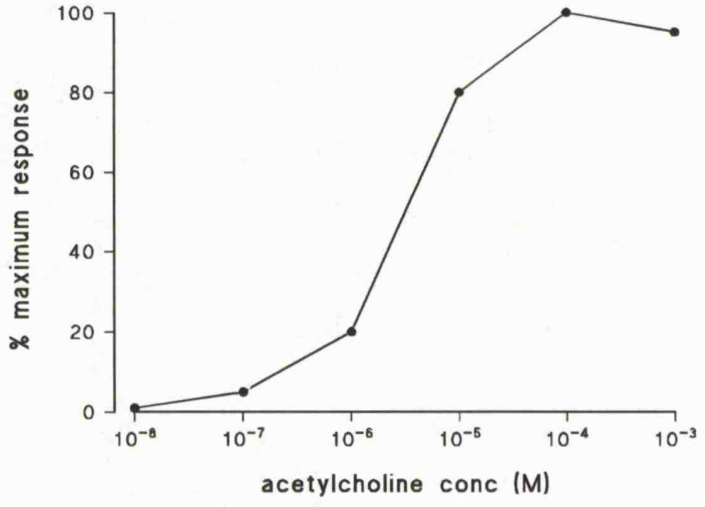


Figure 4. Acetylcholine dose response curve.

Dose response curves were constructed by plotting the response, y axis, against the dose, x axis. The dose was plotted using a logarithmic scale which produced a sigmoidal-shaped curve that accommodated all concentrations of agonists.

Chemically related agonists such as propionylcholine produce curves identical in shape to ACh but which are displaced to the right because of its lower potency (Fig 5) (Burgen & Mitchell 1985). From the curve it can be seen that the lower potency

applies to all doses of propionylcholine, which is a fixed ratio corresponding to 1.3 log units shift along the abscissa. The curves are referred to as parallel and the shift is described as a parallel shift to the right. It is also clear from the curves that butyrylcholine was not only less potent than propionylcholine but it did not reach the same maximum response.

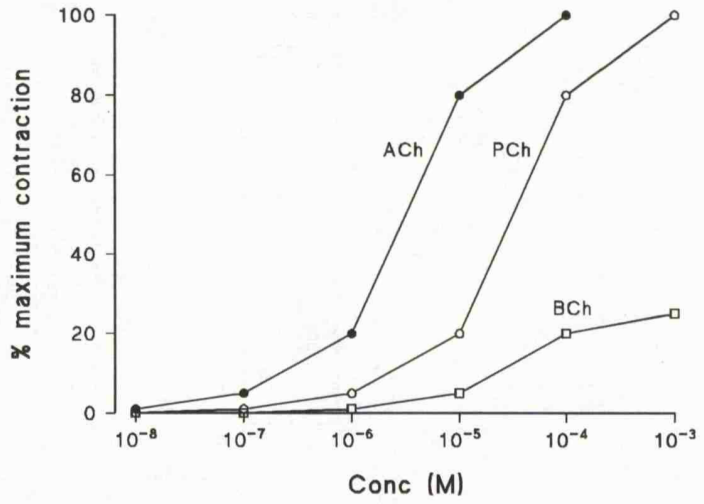


Figure 5. Dose response curves to acetylcholine (ACh), propionylcholine (PCh) and butyrylcholine (Bch). (Taken from Burgen & Mitchell 1985)

The shift in dose response curves was estimated by calculating the concentration of agonist required to produce 50% of maximum response. This is ED_{50} (effective dose at 50%). An increase in ED_{50} resulted in a shift to the right of the dose response curve. Log dose response curves were used for plotting the results of pharmacological measurements for a number of reasons:

(a) Substances acting on the same biological system produce curves of the same form. On a log scale this is easier to distinguish and the parallel position of the curves can be observed.

(b) Ratios of potencies are easily estimated.

(c) Because a logarithmic scale gives equal weighting to all doses it allows a wide range of concentrations to be plotted on the one graph without compressing any part of the curve.

(d) The middle part of the response range is linear and if responses between 20 and 80% of the maximum are obtained a straight line may be drawn through these points. This can be useful when the curve is defined from only two or three observations (Burgen & Mitchell 1985).

The response curve to EFS was plotted using a linear scale and is known as the frequency response curve (Fig 6). The response curve was obtained by stimulating the muscle strip with increasing frequencies, set on a Digitimer stimulator, using 1, 5, 10, 20, 40, 60 and 80 Hz. The maximum response was obtained between frequencies 20 Hz and 60 Hz.

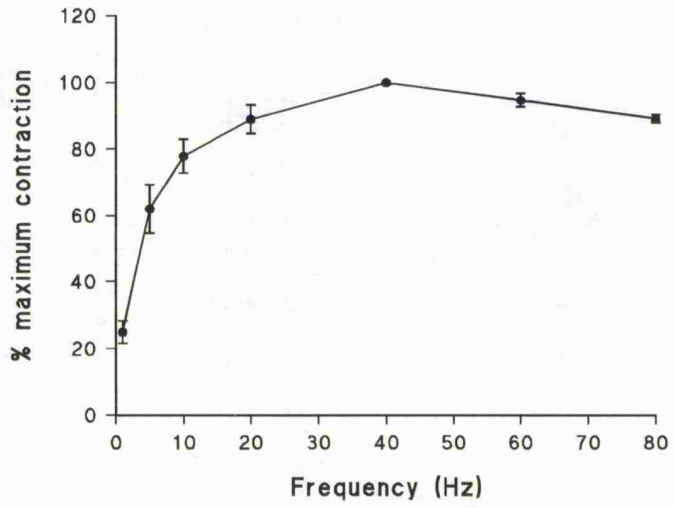


Figure 6. Frequency response curve (n=5) Vertical bars represent standard error of the mean (SEM)

2.2.1 Antagonists

Antagonists do not produce a direct observable response but modify the response to agonists. The 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 ACh in the presence of atropine no contractile response is obtained. To obtain a response much higher concentrations of acetylcholine are required. Where the antagonism can be overcome by increasing the concentration of agonist, the antagonism is known as competitive, if the antagonism cannot be overcome it is known as non-competitive. When the Kreb's solution is replaced and the atropine removed, the normal sensitivity of the muscle to acetylcholine is restored. Therefore the antagonism is reversible. If the log dose response curve is plotted, atropine is shown to cause a parallel shift to the right with the shift being greater depending on the concentration of atropine used. The degree of antagonism and the basis for estimating antagonist affinities by bioassay was first developed by Schild and workers (Schild 1949, Arunlakshana & Schild 1959). The resulting equation is known as the Schild equation: $[H]/[h] = 1 + [A]K_a$

where [H] = concentration of agonist required to yield the same response in the presence of the competitive antagonist.

[h] = concentration of agonist required to yield the same response in the absence of the competitive antagonist.

[A] = free concentration of antagonist.

K_a = equilibrium association constant for the antagonist.

2.3. Receptor theory

Before 1965 information about receptors was deduced from the analysis of dose-response data. Erlich, a physician researching in chemotherapy, stated that for

an agent to act it must be bound and the first receptor models focused on the binding function of the receptor (Yamamura *et al.,* 1985). The receptors for neurotransmitters are membrane localised proteins which recognise a ligand with sensitivity and chemical selectivity. The process of recognition is then converted into a signal that results in cellular activation. In order to account for the dual recognition-activation function of receptors a number of models have been developed that relate receptor occupation to the generation of a cellular signal.

The interaction between a neurotransmitter and its receptor was assumed to be a reversible bimolecular reaction (Clarke 1937). Clarke was the first proponent of the occupancy model of receptor function. The "occupancy" theory states that the magnitude of the biological response is proportional to the amount of ligand-receptor complex formed. A problem with this theory is it is out of keeping with a number of experimental models. It requires a response to be generated at all levels of receptor occupation up to a maximum when all receptors are occupied.

It is known that in many tissues a response can not be detected until an appreciable number of receptors are occupied, "threshold phenomenon" and in others a maximum response is obtained only when a fraction of the available receptors are occupied (Yamamura *et al.,* 1985). If a system has " spare receptors" the ED_{50} is reached at a point where fewer than 50% of the receptors are occupied. In a system exhibiting threshold phenomenon the ED_{50} is reached when more than half of the receptors have been occupied. However, relative ED_{50} values for agonists producing parallel dose-response curves with the same maximum response can be taken as representative of the relative affinity of agonist for receptor (Yamamura *et al.,* 1985).

On the contrary antagonist affinities can be estimated by bioassay. The derivation, by Schild and co workers (1949, 1959), of the relationship for antagonists assumes a simple competitive bimolecular interaction between either agonist or

antagonist and receptor. The principle of the null hypothesis is that when the response to one concentration of agonist in the absence of inhibitor is the same as the response to a higher concentration of agonist in the presence of competitive inhibitor, it is assumed that the amount of agonist reaching the receptor in the two situations is identical.

The occupancy model of drug action does not account for the desensitisation phenomenon observed for many neurotransmitters. Desensitisation can be receptor-specific in that the response of muscle tissue to one agonist is lost whereas the same response to a second series of agonists is retained. The receptor specific phenomenon is termed tachyphylaxis. Paton (1961) suggested that the effect a drug produces depends not on the number of receptors occupied but on the rate of receptor occupation by the drug. Instead of attributing excitation to the occupation of receptor by drug molecules, Paton attributed excitation to the process of receptor occupation, each association providing one quantum of excitation (Yamamura *et al.*, 1985). The rate theory predicts that the maximum response is directly proportional to the dissociation rate constant. Slowly dissociating compounds give small or negligible maximum responses. The rate theory accounts for the stimulator action of agonists and for drug specific fading of an initial response.

The subsequent development of molecular models of receptor function have been described to account for many other aspects of neurotransmitter and hormone action.

General Methods

2.4 Solutions and Chemicals

The physiological Kreb's solution used for all experiments had the following composition: NaCl 119mM, KCl 4.4mM, $NaHCO_3$ 20mM, NaH_2PO_4 1.2mM, $MgCl_2$ 1.2mM, $CaCl_2$ 2.5mM, glucose 11mM made up in distilled water. Where a calcium free, high K Kreb's solution was used the composition was: KCl 127mM, $NaHCO_3$ 20mM, NaH_2PO_4 1.2mM, $MgCl_2$ 1.2mM, EGTA 0.01mM, glucose 11mM, made up in distilled water. Acetylcholine, carbachol, atropine, ATP and tetrodotoxin (TTX) where all supplied by Sigma Chemical Company and made up on the day of the experiment in distilled water. KCl (Fisons) was made up in Kreb's solution, also on the day of the experiment. TTX was stored in aliquots of 1ml at -20°C. All glassware and tubing was kept clean by washing with dilute HCl and distilled water.

2.4.1 Samples

Rats were killed by a blow to the head followed by dislocation of the neck. Bladders were removed and placed into Kreb's solution. Unless otherwise stated the number of samples used for each experiment was five. Human samples were obtained from the bladder dome by cold cup biopsy forceps from male and female patients undergoing routine cystoscopic procedures. All patients had given informed consent and the majority of patients were over 60 years of age. These procedures included Trans Urethral Resection of the Prostate (T.U.R.P) and cystectomy for bladder carcinoma. Local Ethical Committee approval had been granted for the use of biopsies for research purposes. Detrusor muscle samples were prepared for the organ bath by the removal of fat and serosa. Strips of muscle were dissected and mounted in the organ bath chamber. The samples were allowed to equilibrate for up to an hour under a tension of 10 mN before stimulation. This tension was determined previously by length tension experiments. Higher tensions did not allow the strip to contract

maximally and lower tensions did not demonstrate contraction at the lower end of the dose response curve.

2.4.2 Response curves

Dose response curves to acetylcholine (10^{-8}M - $2x10^{-4}$M), carbachol (10^{-8}M - 10^{-4}M) and ATP (10^{-6}M - $2x10^{-3}$M) were obtained by 10 second applications of agonist to the organ bath. The muscle samples were then washed with Kreb's solution and re-stimulated after 3 minutes. Dose response curves to KCl were obtained by the cumulative addition of KCl (10mM-100mM). The concentration of drugs and hormones referred to are all final bath concentrations.

Frequency response curves were obtained by suspending the detrusor muscle samples between parallel circular electrodes. The electrodes were connected to a Digitimer Stimulator delivering single square wave pulses at varying pulse width and voltage. Frequency response curves were obtained by stimulating the bladder strips with 1, 5, 10, 20, 40, 60, and 80 Hz in 10 second trains at 2 minute intervals.

The response of detrusor muscle to stimulation does not alter significantly over the time period of the experiment. The incubation times used for drugs were determined from preliminary experiments (not shown) to produce optimum effect.

2.4.3 Statistical analysis

Statistical analysis was carried out using one way analysis of variance and students t test. A Dunnett's or Bonferroni correction was applied for multiple comparisons. A p value of <0.05 was considered significant. When determined, the EF_{50} (ED_{50} for frequency response curves) estimated from the median effect plot computed using a " Dose Effect Analysis Program" (Biosoft).

CHAPTER 3: OESTROGENS AND PROGESTOGENS

Methods

3.1. Aims

Previous work from this laboratory has shown that *in vivo* oestrogen pre-treatement of rats has a significant inhibitory action on the contractile response of isolated detrusor muscle (Elliott et al 1992b). The *in vitro* administration of diethylstilboestrol (DES) has also been shown to have a significant inhibitory effect on detrusor contractile response (Elliott et al 1992a). The summation of treatments enhances this inhibitory action. Progestogens are known to antagonise the action of oestrogens, therefore, the purpose of this study was to determine what effect the addition of progesterone to the treatment regimen has on rat detrusor contractile response. Work described in this section also examines the direct effect of progesterone on contractile response.

3.2 In vitro treatment

When in the dioestrus phase, virgin female Wistar rats (300-350g) were culled and bladders removed and placed in Kreb's solution. Strips of detrusor muscle were prepared and mounted in the organ bath, as described. Frequency response curves were obtained with the stimulator set at 5 volts and pulse width 1.0 msec. The curves were presented as a percentage of maximum response.

The direct effect of diethylstilboestrol (DES) and progesterone.

Control frequency response curves were obtained and repeated after the addition of either 2μM progesterone, 2μM DES, and 2μM progesterone plus 2μM DES. This experiment was carried out to determine the direct effect of either hormone on the contractile response of isolated rat detrusor muscle and to establish if there was an interaction between progesterone and DES *in vitro*.

Effect of different concentrations of DES or progesterone.

The effects of different concentrations of DES, 0.02μM, 0.2μM, 20μM, or progesterone 0.2μM and 20μM on the contractile response were examined to establish if these hormones affect rat detrusor contraction in a dose dependent manner and at which concentration they become effective. Frequency response curves were obtained in the absence (control) and presence of each concentration of DES or progesterone.

The direct effect of DES and progesterone in the presence of atropine.

The direct effect of progesterone and progesterone plus DES on the contractile response to electrical field stimulation (EFS) in the presence of atropine (10^{-5}M) was examined. Atropine blocks the cholinergic component of the response to EFS, the atropine resistant response is due to the release of adenosine triphosphate (ATP) from nerve terminals. This experiment was to determine the effect of these hormones on the atropine resistant response to EFS and to establish the effect of progesterone with and without DES. Control frequency response curves to EFS were obtained in the absence and presence of atropine (10^{-5}M). Response curves were repeated in the presence of atropine and progesterone (2μM) and atropine plus DES (2μM) and progesterone (2μM).

Effect of progesterone on KCl response curve.

The contractile response to potassium chloride (KCl) is solely dependent on the influx of extra-cellular calcium through voltage dependent calcium channels. The effect of progesterone (2μM) on this response was to establish whether progesterone had an effect on calcium influx. A dose response curve to KCl was constructed by the

cumulative addition of KCl (10-80 mM). This was repeated in the presence of progesterone (2μM).

3.3 In vivo treatment

Virgin female Wistar rats (300-350g) were injected subcutaneously with oestradiol benzoate (150 μg/Kg/d) for 3 days followed by progesterone (160 μg/Kg/d) for 1 day. This cycle was repeated once. Pre-treatment with oestradiol alone for 8 days was also employed. Treatment was commenced when rats were in the dioestrus phase, as judged by vaginal smears. At the end of the treatment period rats were culled and bladders removed and placed in Kreb's solution.

Effect of oestradiol and progesterone pre-treatment.

Frequency response curves were obtained after the treatment period and compared with untreated rat detrusor responses (controls). This was to establish the metabolic effect of progesterone and oestrogen on rat detrusor contractile response compared to controls (no treatment).

Frequency response curves were also obtained after progesterone 2μM alone or with DES 2μM was added directly to the organ bath containing detrusor strips from rats pre-treated with oestradiol and progesterone. This was to determine any difference between the intracellular effects of these hormones and the effect produced in combination with extracellular oestrogen and progesterone. Response curves were presented as an increase in tension (mN).

Effect on atropine sensitivity.

Atropine blocks the cholinergic response of rat detrusor muscle to EFS. The effect of progesterone and oestradiol pre-treatment on atropine sensitivity was

examined. Control frequency response curves were obtained in the absence and presence of atropine (10^{-5}M) in detrusor muscle from untreated rats and in detrusor muscle from rats pre-treated with progesterone and oestradiol. The percentage inhibition of contractile response by atropine in controls and those treated with hormones was compared. Contractile responses were presented as a percentage of maximum response.

Effect on tetrodotoxin resistance.

Tetrodotoxin blocks the conduction of action potentials without affecting membrane potentials, thereby preventing the release of neurotransmitters. It is used as an experimental tool to establish the neurogenic origin of EFS. The TTX-resistant component of EFS is due to direct muscle stimulation. The effect of 8 days treatment with oestradiol or oestradiol and progesterone on the TTX-resistant component of EFS on rat detrusor muscle was examined.

Control frequency response curves were obtained then repeated in the presence of 1.6 x 10^{-6}M TTX. Further frequency response curves were obtained in detrusor muscle from rats pre-treated with oestradiol or oestradiol and progesterone with TTX in the bath chamber. These responses were compared to control responses plus TTX to see if hormone treatment altered the TTX-resistant response.

Responses were recorded as an increase in tension (mN). The concentration of hormones and TTX described are bath concentrations, and these were left in the organ bath for at least 15 minutes before stimulation.

Solutions and chemicals

Oestradiol benzoate and progesterone were supplied by Paines and Byrne in 1ml ampoules for injection. DES (Sigma) and progesterone (Sigma) were dissolved in ethanol and made up on the day of the experiment. The concentration of ethanol in the organ bath did not exceed 20mM.

Results

The responses of isolated detrusor muscle to EFS did not significantly alter over the time period of the experiment having less than 10% variation. Ethanol alone had a slight, but not significant, potentiating effect on contractile response. The final concentration of ethanol in the bathing solution did not exceed 20 mM.

3.4 In vitro treatment

The direct effect of DES and progesterone.

The mean frequency-response curves obtained from 6 different bladder muscle preparations are shown in Fig 7. The EF_{50} (6.2±1.1 Hz) obtained after the addition of 2μM progesterone was significantly different from the control EF_{50} (2.4±0.43 Hz) ($p<0.01$). The maximum contractile response to EFS was reduced significantly by 12% after the addition of progestergone 2μM to the bath ($p<0.01$), and by a further 30% after the addition of DES 2μM ($p<0.01$). In 2 out of 6 samples the addition of DES resulted in the maximum contractile response to EFS being reduced by 50% compared to controls. There was also a significant difference between the maximum contractile response obtained after the addition of progesterone and progesterone plus DES ($p<0.01$).

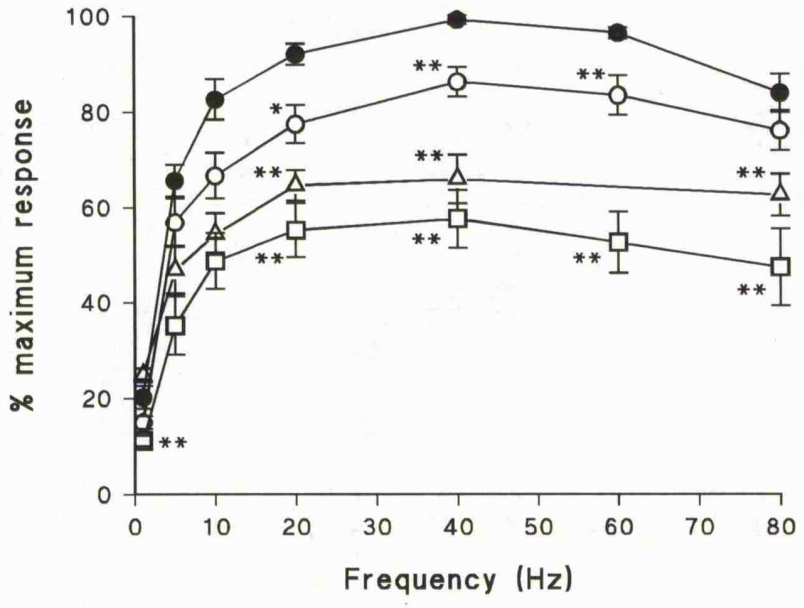
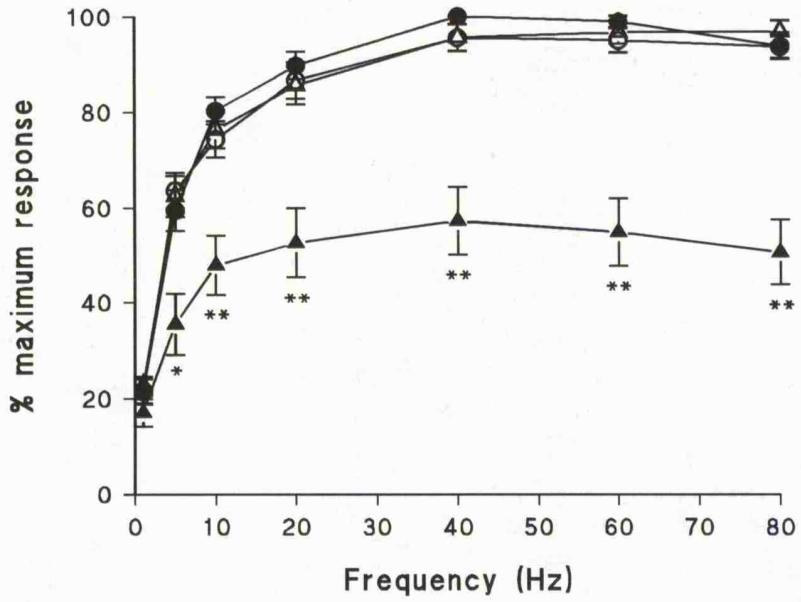


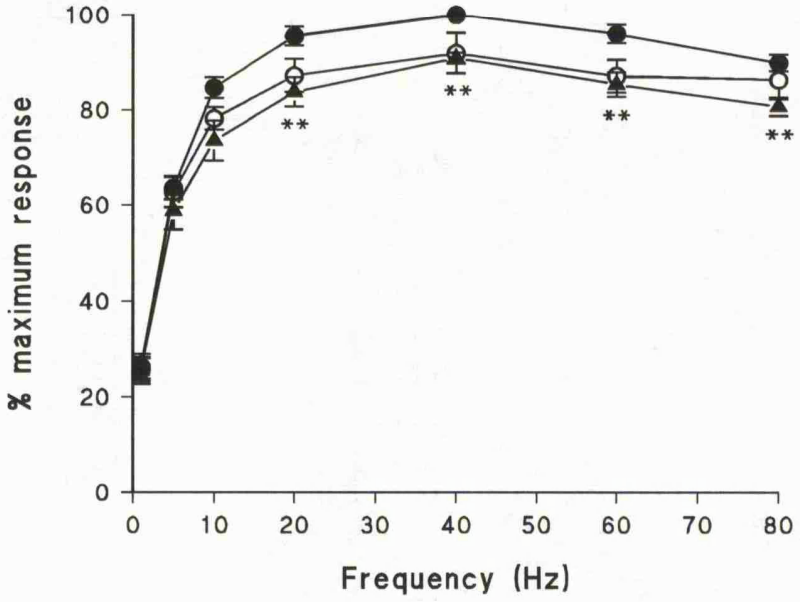
Figure 7. The effect of DES and progesterone in vitro on frequency response curves *● Control, ○ after the addition of 2μM progesterone, Δ after the addition of DES 2μM, □ after the addition of 2μM progesterone and 2μM DES. Vertical bars represent SEM (n=6) *p<0.05, **p<0.01 compared to control*

Effect of different concentrations of DES or progesterone.

Very low concentrations of DES, 0.02μM and 0.2μM, did not have a significant effect on detrusor contractile response to EFS (Fig 8a). However high concentrations of DES, 20μM, had a significant inhibitory effect on contractile response, reducing the response by 43%, ($p<0.01$). Low concentrations of progesterone, 0.2μM, did not have an effect on detrusor contractile response to EFS (Fig 8b). Progesterone, 20μM, did however significantly reduce the maximum contractile response to EFS by 8% ($p<0.01$). This inhibition was similar in magnitude to the reduction of contractile response produced by 2μM progesterone, suggesting the direct inhibitory action of progesterone may be maximal at this dose.



8(a)



8(b)

Figure 8. (a) The effect of DES, in vitro, on the frequency response curve to EFS in isolated rat detrusor muscle. *● control; ○ after the addition of 0.02μM DES; Δ after the addition of 0.2μM, ▲ after the addition of 20μM DES.* ***(b) The effect of progesterone, in vitro, on the frequency response curves to EFS in isolated rat detrusor muscle.*** *● control; ○ after the addition 0.2μM progesterone; Δ after the addition of 20μM progesterone. Vertical bars represent the SEM (n=5) * $p<0.05$ ** $p<0.01$ compared to control.*

The direct effect of DES and progesterone in the presence of atropine.

The addition of atropine, 10μM, to the bath chamber significantly reduced the maximum contractile response to EFS by 37% compared to controls ($p<0.01$) (Fig 9). Atropine, therefore, blocked the cholinergic response to EFS. The atropine resistant response was not affected by the further addition of progesterone, 2μM, to the bath. However the addition of DES, 2μM, significantly reduced the atropine resistant response by a further 16% ($p<0.05$).

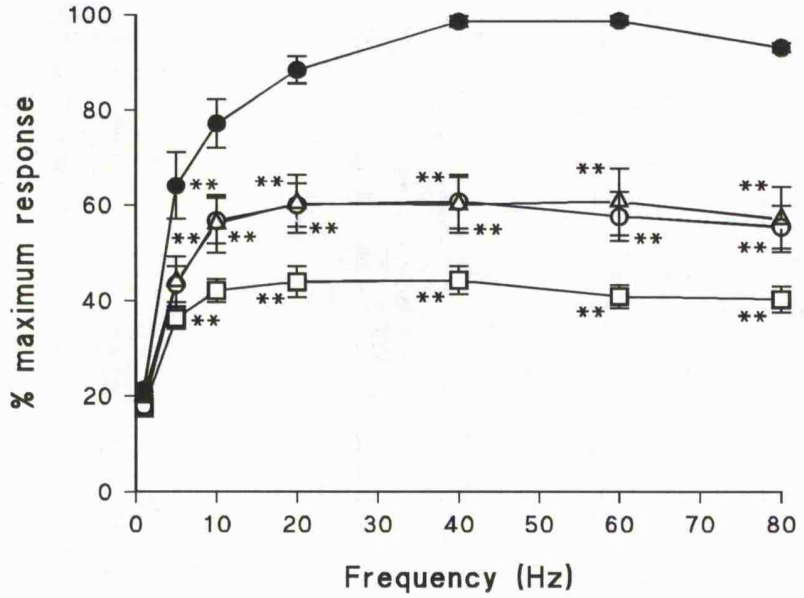


Figure 9. The effect of DES, progesterone and atropine, in vitro, on EFS in rat detrusor muscle. *● control, ○ after the addition of 10μM, atropine, Δ after the addition of 10μM atropine and 2μM progesterone, □ after the addition of 10μM atropine and 2μM DES. Vertical bars represent SEM (n=5) **p<0.01 compared to control.*

Effect of progesterone on K^+ response curve.

The cumulative addition of KCl, produced a dose-dependent contractile response which reached a maximum at 70mM (Fig 10). The addition of progesterone, 2μM, to the bath reduced rat detrusor contractile response to KCl at all

concentrations except 10mM, but the inhibition was only significant at 40 ($p<0.01$), 60 ($p<0.05$) and 70 mM KCl ($p<0.01$)

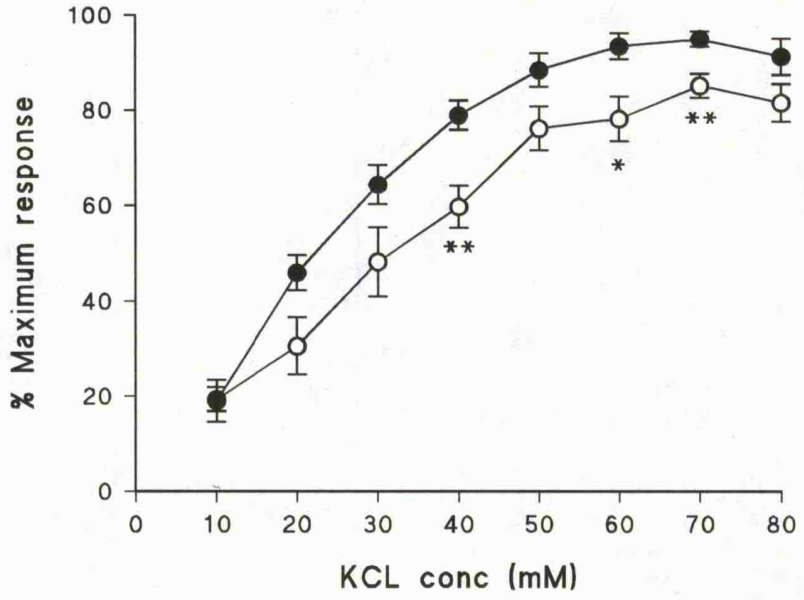


Figure 10. The effect of progesterone, in vitro, on the contractile response to KCl. *● control, ○ after the addition of 2μM progesterone. Vertical bars represent SEM (n=5) *P<0.05, **p<0.01 compared to control.*

3.5 In vivo treatment

Effect of oestradiol and progesterone pre-treatment.

Frequency response curves to EFS obtained in detrusor muscle samples from rats treated with oestradiol and progesterone for 8 days, is shown in Fig 11. There was a non significant increase in the maximum contractile response to EFS in detrusor muscle from rats pre-treated with oestradiol and progesterone compared to the response in untreated controls (n=10). The addition of progesterone, 2μM, to the bath chamber resulted in a small, non significant, decrease in maximum contractile response. The further addition of DES, 2μM, with progesterone still present in the

bath chamber, caused a further reduction in contractile response, bringing the response back to control levels.

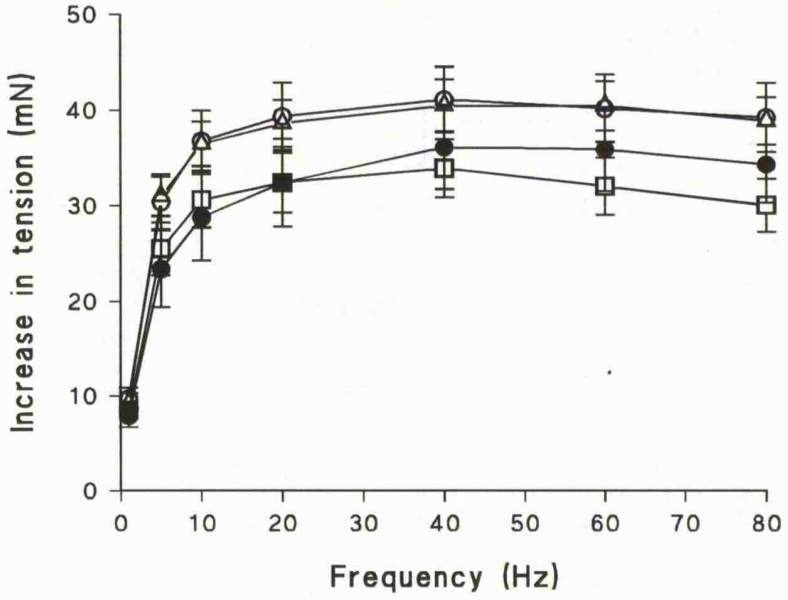


Figure 11. Frequency-response curves to EFS in detrusor muscle from in vivo pre-treated rats. *● control (untreated), Δ after pre-treatment with progesterone and oestradiol (n=10) ○ after the addition of 2μM progesterone, □ after the addition of 2μM progesterone and 2μM DES. Vertical bars represent SEM (n=6).*

Effect on atropine sensitivity

In control rats the maximum contractile response of detrusor muscle to EFS was reduced by 38% after the addition of 10μM atropine to the bath chamber. The frequency response curve showed an increase in the mean EF_{50} from 3 Hz to 16.2 Hz (Fig 12a). In rats pre-treated with oestradiol and progesterone, the atropine sensitivity of detrusor muscle to EFS was similar to the sensitivity in control detrusor muscle. The maximum contractile response of detrusor muscle from pre-treated rats was reduced by 35% after the addition of 10μM atropine and the mean EF_{50} increased from 2.3 Hz to 12.3 Hz (Fig 12b). Responses after pre-treatment with progesterone were statistically indistinguishable from those of control animals.

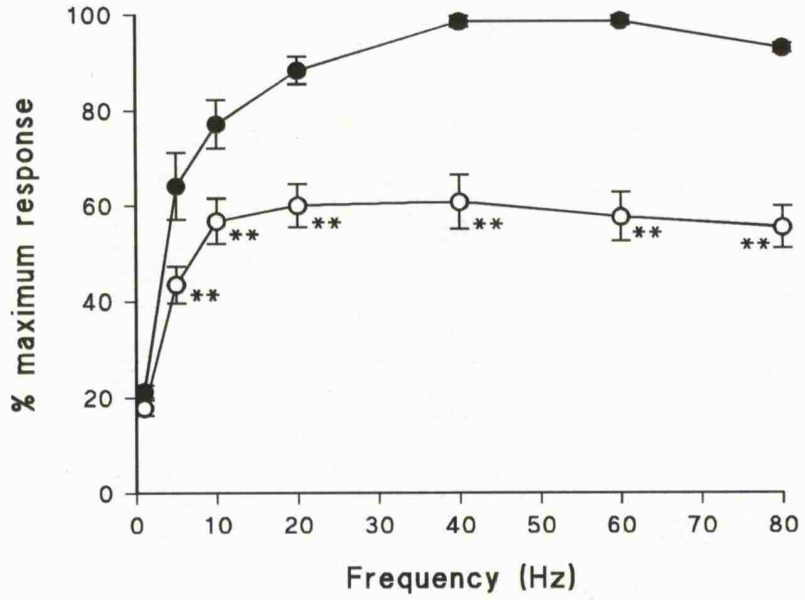


Figure 11a. Effect of atropine on the frequency-response curves in control rats.

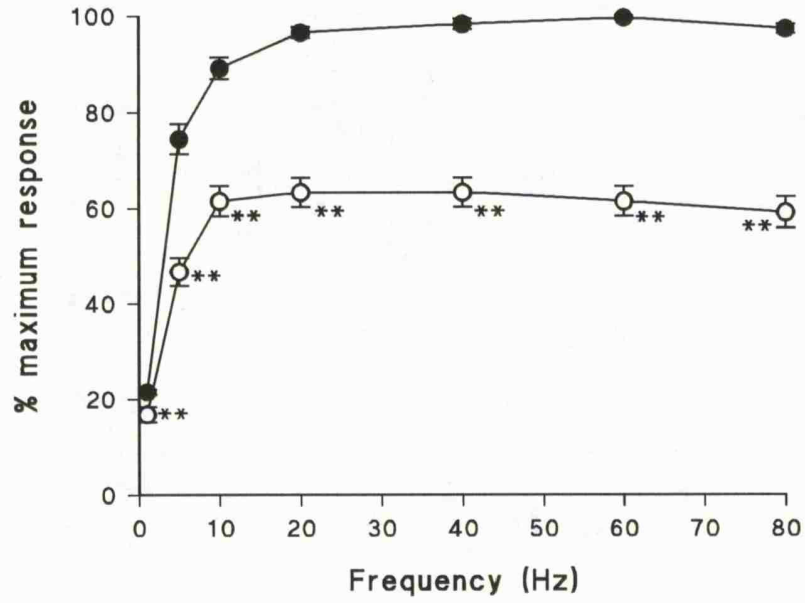


Figure 11b. Effect of atropine, in vitro, on the frequency-response curves in rats pretreated with progesterone and DES. ● *control,* ○ *after the addition of 10μM atropine. Vertical bars represents the SEM (n=5)* ***p<0.01 compared to control.*

Effect on tetrodotoxin resistance.

At the parameters used for EFS, TTX blocked 50% of the maximum contractile response, and more than 50% of the response at lower frequencies. The TTX resistant response is due to direct muscle stimulation. Pre-treatment of rats with oestradiol and progesterone had a negligible but non1 significant effect on the TTX resistant response (Fig 13). However, pre-treatment with oestradiol alone did significantly reduce the TTX resistant response ($p<0.05$).

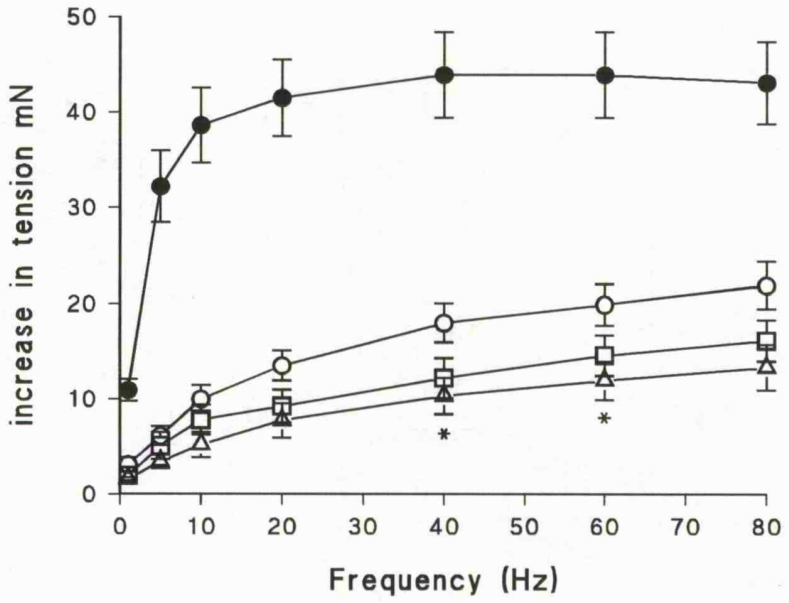


Figure 13. The effect of oestradiol and progesterone pre-treatment on the TTX resistant response to EFS. *● control, ○ after TTX, Δ after oestradiol treatment plus TTX, □ after oestradiol and progesterone pre-treatment plus TTX. Vertical bars represent SEM (n=5) *p<0.05 compared to control plus TTX.*

3.6 Summary

Very low concentrations of DES and progesterone had no direct effect on the contractile response of rat detrusor muscle to EFS. Higher concentrations inhibited significantly the response to EFS. The direct inhibitory effect of high concentrations of progesterone was probably due to the inhibition of calcium influx as the response to KCl was significantly reduced. Pre-treatment with oestradiol and progesterone had no effect on isolated rat detrusor muscle contraction. Pre-treatment also had a minimum effect on the cholinergic and TTX resistant component to EFS.

CHAPTER 4: CALCIUM ANTAGONISTS

Methods

4.1. Aims

Calcium antagonists have a significant inhibitory effect, *in vitro*, on the contractile response of isolated human and animal detrusor muscle (Forman *et al.*, 1978, Zar *et al.*, 1990, Elliott *et al.*, 1992a). However, the clinical effects of these drugs on urge incontinence are inconsistent (Rud *et al.*, 1979, Laval & Lutzeyer 1980). These results are surprising in view of the inhibitory *in vitro* effects of calcium antagonists on isolated detrusor samples. A possible explanation for this discrepancy is the method and duration of drug administration. In man treatment with calcium antagonists is chronic whereas *in vitro* treatment is acute. It is possible the chronic administration of calcium antagonists could be inducing tolerance. The aim of the work described in this section was to investigate the effect of different methods of treatment with nimodipine on the contractile response of isolated rat detrusor muscle. The effect of calcium antagonists *in vitro* on human isolated detrusor was also examined.

4.2 In vitro rat detrusor muscle treatment.

Male Wistar rats (300-400g) were killed and bladders removed then placed into Kreb's solution. Tissues were dissected free of fat and serosa and strips of muscle (4mm x 1mm x 1mm) were suspended in a 50ml organ bath chamber as previously described in section 2.1. Frequency response curves were obtained as described (section 2.2) except the Digitimer Stimulator was set at the following parameters to reduce direct muscle stimulation: Pulse width 0.5 msec and voltage 10 volts. Under these conditions the contractile response of rat detrusor muscle to EFS was abolished by 1.6×10^{-6} tetrodotoxin, indicating its neurogenic origin.

Time response curves for calcium antagonists

Strips of rat detrusor muscle were stimulated with 40 Hz EFS. Samples were incubated with either verapamil 1.5μM, nifedipine 0.25μM or nimodipine 0.1μM and stimulated at intervals of 5 mins. This was to evaluate the incubation time required for each calcium antagonist to reach maximum effect.
The concentrations of drugs used were similar to human plasma levels (46ng/ml after taking 40mg t.d.s. Bath concentration 0.1μM = 42ng/ml) obtained after chronic oral administration (Drug Information Service, Leicester Royal Infirmary).

Effect of nimodipine on EFS

When consistent frequency response curves had been obtained the tissues were washed and re-equilibrated. Nimodipine 0.1μM was added to the bath and after 15 minutes incubation a second frequency response curve was obtained. Nimodipine 0.1μM was used for rat *in vitro* and *in vivo* experiments because it was found in these experiments to have the greatest inhibitory action on human isolated detrusor muscle contractile response. Responses were recorded as an increase in tension (mN).

Effect of washing after the addition of nimodipine

To establish the relative stability of the inhibitory effect of nimodipine on the contractile response of rat detrusor muscle, the following experiment was performed. Frequency response curves were obtained before and after the addition of nimodipine 0.1μM to the organ bath. The tissues were then washed and stimulated again at intervals of 30, 45 and 60 minutes, without further additions of nimodipine.
Contractile responses were presented as a percentage of maximum response.

Effect of nimodipine on carbachol stimulation

After consistent dose-response curves to carbachol had been obtained the dose response curve was repeated after the addition of nimodipine 0.1μM to the bath. It was not necessary to re-apply nimodipine after each stimulation and wash, because of the stability of nimodipine in the tissue samples.

4.3 In vitro human detrusor muscle treatment

Bladder biopsy samples were dissected free of fat and serosa and strips of muscle (4mm x 1mm x 1mm) were suspended in a 50ml organ bath chamber (as above) and allowed to equilibrate for an hour before stimulation.

Effect of calcium antagonists

Dose response curves to carbachol were obtained before and after 15 minutes incubation with either nimodipine 0.1μM, nifedipine 0.1μM and 0.25μM or verapamil 0.1μM and 1.5μM. These concentrations were chosen because they are similar to plasma levels achieved after chronic oral administration in man.

The response to carbachol was investigated because human detrusor muscle contraction is principally mediated via cholinergic mechanisms (Sibley 1984). Responses were recorded as a percentage of the maximum control response.

4.4 In vivo treatment

A group of 6 male Wistar rats weighing 350-400g were treated for 8 days with nimodipine 5.14 mg Kg^{-1} daily, administered orally by gastric intubation. The daily

dose was dissolved in 0.5ml vehicle which was prepared by mixing together 96.6g polyethylene glycol 400, 6.0g glycerine and 10ml of water. Two other groups of 6 rats received either the vehicle only or no treatment and one group of 5 rats received only one dose of nimodipine (there were only 5 rats in this group because one rat, which received no treatment, had been used in the middle of the experiment to standardise the organ bath and equipment. This was to ensure that the results were not due to an artefact). One hour following the last dose of acute and chronic nimodipine pre-treatment, rats were killed and bladders were removed and placed in 10ml Kreb's solution. The tissue was dissected free of fat and serosa and strips of muscle from the bladder body (4mm x 1mm x 1mm) was mounted in the organ bath as described (section 2.1). Samples were allowed to equilibrate for 45 minutes before being stimulated.

Serum nimodipine levels

Serum concentrations of nimodipine from the acute and chronic treatment groups were measured by high-performance liquid chromatography (H.P.L.C.), with the method of Ferguson *et al* (1989). They were performed by Mr Paul Whitaker, Toxicology Lab, Leicester Royal Infirmary.

Effect of nimodipine pre-treatment on EFS evoked response

To compare the effect of different treatment regimes on isolated bladder muscle contraction, frequency response curves were obtained in detrusor samples from rats treated with nimodipine for either 8 days or with a single dose. These were compared with response curves from rats treated with only the vehicle for 8 days or no treatment. The dose response curves were presented as an increase in tension (mN).

Effect of nimodipine pre-treatment plus nimodipine in the bath

Frequency response curves were obtained in detrusor muscle from rats treated with nimodipine for 8 days before and after the addition of nimodipine 0.1μM to the organ bath. This experiment was carried out to establish the presence of functional calcium channels after the tissue had been exposed to nimodipine for 8 days.

Effect of nimodipine treatment and bath applied nimodipine on the carbachol evoked response

Carbachol response curves were obtained from detrusor muscle taken from rats treated with nimodipine for 8 days and compared with control dose response curves (no treatment). Another dose response curve was obtained after the addition of nimodipine 0.1μM in the bath containing samples pre-treated with nimodipine for 8 days. This was to establish the presence of receptor operated calcium channels in our samples after 8 days nimodipine treatment.

Effect of nimodipine pre-treatment on the response to $\mathbb{K}^+$

To investigate the effect of nimodipine treatment on calcium influx via voltage-sensitive calcium channels, response curves to K^+ were obtained in rats pre-treated with nimodipine for 8 days and after a single dose, these were compared to response curves from untreated rats (controls).

Effect of nimodipine pre-treatment plus bath applied nimodipine on the response to K^+.

Dose response curves to K^+ were obtained in detrusor muscle strips from rats pre-treated with nimodipine for 8 days and after the addition of nimodipine 0.1μM to the bath. This was to determine the presence of functioning voltage-sensitive calcium channels after the treatment period.

Solutions and chemicals

Nimodipine (Bayer) and nifedipine (Sigma) were stored in the dark and made up in ethanol on the day of the experiment. Verapamil (Sigma) was made up in ethanol on the day of the experiment. For *in vivo* treatment nimodipine was dissolved in polyethylene glycol (Sigma), glycerine (Fisons) and distilled water prior to oral administration.

Results

4.5 In vitro rat detrusor muscle treatment.

Time response curves for calcium antagonists

When nimodipine, 0.1μM, had been in contact with rat detrusor muscle for 5 minutes, the contractile response to 40 Hz EFS was reduced by 36% compared to control (Fig 14a). After 10, 15, 20, 25 and 30 minutes incubation the contractile responses were reduced by 50%, 55%, 55%, 57% and 56% respectively.

Nifedipine, 0.25μM, reduced the contractile response to 40 Hz EFS by 29% compared to control, after 5 minutes incubation in the organ bath (Fig 14b). After 10, 15, 20, 25, and 30 minutes incubation the contractile responses were reduced by 47%, 57.1%, 57%, 62% and 62% respectively.

Verapamil, 1.5μM, reduced the contractile response to 40 Hz EFS by 12%, 30%, 38%, 38%, 40% and 40% after 5, 10, 15, 20, 25 and 30 minutes respectively (Fig 14c). The calcium antagonists studied demonstrated an increase in inhibitory effect up to 15 minutes incubation time in the organ bath. After this time a plateau was reached in which the inhibitory effect on contractile response remained the same. Therefore, 15 minutes incubation time for these drugs was chosen.

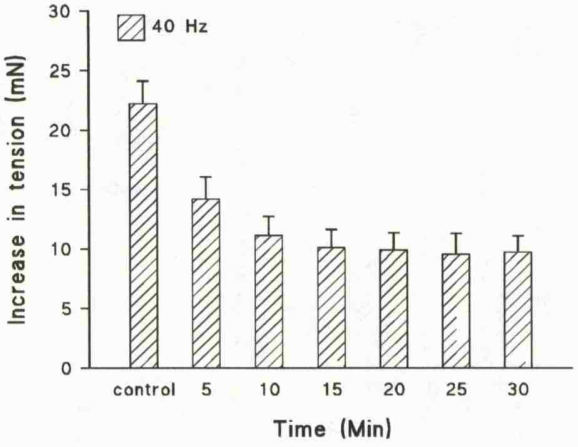


Figure14a. Time response curve to 0.1μM nimodipine, in vitro.

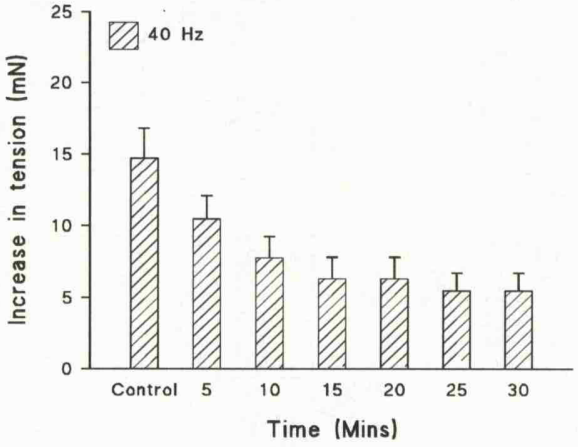


Figure 14b. Time response curve to 0.25μM nifedipine, in vitro.

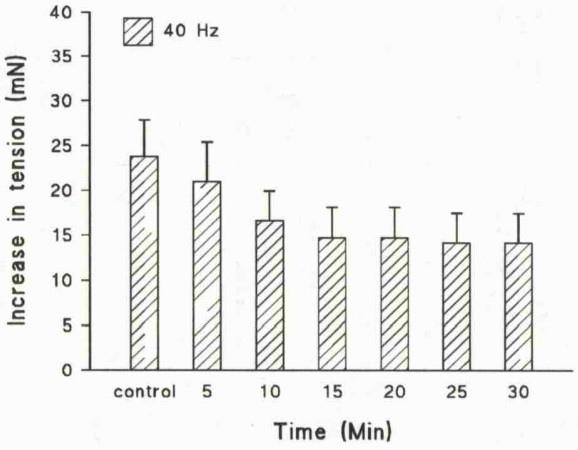


Figure 14c. Time response curve to 1.5μM verapamil, in vitro.

Effect of nimodipine on EFS evoked response

The addition of 0.1μM nimodipine to the organ bath chamber reduced the spontaneous contractions of isolated rat detrusor muscle. Nimodipine also reduced the maximum contractile response, of the frequency response curve, to EFS by 51% ($p<0.01$, Fig 15).

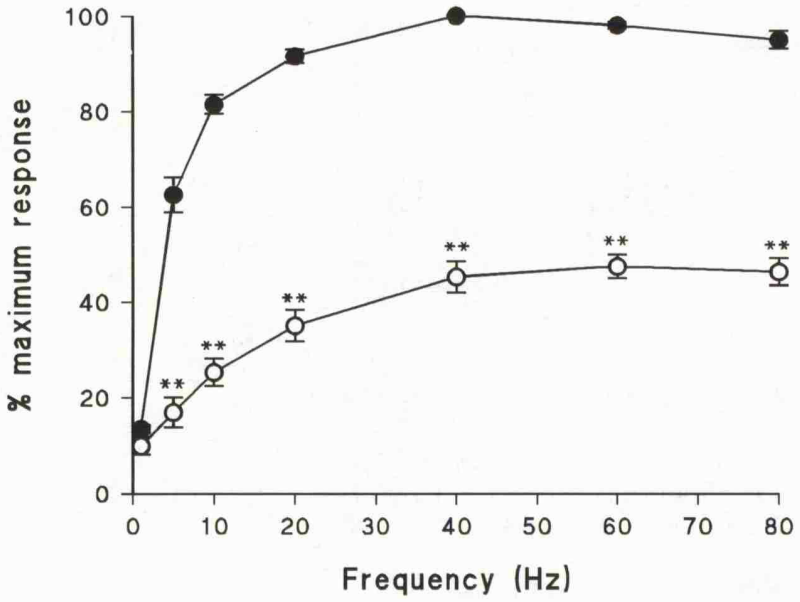


Figure 15. The effect of 0.1μM nimodipine,in vitro, on the contractile response of rat detrusor muscle to EFS. *● control, ○ after 0.1μM nimodipine. Vertical bars represent SEM (n=4) **p<0.01*

Effect of washing after the addition of nimodipine

After the addition of 0.1μM nimodipine to the organ bath the tissues were washed and re-stimulated with 20, 40, 60 and 80 Hz at intervals of 30, 45 and 60 minutes. The maximum contractile response was reduced by 44%, 34% and 31%, respectively, compared to controls (Fig 16). Although still significantly different from control ($p<0.01$) 22% of the original inhibition following the addition of nimodipine was lost after 60 minutes and 3 washes.

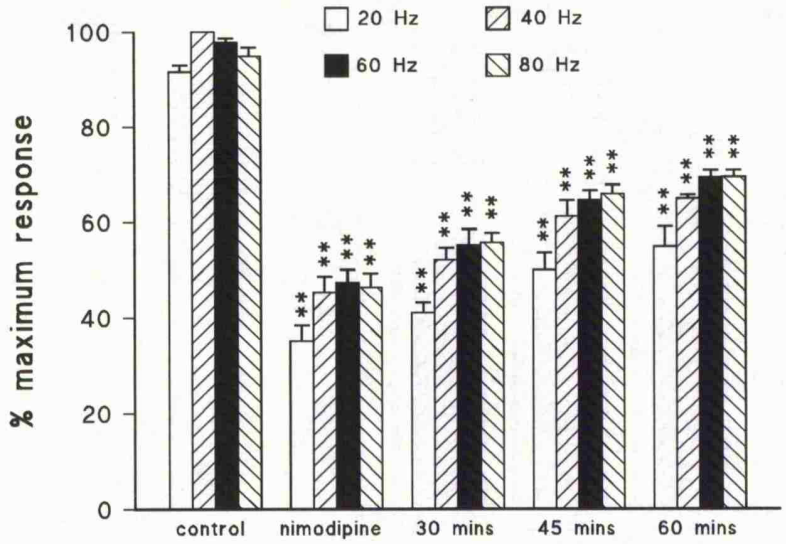


Figure 16. The effect of washing after the addition of nimodipine 0.1μM, in vitro.

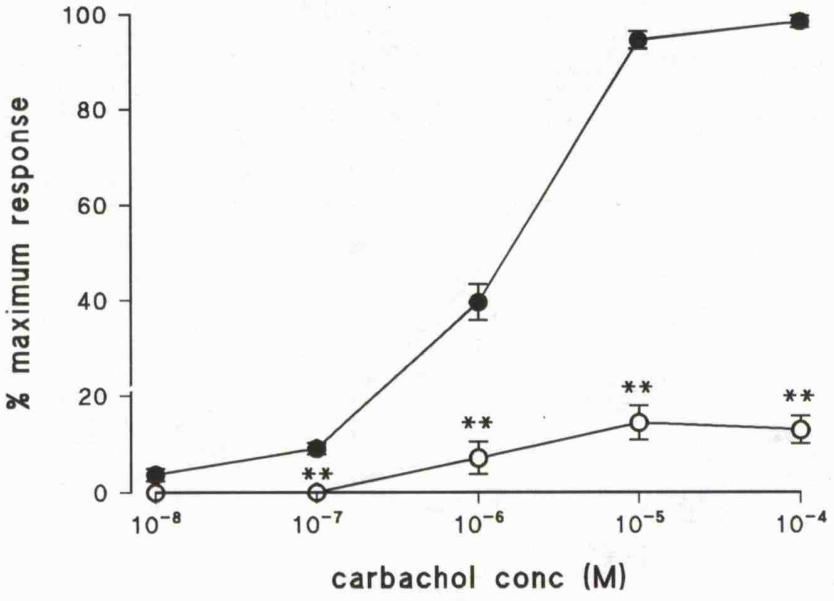


Figure 17. The effect of nimodipine, in vitro, on rat detrusor contractile response to carbachol. *● control, ○ after 0.1μM nimodipine. Vertical bars represent SEM (n=4) **p<0.01*

Effect of nimodipine on the carbachol evoked response.

The maximum contractile response of rat detrusor muscle to carbachol stimulation was reduced by 84% after the addition of nimodipine, 0.1μM, to the bath chamber ($p<0.01$, Fig 17). Nimodipine was added only once to the bath chamber and not re-applied after each stimulation with carbachol. The inhibitory action of nimodipine was maintained after washing the samples.

4.6 In vitro human detrusor muscle treatment

Effect of calcium antagonists.

Nimodipine, 0.1μM, reduced the maximum contractile response of isolated human detrusor muscle to carbachol by 42% compared to control ($p<0.01$, Fig 18a).

The addition of nifedipine 0.1μM and 0.25μM to the organ bath chamber reduced the maximum contractile response by 35% ($p<0.05$) and 41% ($p<0.01$), respectively (Fig 18b). Increasing the concentration of nifedipine did not produce a further dose related inhibitory effect.

Verapamil, 0.1μM, did not produce a significant inhibitory effect on contractile response whereas 1.5μM reduced the maximum contractile response of human detrusor muscle to carbachol by 28% ($p<0.01$, Fig 18c). At the concentrations studied, nimodipine 0.1μM had the most inhibitory action *in vitro* on the contractile response of human isolated detrusor muscle compared to nifedipine and verapamil.

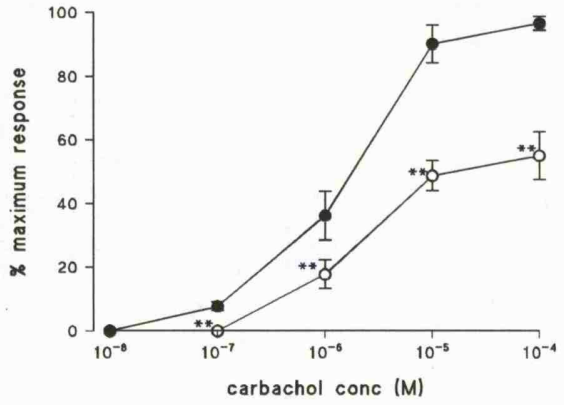


Figure 18a. The effect of nimodipine, in vitro, on the contractile response of human detrusor muscle to carbachol

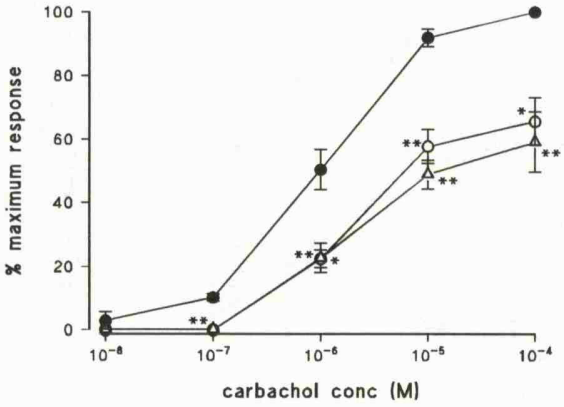


Figure 18b. The effect of nifedipine, in vitro, on the contractile response of human detrusor muscle to carbachol.

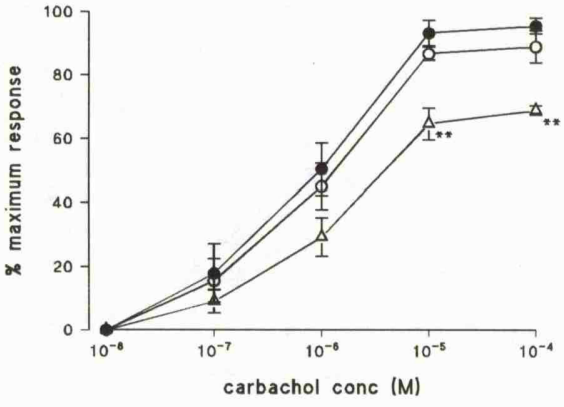


Figure 18c. The effect of verapamil, in vitro, on the contractile response of human detrusor muscle to carbachol. *● control, (18a) ○ after nimodipine 0.1μM. (18b) ○ after nifedipine 0.1μM Δ after nifedipine 0.25μM. (18c) ○ after verapamil 0.1μM, Δ after verapamil 1.5μM. Vertical bars represent SEM (n=5) *p<0.05, **p<0.01 Compared to control*

4.7 In vivo treatment

Effect of nimodipine pre-treatment on EFS evoked response.

The maximum contractile response of rat detrusor muscle to EFS, after pre-treatment with nimodipine for 8 days or with the vehicle, showed no significant difference from control (Fig 19). However, the contractile response of detrusor muscle after the rats had received only one dose of nimodipine was significantly reduced by 66% compared to controls ($p<0.05$).

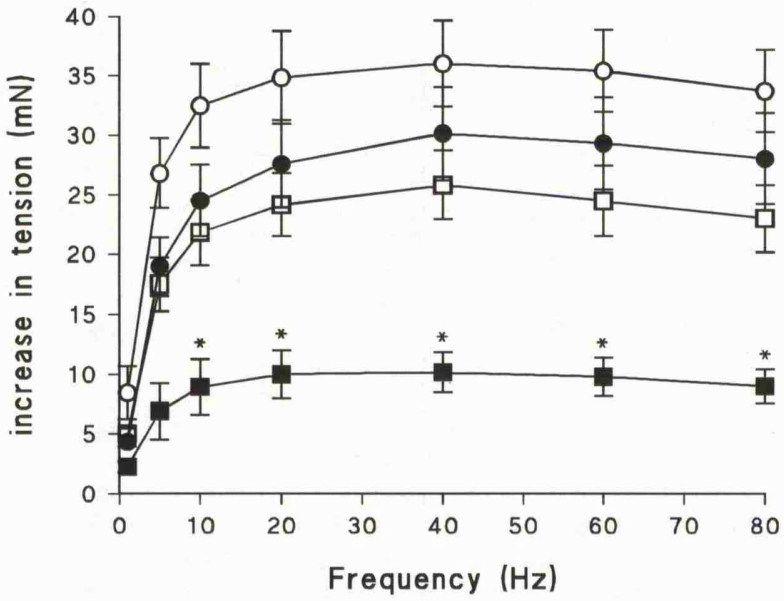


Figure 19. The effect of nimodipine pre-treatment, in vivo, on the contractile response of rat detrusor muscle. *● control, ○ after 8 days nimodipine treatment, □ after pre-treatment with the vehicle, ■ after a single dose of nimodipine. Vertical bars represent SEM (n=5) *p<0.05 compared to control*

Serum nimodipine levels

The serum nimodipine concentration in rats treated with a single dose was 35ng/ml (SEM ± 2.72) and for those treated for 8 days was 46ng/ml (SEM ± 1.84) (Table II). Rats treated for 8 days had a significantly higher serum nimodipine concentration than those treated with a single dose ($p<0.01$).

Rat Number	Single dose ng/ml	Rat number	Eight days ng/ml
1	27	7	40
2	35	8	45
3	40	9	52
4	32	10	47
5	45	11	51
6	30	12	44

Table II. Serum nimodipine concentrations from rats pre-treated with either a single dose or for 8 days.

Effect of nimodipine pre-treatment plus nimodipine in the bath.

When nimodipine 0.1μM was added to the bath chamber containing detrusor muscle from rats treated for 8 days with nimodipine, the contractile response to EFS was significantly reduced by 60% ($p<0.01$, Fig 20). This was not significantly different to responses obtained after bath applied nimodipine in control rats..

Effect of nimodipine treatment and bath application on carbachol evoked responses.

The contractile response of detrusor muscle to carbachol, from rats treated with nimodipine for 8 days, showed a non significant increase compared to controls (no treatment) (Fig 21). The addition of nimodipine, 0.1μM, to the bath chamber containing detrusor muscle from rats treated for 8 days with nimodipine, reduced the maximum contractile response by 91% ($p<0.01$).

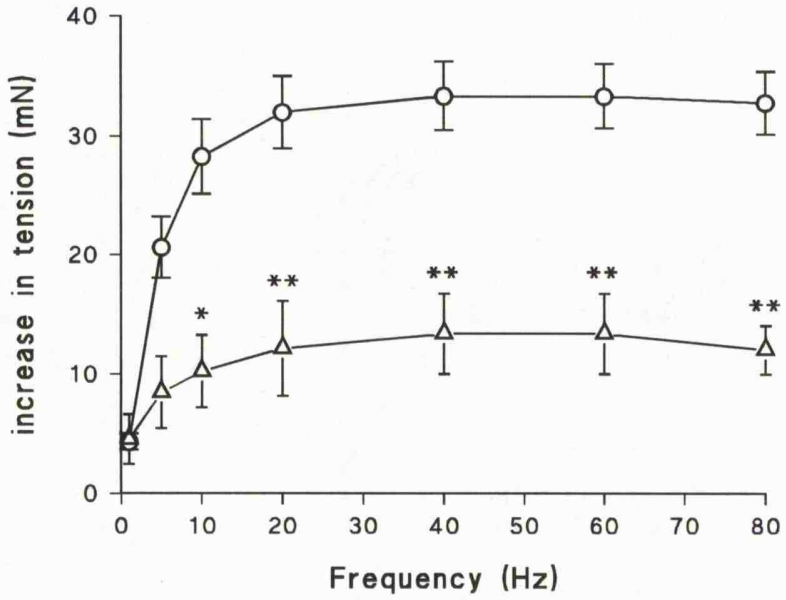


Figure 20. The effect of nimodipine pretreatment, in vivo, and nimodipine in the bath on the contractile response of rat detrusor muscle to EFS. *○ after 8 days nimodipine treatment, Δ after nimodipine 0.1μM. Vertical bars represent the SEM. (n=4) **p<0.01*

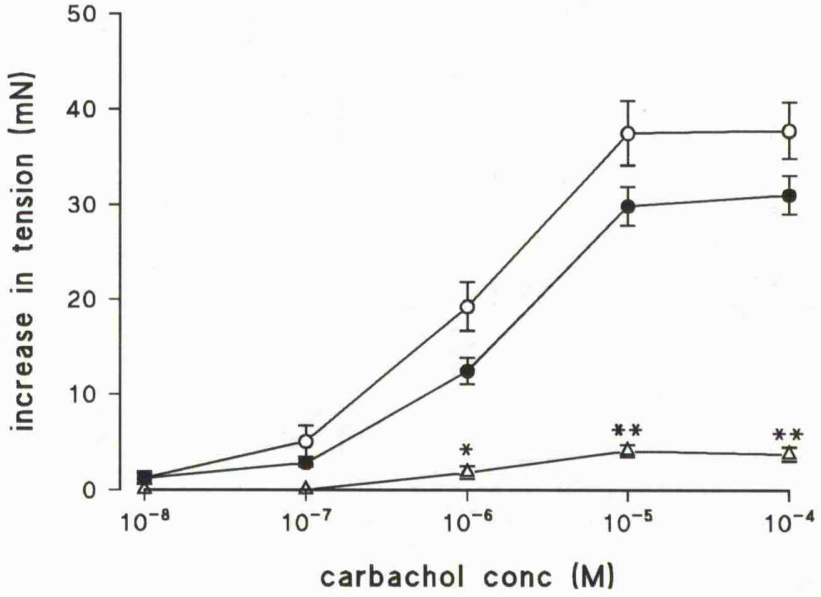


Figure 21. The effect of nimodipine pretreatment, in vivo, and nimodipine in the bath on the contractile response of rat detrusor muscle to carbachol. *● control, ○ after 8 days nimodipine treatment, Δ after the addition of nimodipine 0.1μM. Vertical bars represent SEM (n=5) *p<0.05, **p<0.01.*

Effect of nimodipine pre-treatment on the response to K^+

In rats pre-treated with nimodipine for 8 days, the contractile response of isolated detrusor muscle to K^+ showed no significant difference from control (untreated) (Fig 22). When the rats had received only 1 dose of nimodipine the contractile response of detrusor muscle was significantly reduced by 51% compared to controls (untreated).

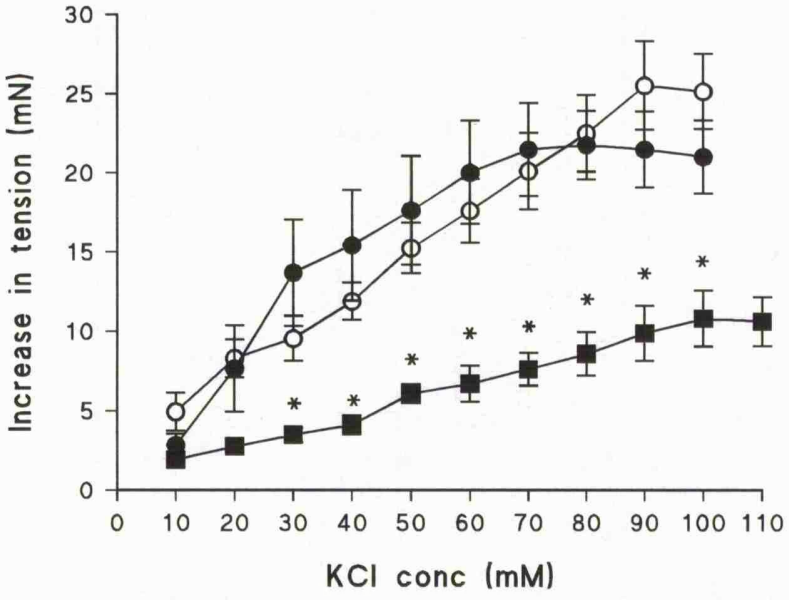


Figure 22. The effect of nimodipine pre-treatment, in vivo, on the contractile response of rat detrusor muscle to KCl. *● control, ○ after 8 days nimodipine treatment, ■ after a single dose of nimodipine. Vertical bars represent SEM. (n=5) * $p<0.05$*

Effect of nimodipine treatment plus bath applied nimodipine on the response to K^+

The addition of nimodipine 0.1μM to the organ bath, containing detrusor muscle from rats pre-treated with nimodipine for 8 days, abolished the contractile response to K^+ (Fig 23).

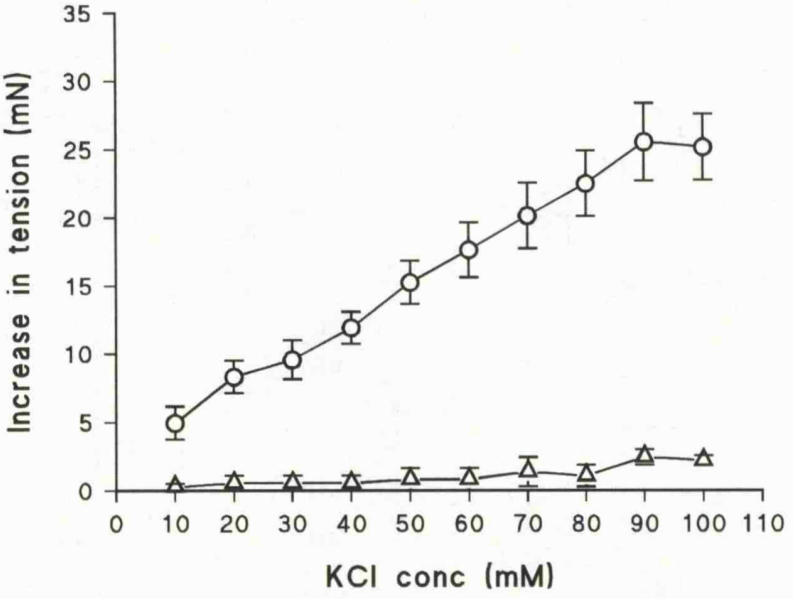


Figure 23. The effect of nimodipine pre-treatment, in vivo, and nimodipine in the bath on the contractile response of rat detrusor muscle to KCl. *○ after8 days nimodipine treatment. Δ after the addition of 0.1μM nimodipine Vertical bars represent SEM (n=5)*

4.8 Summary

At the concentrations of calcium antagonists studied, nimodipine was found to have the most inhibitory action on the contractile response of isolated human detrusor muscle. Nimodipine had a significant inhibitory effect on the contractile response of rat detrusor muscle *in vitro.* This inhibitory effect was stable and not easily washed out of the tissue samples. The chronic *in vivo* treatment of rats with nimodipine had no effect on the contractile response of isolated detrusor muscle. However, treatment with a single dose of nimodipine significantly reduced detrusor muscle contractile response. Nimodipine abolished the contractile response to K^+, thus confirming a blockade of calcium uptake.

CHAPTER 5: CAFFEINE

Methods

5.1. Aims

The diuretic effect of caffeine is well known and caffeine consumption has been shown to have a detrimental effect on bladder function in those suffering from incontinence. It is possible that this effect is not only due to the diuretic action of caffeine but to a direct effect on detrusor muscle contraction. In a group of women with confirmed detrusor instability it was found that the administration of caffeine caused a significant increase in detrusor pressure on bladder filling (Creighton & Stanton 1990). The aim of work described in this section was to determine the effect of caffeine on detrusor muscle contraction with a view to advising people with detrusor instability on caffeine consumption.

5.2 In vitro human detrusor muscle treatment

Human detrusor muscle samples were placed immediately into Kreb's solution and transported to the laboratory. Muscle strips (4mm x 1mm x 1mm) were dissected free of fat and serosa and mounted in the organ bath as described in section 2.1.

Effect of caffeine on the acetylcholine evoked response

When consistent dose response curves to acetycholine had been achieved (control) caffeine 50μM was added to the bath chamber. This concentration was used as it is equivalent to plasma concentrations obtained after the ingestion of 3 cups of coffee (250mg) (Gillman *et al.*, 1985). Following 10 minutes incubation a second dose response curve was obtained. Responses were presented as an increase in tension (mN) and as a percentage of maximum response.

Effect of caffeine on carbachol evoked response

When consistent dose response curves to carbachol had been obtained, caffeine 50μM was added to the chamber. After 10 minutes incubation a second dose response curve was obtained. Responses were presented as an increase in tension (mN) and as a percentage of maximum response.

5.3 In vitro rat detrusor muscle treatment

Male Wistar rats (200-300g) were killed and bladders were removed and placed in Kreb's solution. Strips of muscle from the bladder body were suspended in the organ bath chamber as described in section 2.1.

Effect of caffeine on acetylcholine and carbachol evoked responses

Carbachol is an ester of choline which is not hydrolysed by cholinesterase. Because of this the effect of caffeine on the contractile response to acetylcholine and carbachol was investigated. When consistent dose response curves had been achieved to acetylcholine and carbachol, caffeine 50μM was added to the bath chamber. After 10 minutes incubation a second dose-response curve was obtained. Responses were presented as an increase in tension (mN) and as a percentage of maximum response. Control responses were compared to responses obtained after the addition of caffeine.

Effect of caffeine on the response to adenosine triphosphate (ATP)

The response to ATP was investigated because it is the neurotransmitter responsible for the non-adrenergic non-cholinergic (NANC) response to nerve stimulation in the rat. When consistent dose response curves had been achieved, caffeine 50μM was added to the bath chamber. After 10 minutes incubation a second

dose-response curve was obtained. Responses were presented as an increase in tension (mN) and as a percentage of maximum response.

Effect of caffeine on EFS evoked response

The Digitimer Stimulator was set at 20 volts, pulse width 0.05msec. When consistent frequency response curves had been achieved the tissues were washed and re-equilibrated. Caffeine 50μM was then added to the bath and after 10 minutes incubation a second frequency response curve was obtained. Responses were recorded as an increase in tension (mN) and as a percentage of maximum response. The response to EFS was abolished by tetrodotoxin 1.6×10^{-6}M, indicating its neurogenic origin.

Effect of caffeine and atropine on EFS evoked response

When consistent responses had been obtained, atropine 1μM was added to the chamber to block the cholinergic response to EFS. After 10 minutes incubation a second frequency response curve was obtained. With atropine still present in the organ bath chamber, caffeine 50μM was added and a third frequency response curve obtained. This was to establish if caffeine only influenced the cholinergic response to EFS. Responses were presented as an increase in tension (mN) and as a percentage of maximum response.

Effect of different concentrations of caffeine on the response to acetylcholine

Rat detrusor samples were stimulated repeatedly with 10 second applications of acetylcholine 10^{-3}M, until consistent responses were obtained. This was considered the control response. Different concentrations of caffeine, 1μM, 10μM, 50μM, 100μM and 1mM were added to the bath chamber for 10 minutes prior to

stimulation with acetylcholine 10^{-3}M. This was to determine whether the action of caffeine was dependent on the concentration used. The responses obtained were presented as an increase in tension (mN) and compared to the control response.

Effect of caffeine on acetylcholine response in high K^+, Ca^{2+} free medium

The contractile response of detrusor muscle to acetylcholine in high K^+, calcium free Kreb's solution fades after repeated stimulation, as internal calcium stores are depleted. The response can be restored by the addition of calcium to the bathing medium, which replenishes internal calcium stores.

After stimulation with acetylcholine 10^{-3}M the tissue samples were washed with high K^+, Ca^{2+} free Kreb's solution then calcium 2.5mM was added to the bath chamber. After 5 minutes the tissues were washed again and left for 3 minutes before being stimulated with acetylcholine 10^{-3}M. This was repeated until consistent responses were obtained which were taken as the control response. After the samples had been incubated with calcium for 5 minutes , then washed, caffeine 50μM was added to the chamber. After 3 minutes incubation the samples were stimulated with acetylcholine 10^{-3}M. This experiment investigated the influence of extracellular calcium depletion on the effect of caffeine on contractile response. The responses were presented as an increase in tension (mN).

Effect of caffeine and ryanodine on the acetylcholine evoked response

The detrusor samples were exposed to 10 second applications of acetylcholine 10^{-3}M until consistent responses were obtained, this was taken as the control response. Caffeine 50μM was added to the bath and after 10 minutes incubation the sample was restimulated. After washing, the sample was incubated with ryanodine alone, 10^{-5}M, and with caffeine 50μM plus ryanodine 10^{-5}M for 10 minutes, and then

stimulated with acetylcholine. Ryanodine depletes internal calcium stores and, therefore, it may influence the effect of caffeine on the contractile response of detrusor muscle.

The contractile responses were presented as an increase in tension (mN) and the response in the presence of caffeine alone was compared to the response in the presence of caffeine and ryanodine.

Solutions and chemicals

Caffeine (Pharmacy, Leicester General Hospital), acetylcholine chloride, atropine, carbamylcholine chloride, ATP (all Sigma) and Ryanodine (ICN) were all dissolved in distilled water and made up on the day of the experiment.

Results

5.4 In vitro human detrusor muscle treatment

Effect of caffeine on acetylcholine evoked response

Human detrusor isolated muscle strips showed a concentration dependent contractile response to the application of ACh (10^{-8}M - 2 x 10^{-3}M) and carbachol (10^{-8}M - 10^{-4}M). The maximum contractile response to ACh was obtained at a high concentration (10^{-3}M) compared to carbachol (10^{-5}M - 10^{-4}M). Caffeine, 50μM, increased significantly the maximum contractile response to ACh by 15% ($p<0.05$) compared to control, but had no effect on the contractile response to carbachol (Fig's 24a & 24b)

5.5 In vitro rat detrusor muscle treatment

Effect of caffeine on acetylcholine and carbachol evoked response.

Concentration-dependent contractile responses of rat detrusor muscle were seen after the application of ACh and carbachol to the organ bath. The dose response curves were presented as an increase in tension, expressed as mN, and as a percentage of maximum response. When expressed as a percentage of maximum response, the dose response curve to ACh after the application of caffeine, 50μM, was similar to the control response curve. However, the maximum contractile response achieved after the addition of caffeine was increased by 9% compared to controls ($p<0.05$, Fig 25a). When expressed as an increase in tension, the increase in maximum response after the addition of caffeine was not significant (Fig 25b). This was due to the wide variation in tension encountered during stimulation, resulting in a high SEM.

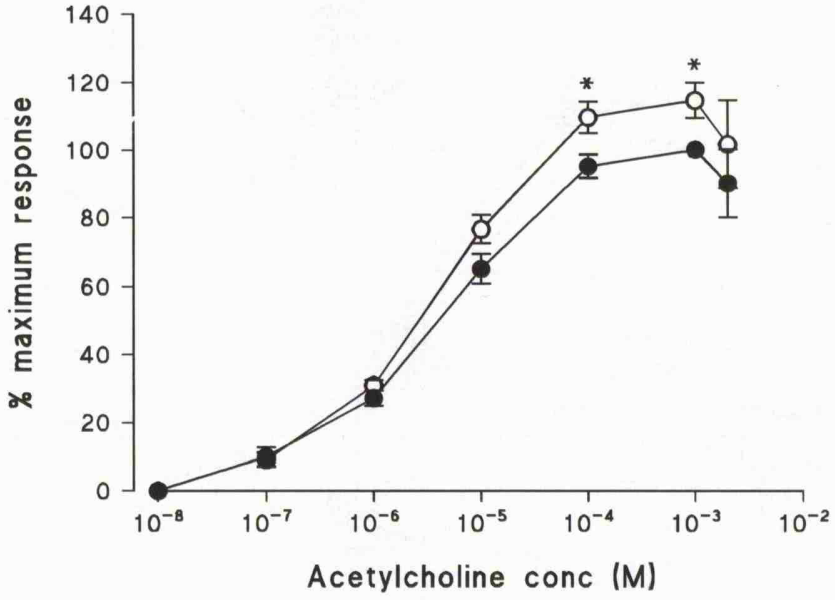


Figure 24a. The effect of caffeine, in vitro, on the contractile response of human detrusor muscle to acetylcholine. *● control, ○ after the addition of caffeine 50μM. Vertical bars represent SEM (n=8) *p<0.05*

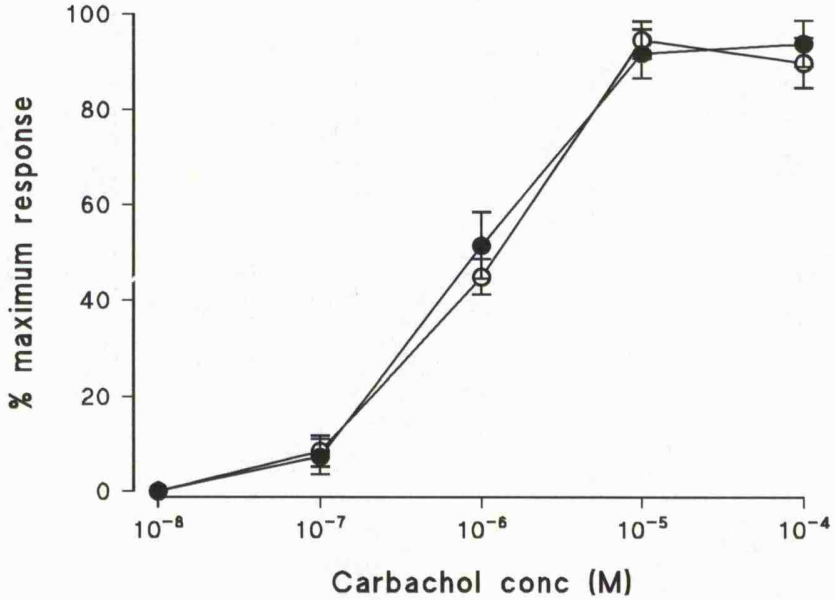


Figure 24b. The effect of caffeine, in vitro, on the contractile response of human detrusor muscle to carbachol. *● control, ○ after the addition of caffeine 50μM. Vertical bars represent SEM (n=8)*

The dose response curves to carbachol, before and after the addition of caffeine, 50μM, were similar. When expressed as a percentage of maximum response, the maximum response to carbachol was not altered by the addition of caffeine, 50μM, (Fig 26a). The dose response curves were also similar when expressed as an increase in tension (Fig 26b). As with ACh, the SEM's were higher when the contractile response was expressed as an increase in tension.

Effect of caffeine on the response to adenosine triphosphate (ATP)

Rat detrusor muscle contractile response to ATP is dose-dependent. However, the amplitude of response was considerably less than to ACh and carbachol. The addition of caffeine, 50μM, increased, non significantly, the maximum response to ATP when expressed as a percentage of maximum response and as an increase in tension (Fig's 27a & 27b).

Effect of caffeine on EFS evoked response.

Stimulation of intrinsic nerves in the muscle strips resulted in a frequency-dependent increase in tension. The maximum response to EFS was less than the maximum response to ACh and carbachol. When expressed as a percentage of maximum response, caffeine, 50μM, increased significantly the maximum response to EFS by 11% ($p<0.01$, Fig 28a). When the contractile response to EFS was expressed as an increase in tension, the maximum response was not significantly increased by caffeine (Fig 28b).

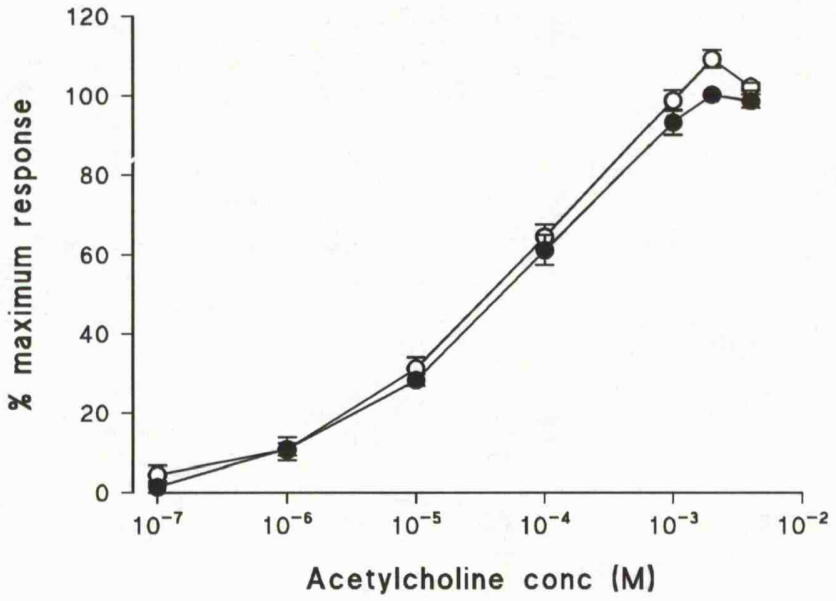


Figure 25a. The effect of caffeine, in vitro, on the contractile response of rat detrusor muscle to acetylcholine. *● control, ○ after the addition of caffeine 50μM. Vertical bars represent SEM. (n=5) *p<0.05*

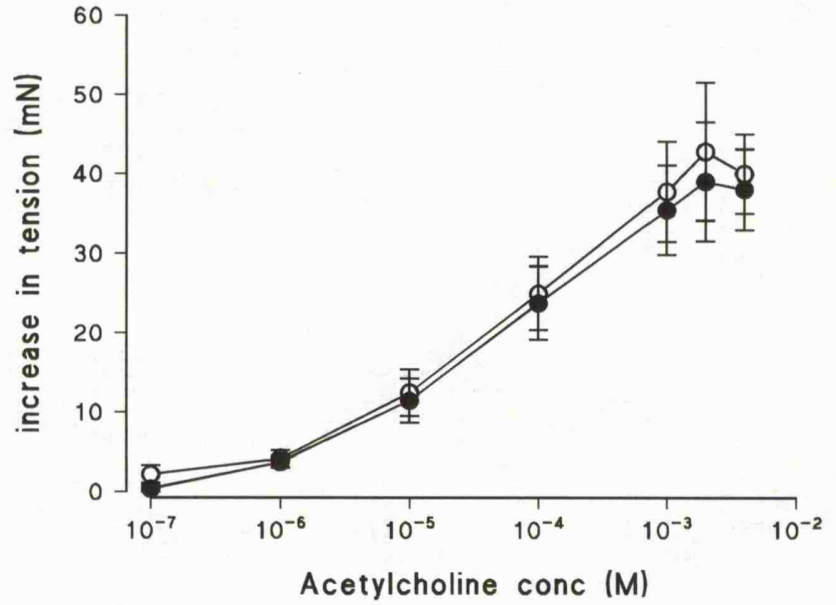


Figure 25b. The effect of caffeine, in vitro, on the contractile response of rat detrusor muscle to acetylcholine. *● control, ○ after the addition of caffeine 50μM. Vertical bars represent SEM. (n=5)*

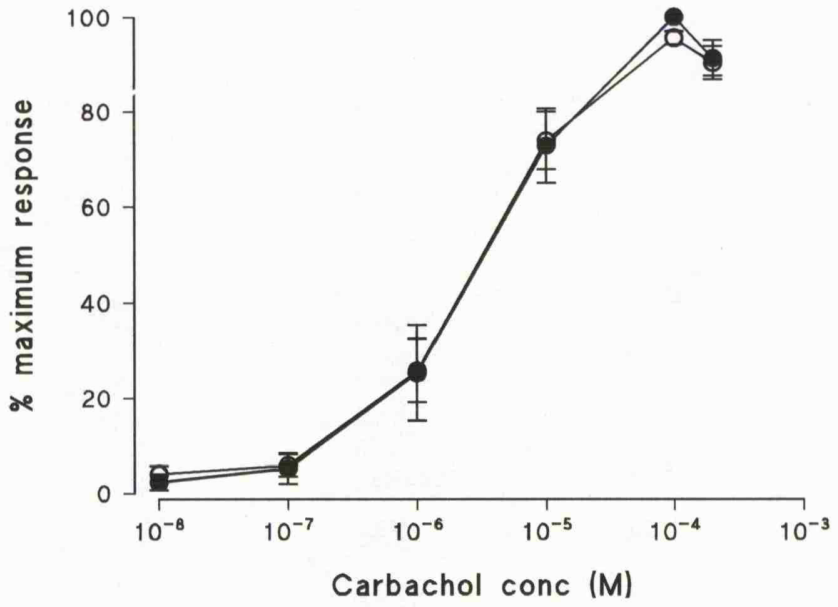


Figure 26a. The effect of caffeine, in vitro, on the contractile response of rat detrusor muscle to carbachol. *● control, ○ after the addition of caffeine 50μM. Vertical bars represent SEM (n=5).*

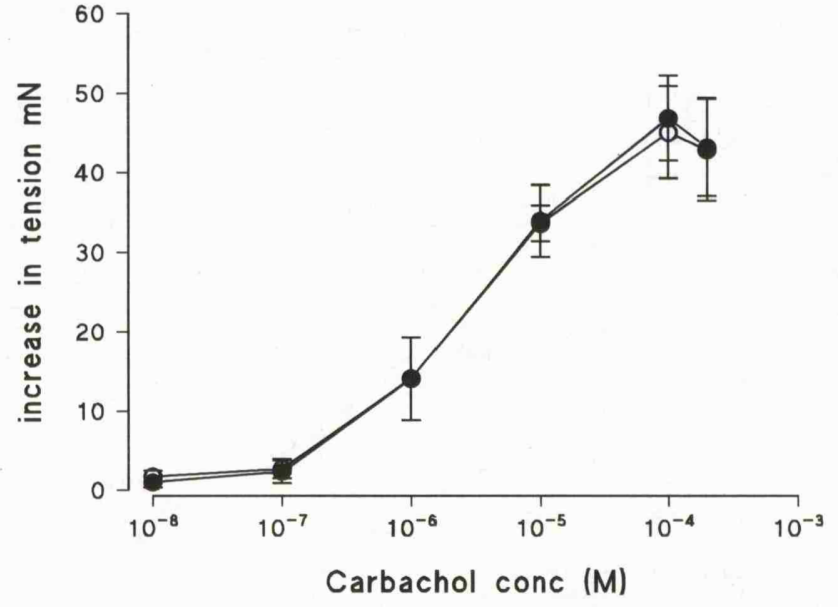


Figure 26b. The effect of caffeine, in vitro, on the contractile response of rat detrusor muscle to carbachol. *● control, ○ after the addition of caffeine 50μM. Vertical bars represent SEM. (n=5)*

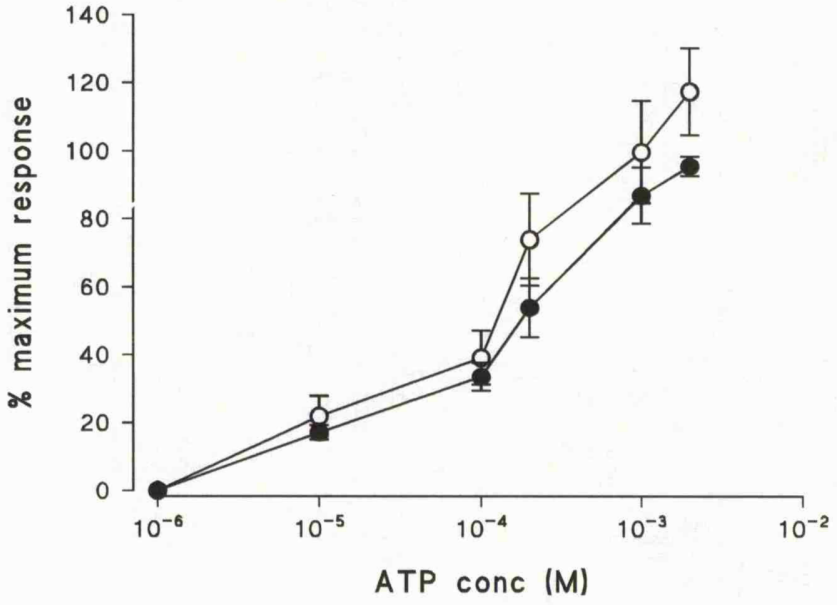


Figure27a. The effect of caffeine, in vitro, on the contractile response of rat detrusor muscle to ATP. *● control, ○ after the addition of caffeine 50μM. Vertical bars represent SEM. (n=5)*

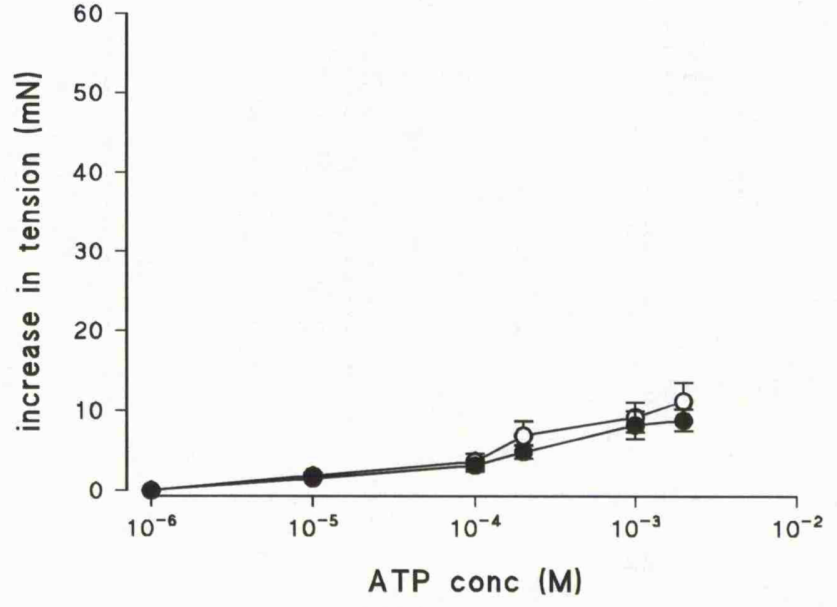


Figure 27b. The effect of caffeine, in vitro, on the contractile response of rat detrusor muscle to ATP. *● control, ○ after the addition of caffeine 50 μM. Vertical bars represent SEM (n=5)*

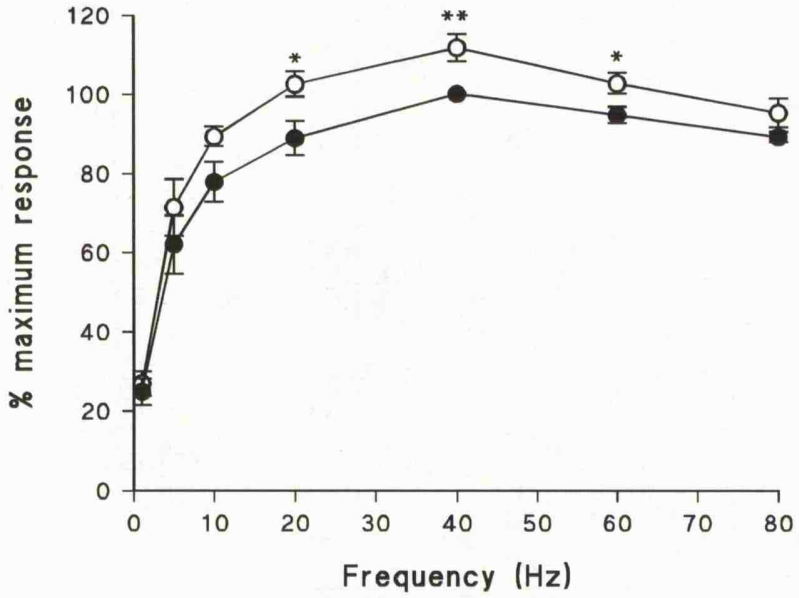


Figure 28a. The effect of caffeine, in vitro, on the contractile response of rat detrusor muscle to EFS. *● control, ○ after the addition of caffeine 50μM Vertical bars represent SEM (n=5). *p<0.05 **p<0.01*

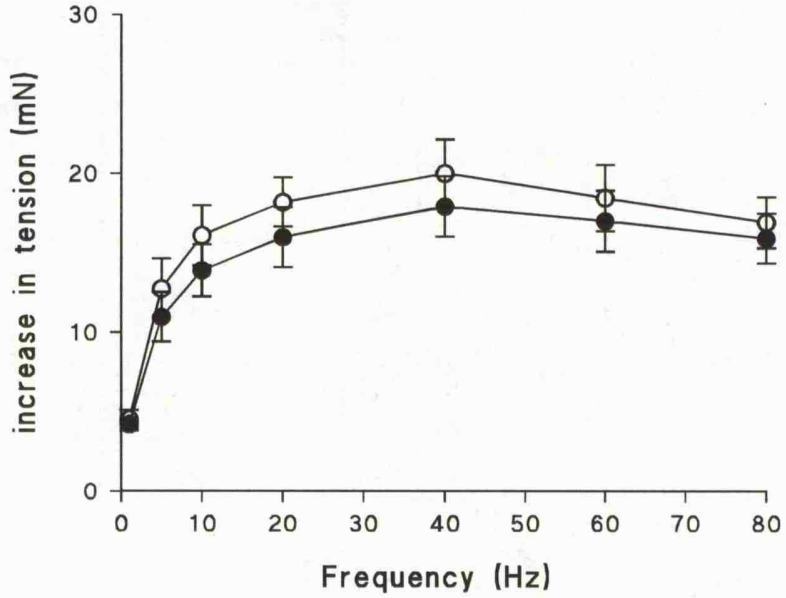


Figure 28b. The effect of caffeine, in vitro, on the contractile response of rat detrusor muscle to EFS. *● control, ○ after the addition of caffeine 50μM. Vertical bars represent SEM (n=5)*

Effect of caffeine and atropine on EFS evoked response

Atropine, 1μM, reduced the maximum response to EFS by 30% ($p<0.01$) thereby blocking the cholinergic response. The addition of atropine, 1μM, and caffeine, 50μM, reduced the response by 25% (Fig 29). The difference between the response after atropine alone and atropine plus caffeine was not significant. The slight increase in maximum response to EFS by caffeine was therefore abolished by atropine.

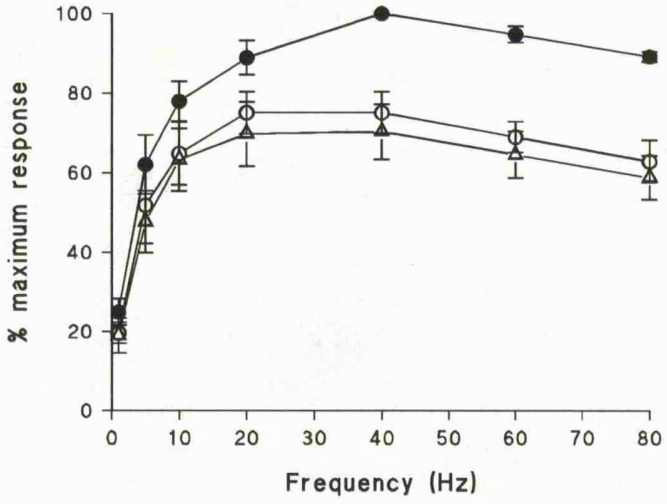


Figure 29. The effect of caffeine, in vitro, on the contractile response of rat detrusor muscle to EFS in the presence of atropine 1μM. *● control, ○ after the addition of caffeine 50μM and atropine 1μM. Δ after the addition of atropine 1μM. Vertical bars represent SEM (n=5).*

Effect of different concentrations of caffeine on acetylcholine evoked response.

At the concentration of ACh, 10^{-3}M, used for this experiment there was no significant difference between the control response and responses after the addition of different concentrations of caffeine. However, after the addition of caffeine, 10μM and 50μM there was an increase of 5% in contractile response compared to control

(Fig 30). The responses after the addition of caffeine, 1μM and 100μM, were almost the same as control whereas 1mM caffeine slightly reduced the contractile response to ACh 10^{-3}M by 8%.

Effect of caffeine on acetylcholine response in high K^+, Ca^{2+} free medium.

In a calcium free medium the contractile response of rat detrusor muscle to ACh, 10^{-3}M, before and after the addition of caffeine, 50μM, was the same (Fig 31).

Effect of caffeine and ryanodine on acetylcholine evoked response.

After the addition of ryanodine 10^{-5}M to the organ bath the dose response curve to ACh was shifted to the right. The samples were allowed to re-equilibrate then caffeine, 50μM, and ryanodine, 10^{-5}M, was added to the bath. The dose response curve to ACh was slightly shifted to the left, back towards the control response (Fig 32). The addition of ryanodine to the bathing medium, therefore, abolished the slight but non significant effect of caffeine on rat detrusor contractile response to ACh.

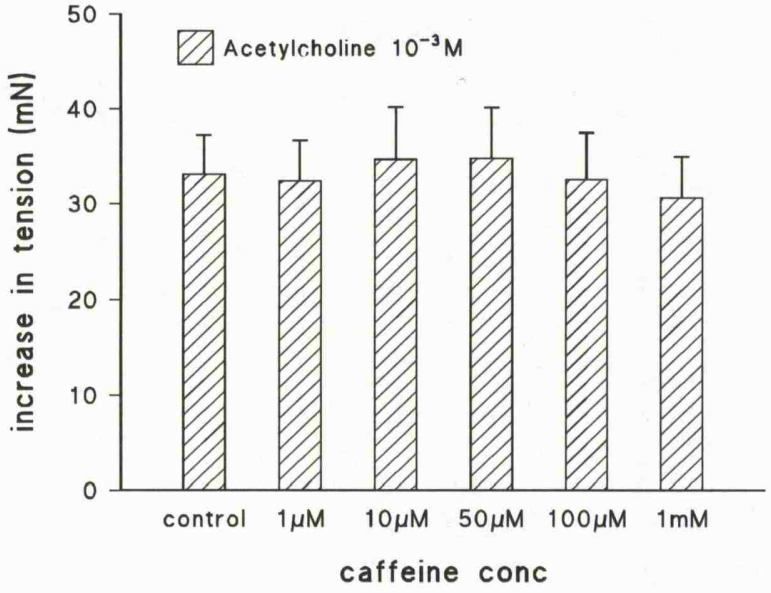


Figure 30. The effect of different concentrations of caffeine, in vitro, on the contractile response of rat detrusor muscle to acetylcholine 10^{-3}M.
Vertical bars represent SEM (n=5).

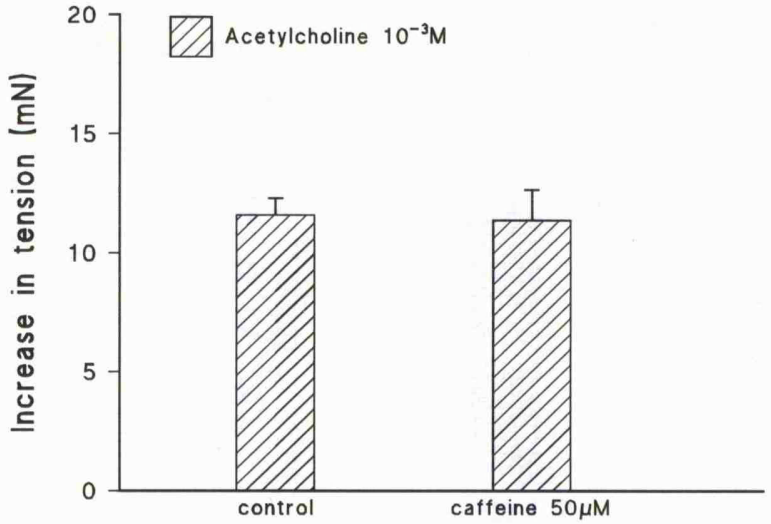


Figure 31. The effect of caffeine, in vitro, on the contractile response of rat detrusor muscle to acetylcholine 10^{-3}M in high K^{+} Ca^{2+} free medium.
Vertical bars represent SEM

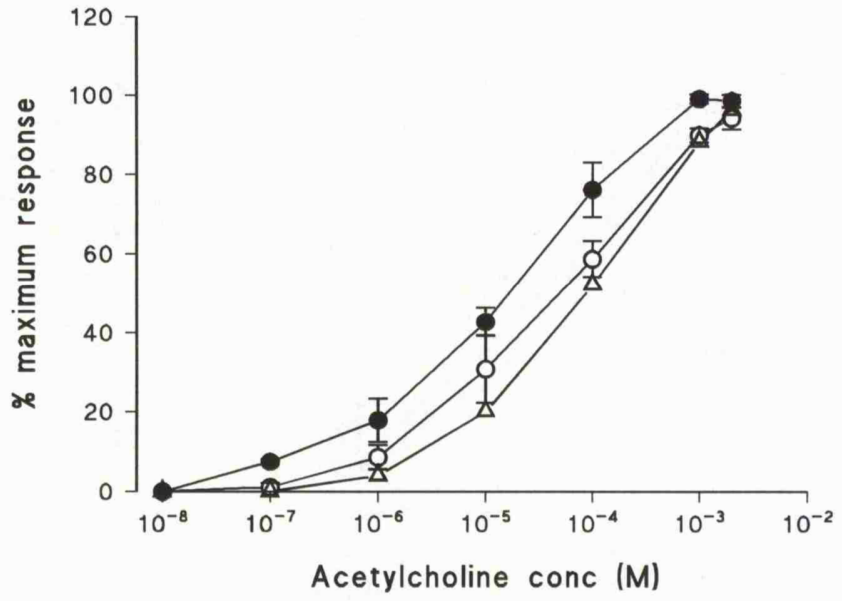


Figure 32. The effect of caffeine and ryanodine, in vitro, on the contractile response of rat detrusor muscle to acetylcholine. *● control, Δ after the addition of ryanodine 10μM, ○ after the addition of caffeine 50μM and ryanodine 10μM. Vertical bars represent SEM (n=4)*

5.6 Summary

Caffeine had a slight potentiating effect on the contractile response of rat detrusor muscle to ACh and EFS which was not significant when contraction was expressed in tension (mN). The response to carbachol was not altered by caffeine. Caffeine also slightly potentiated the response to ACh in human isolated detrusor muscle which was of greater magnitude than in rat detrusor muscle. The slight potentiating effect of caffeine on ACh was abolished by the removal of calcium from the bathing medium and by the addition of ryanodine. Overall the effect of caffeine was small and probably clinically insignificance.

CHAPTER 6: DISCUSSION

" Psychologically and sociologically the consequences of loss of continence in our society should never be underestimated. The loss of dignity and self-esteem precipitated by losing control and wetting bed, chair or clothing can have devastating effects upon the individual concerned" (Garrett 1983).

These words by Garrett (1983) describe eloquently the discomfort, shame and loss of self-confidence endured by those with urinary incontinence. They also serve as a poignant reminder of the purpose of research.

A concise brief summary of the results obtained in this thesis is as follows: (a) bath applied female sex hormones reduced the contractility of isolated rat detrusor muscle, (b) pre-treatment of rats with oestradiol and progesterone had no significant effect on isolated detrusor muscle contractile response, (c) bath applied calcium antagonists had a significant inhibitory effect on isolated human detrusor muscle contractile response, (d) chronic pre-treatment of rats with nimodipine had no effect on the contractile response of isolated detrusor muscle, (e) there was a significant reduction in the contractile response of isolated detrusor muscle from rats pre-treated with a single dose of nimodipine, and (f) caffeine slightly enhanced the contractile response of human and rat detrusor muscle to acetylcholine and electrical stimulation.

6.1 Oestrogens and progestogens.

Previous work from this laboratory investigated the effect of bath applied DES and pre-treatment *in vivo* of rats with oestradiol on the contractile response of isolated detrusor muscle (Elliott *et al.*, 1992a, 1992b). Each method of treatment had a significant inhibitory effect on detrusor contraction. When both treatments were applied they summated to produce a highly significant decrease in contractile response

to cholinergic and EFS. It was proposed that the direct effect of DES inhibited calcium uptake (Elliott *et al.*, 1992a) and pre-treatment with oestradiol altered muscarinic receptor densities (Elliott *et al.*, 1992b).

In humans it is usual to administer oestrogens with progestogens, to protect the uterus. This led to the present investigation which demonstrated that pre-treatment with oestradiol and progesterone had no significant effect on the contractile response of isolated rat detrusor muscle to EFS. This result suggested progesterone antagonised the inhibitory action of oestrogen on rat detrusor muscle contraction. Oestradiol and progesterone pre-treatment in anaesthetised rats has also been found to have no effect on bladder contraction after stimulation of hypogastric and pelvic nerves (Sato *et al.*, 1989).

The urinary bladder is clearly a target for hormonal action with oestrogen receptors identified in the human bladder body, urethra and trigone (Iosif *et al.*, 1981). A comparative study identified oestrogen receptors in the uterus, vagina and urethra of ovariectomised rabbits with the uterus having the highest concentration. However, receptors were clearly evident in the urethra and bladder (Batra & Iosif 1983).

Progesterone receptors have also been found in the urethra, urinary bladder and vagina of rabbits previously treated with oestrogen (Batra & Iosif 1983, Batra & Iosif 1987). In most instances the interaction between progesterone and oestrogen is antagonistic, mediated by a reduction of oestrogen receptors. This antioestrogenic

effect of progesterone has been demonstrated in the uterus but not in the vagina or urethra of female rabbits (Batra & Iosif 1989).

In the present results, progesterone and oestrogen treatment of rats had no effect on detrusor contractile response whereas oestrogen treatment alone has been shown to have a significant inhibitory effect on contractile response and cholinoceptor densities (Elliott *et al.*, 1992b, Batra & Andersson 1989, Shapiro 1986). It is therefore possible that an antioestrogenic effect of progesterone has been demonstrated in rat detrusor muscle.

The mechanism for such an effect has been more extensively investigated in uterine tissue. Oestrogen is able to penetrate the cell membrane and combine in the cytoplasm with a specific receptor (Batra 1980). The receptor complex then moves into the cell nucleus which, after modification, leads to DNA-dependent RNA synthesis and finally the synthesis of proteins (Batra 1980). It seems that the progesterone-receptor complex influences the replenishment of the cytoplasmic oestrogen receptor by interfering with its resynthesis or recycling, thereby reducing the quantity of oestrogen receptors (Hsueh *et al.*, 1975). Batra and Iosif (1989) suggested that progesterone induces a turnover of oestrogen receptors in the uterus, and not in the vagina and urethra of the rabbit, because of the organs high sensitivity to progesterone. Also it is possible that a minimum number of progesterone receptors is required to induce down regulation of oestrogen receptors.

The results in this thesis would suggest that rat detrusor muscle fulfils either one or both of these criteria. This is supported further by the lack of effect of progesterone and oestrogen treatment on the weight of rat bladders. Oestrogen treatment alone significantly increased the mass of rabbit and rat bladders (Shapiro 1986, Elliott *et al.,* 1992b) possibly due to mucosal hyperplasia or an increase in smooth muscle mass (Batra & Iosif 1983).

Oestrogen and progesterone pre-treatment also had no effect on the cholinergic component of nerve stimulation in rat detrusor muscle strips. The contractile response to EFS was approximately 38% cholinergic and 62% purinergic. Oestrogen pre-treatment has been shown to increase significantly the purinergic component and decrease the cholinergic response to nerve stimulation (Elliott *et al.,* 1992). In the present study this effect was abolished by the addition of progesterone to the treatment regimen. Atropine sensitivity has also been found to increase in rat detrusor muscle from ovariectomised animals, suggesting that a lack of oestrogen increases cholinergic responsiveness (Eika *et al.,* 1988).

These results clearly demonstrate that female hormones can cause a substantial shift of the contractile response to field stimulation from cholinergic to purinergic, although the contractile response to ATP has been shown to be incapable of substantially emptying the bladder (Levin *et al.,* 1983).

Pregnancy in animals also decreases the contractile response of detrusor muscle to cholinergic stimulation and significantly increases the response to ATP

(Levin *et al.*, 1991, Tong *et al.*, 1995). Tong *et al* (1995) examined the effect of pregnancy and daily intramuscular injections of progesterone in non pregnant animals on the contractile response and muscarinic receptor density in rat urinary bladders. The maximum contractile response to ACh was significantly reduced in the pregnant rats and in those treated with progesterone, as were muscarinic receptor densities. The response to ATP was found to be increased in pregnant rats.

These results oppose previous findings where oestrogen administration, not progesterone, inhibited the contractile response of rat detrusor muscle to ACh and decreased muscarinic receptor density (Ekstrom *et al.*, 1993, Elliott *et al.*, 1992b, Batra & Andersson 1989, Shapiro 1986). It is interesting to note that Tong *et al* (1995) found the wet weight of empty bladders to be greater in the pregnant group than in any of the others, including the progesterone treated group. Oestrogen, but not progesterone, is known to cause mucosal hyperplasia and has been shown to increase the weight of urinary bladders in previous studies (Ekstrom *et al.*, 1993, Batra & Andersson 1989, Shapiro 1986). Unfortunately Tong *et al* (1995) did not estimate oestrogen levels in their pregnant rabbits and could therefore not exclude the influence of oestrogen on their results

It is clear that modulation of oestrogen and progesterone can significantly alter bladder autonomic receptor density and response to stimulation. However, some reports in the literature have conflicting opinions regarding the effect of female hormones on detrusor muscle. Levin *et al* (1980, 1981) reported acute oestradiol administration to sexually immature rabbits induced an increase in muscarinic and α-

adrenergic receptor density in the bladder body and increased the response to muscarinic and α-adrenergic stimulation. Shapiro (1986) found that chronic oestradiol treatment for three weeks decreased muscarinic receptors in the rabbit bladder body. Levin (1980) treated immature rabbits with oestradiol for only four days whereas Shapiro (1986) treated mature ovariectomised rabbits for three weeks. The difference in duration of treatment and maturity of rabbits may account for these conflicting results.

Treatment with progesterone alone was not investigated in the present study as it is unlikely to be given without oestrogen in clinical practice, except for certain gynaecological abnormalities or in women unable to take oestrogen-containing birth control pills. However, a study by Ekström *et al* (1993) demonstrated that progesterone treatment alone in castrated rabbits, for four to six months, increased the sensitivity of bladder muscle strips to parasympathomimetics and increased the maximal nerve-induced contraction of the bladder. In contrast oestrogen treatment shifted the frequency response curve of the bladder to the right. Progesterone treatment alone therefore opposes the effect of oestrogen on detrusor muscle contractile response, this could have been further clarified by the inclusion of a progesterone and oestradiol treated group in the Ekström *et al* (1993) study.

Tetrodotoxin (TTX) selectively inhibits sodium channels which mediate action potentials in the intramural nerves, which in turn are responsible for the depolarisation of the synapse and stimulation of the release of neurotransmitters (Gershon 1967, Nakashima *et al.*, 1990). Tetrodotoxin therefore abolishes the response to nerve

stimulation (Brading & Williams 1990). It is used as an experimental tool to establish the neurogenic origin of electrical field stimulation (EFS). The TTX resistant component of EFS is due to direct muscle stimulation and the magnitude of this component is related to the duration of the electrical impulse. The higher the pulse width the more direct muscle stimulation. In this section of the thesis a pulse width of 1.0 msec was utilised resulting in a 50% block of the maximum response to EFS by TTX and the remaining 50% of the contractile response being due to direct muscle stimulation. At lower frequencies (10 Hz) 27% of the response was due to direct stimulation of the muscle. Oestrogen treatment alone has been shown to increase significantly the sensitivity of rat detrusor muscle to TTX, thereby increasing the inhibition of sodium channels and reducing direct muscle stimulation (Elliott *et al.*, 1992b). However, treatment with oestrogen and progesterone had no significant effect on the sensitivity to TTX, being similar to control response plus TTX. This observation is another example of the ability of progesterone to alter an effect produced by oestrogen treatment alone. The mechanism by which oestrogen treatment increases the sensitivity of detrusor muscle to TTX, and progesterone plus oestrogen treatment restores the sensitivity to near control levels, is unclear. This phenomenon requires further investigation.

Overall the effect of pre-treatment with oestradiol and progesterone on rat detrusor contractile responses was found to oppose the action of oestradiol treatment alone. On the contrary, the effect of direct administration of progesterone to the organ bath had an inhibitory effect on rat detrusor contractile response, similar but less potent to bath applied DES. There is nothing in the literature regarding the direct

effect of progesterone on isolated detrusor muscle contraction. However, a study by Batra & Bengtsson (1978) found that both oestrogens and progesterone were able to decrease calcium entry into uterine cells which probably accounted for their inhibitory effect on uterine smooth muscle contractility. In isolated rat detrusor muscle low concentrations of progesterone, 0.2μM, had no effect on the contractile response whereas higher concentrations, 2μM and 20μM, had a significant inhibitory effect which was not dose-dependent. Diethylstilboestrol (DES) had a greater inhibitory effect on contractile response, with 20μM almost halving the magnitude of response to EFS. The addition of both progesterone and DES to the organ bath, each at 2μM, resulted in a greater inhibitory effect than either alone, suggesting no antagonistic action between these hormones *in vitro*. The inhibitory action of bath applied progesterone was possibly due to the inhibition of calcium influx, as demonstrated by a reduction in the response to KCl. The contractile response to KCl is entirely sustained by the influx of calcium via voltage-sensitive calcium channels (Maggi *et al.*, 1989). A reduction of this response therefore suggests an inhibitory effect on calcium uptake.

The direct inhibitory action of progesterone and DES differed by their effect on the cholinergic and non cholinergic component of the response to EFS. After the addition of atropine to the bath, thus blocking the cholinergic response, the further addition of progesterone had no additional effect on contractile response. Whereas the addition of DES, in the presence of atropine, inhibited the cholinergic resistant response to EFS. Progesterone therefore only acts on the cholinergic response to EFS whereas DES also had an inhibitory effect on the purinergic component.

The reason for the contradictory effect of *in vitro* and *in vivo* treatment with progesterone on rat detrusor contractile response is unclear. It is possible that after *in vivo* treatment with progesterone the concentration of the hormone in detrusor smooth muscle cells is too low to have a direct effect on contractile response but is high enough to affect oestrogen receptor turnover.

Overall, the results from this and previous studies indicate that female hormones may have a role to play in the treatment of detrusor instability. Treatment with oestrogens alone is preferable as progesterone has been shown in this study to alter the functional effect of oestrogen on rat detrusor contractile response and may antagonise the beneficial effects of oestrogen on urinary symptoms. Furthermore it has been suggested that progestogens could play a role in the causation of some lower urinary tract symptoms (Benness *et al.*, 1991). In some women urinary incontinence was exacerbated during the progestogen phase of hormone replacement therapy, possibly due to an effect on urethral function (Benness *et al.*, 1991). Fluctuations in progesterone and oestradiol levels during the normal menstrual cycle were found to have no effect on urodynamic parameters (Sorensen *et al.*, 1988). The levels of these hormones might not be high enough or lack adequate exposure time to significantly effect urodynamic parameters. The lack of effect could also be caused by opposite hormonal actions on the different tissues of the urethra and bladder (Sorensen *et al.*, 1988).

Clinically, oestrogen replacement has been shown to alleviate urgency, urge incontinence, frequency, nocturia and dysuria. In the few properly placebo-controlled studies of oestrogen therapy in the management of urinary incontinence there is no conclusive evidence that oestrogen improves stress incontinence (Judge 1969, Walter *et al.,* 1978, Samsioe *et al.,* 1985, Wilson *et al.,* 1987). Wilson *et al.,* (1987) treated 36 women, with genuine stress incontinence, with cyclical oral oestrogen for three months. Although there was symptomatic improvement there was no significant difference in subjective response, urethral pressure profiles, or Urilos test (test of urine loss). Samsioe *et al* (1985) treated 34 women aged 75 years with 3mg oral oestriol and found that this was more effective than placebo in improving women with urge incontinence and mixed incontinence. There was no difference between oestriol and placebo in those women with stress incontinence.

The clinical role of oestrogens in the management of postmenopausal urinary incontinence requires clarification. On the basis of results from this thesis, and previous studies, clinical trials examining the effect of female hormones on urinary incontinence will be incorporated into an MRC funded survey. This survey will investigate the prevalence of urinary incontinence in Leicestershire. The possible existence and or nature of the anti-oestrogenic effect of progesterone on animal and human detrusor muscle function requires further investigation. This should include investigation of the sensitivity and density of progesterone receptors in detrusor muscle plus the levels required to have a possible anti-oestrogenic effect on the urinary bladder. The results of such experiments would help to adjust hormone

treatment levels, particularly progesterone, when used to treat urinary incontinence and particularly detrusor instability.

6.1.1 Conclusions.

Oestrogen treatment alone for detrusor instability may be effective in reducing uninhibited detrusor contractions. The addition of a progestogen to the treatment regimen, which would be required in patients with a uterus, may diminish the effectiveness of oestrogen in reducing detrusor contractile response.

6.2 Calcium antagonists.

Multiple sources of calcium are mobilised for human detrusor muscle contraction induced by different stimulants, but the sole source of calcium for contraction induced by KCl is entry of extracellular calcium through dihydropyridine and voltage-sensitive calcium channels (Maggi *et al.*, 1989). In the guinea-pig, and probably in other small mammals, acetylcholine stimulates muscarinic receptors on the surface of smooth muscle cells which are linked by a guanine-nucleotide binding protein to the enzyme phospholipase C (PLC). Stimulation of the receptor activates the enzyme which catalyses the hydrolysis of phosphatidylinositol 4,5 bisphosphate (PIP_2) resulting in the release of inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol, which are both second messengers. IP_3 diffuses into the cell and releases calcium from a store within the sarcoplasmic reticulum resulting in contraction and the influx of extra cellular calcium (Iacovou *et al.*, 1990). In contrast, ATP, which is the co-neurotransmitter mediating detrusor contraction in mammals, did not induce an accumulation of inositol phosphates (Iacovou *et al.*, 1990). It was postulated by Iacovou *et al.*, (1990) that ATP stimulates the P_{2x} receptor which opens ion channels resulting in an influx of extracellular calcium ions. The response to ATP is therefore more dependent on extracellular calcium than the response to carbachol (Iacovou *et al.*, 1990). However, calcium channel blockers, *in vitro*, have been shown to inhibit both the non-cholinergic (ATP) and cholinergic components (Bhat *et al.*, 1989) of the neurogenic response in the rat urinary bladder. There is also further evidence that these drugs have a significant inhibitory effect on both human and animal detrusor muscle contractile response *in vitro* (Forman *et al.*, 1978; Hassouna *et al.*, 1986; Bo

& Burnstock 1990; Fovaeus *et al.,* 1987; Zar *et al.,* 1990; Scultety 1991; Elliott *et al.,* 1992).

In this thesis the effect of nifedipine, nimodipine and verapamil on the contractile response of isolated human detrusor muscle was investigated to confirm the direct actions of nifedipine and verapamil, reported previously (Fovaeus *et al.,* 1987, Elliott *et al.,* 1992), and to establish the direct effect of nimodipine. Time response curves determined the incubation time for a steady state effect of each calcium antagonists investigated, to be 15 minutes. At the concentrations employed, which were equivalent to those achieved in human plasma after chronic oral administration (Drug Information Service, Leicester Royal Infirmary), nimodipine had the greatest inhibitory action on isolated human detrusor contractile response to carbachol, and verapamil the least. The present *in vitro* experiments therefore established for the first time that human detrusor muscle was sensitive to nimodipine.

The majority of *in vivo* animal studies investigating the effects of calcium antagonists on bladder contraction have been performed after intravenous administration or after a single high oral dose. Sjogren & Andersson (1979) examined the effects of verapamil and nifedipine *in vitro* and *in vivo* on bladder contractions in the guinea-pig. Verapamil showed weak inhibitory effects *in vitro* and no consistent effect *in vivo.* Intravenous administration of calcium antagonists such as nifedipine, nicardipine and verapamil, plus oral doses of nicardipine and verapamil all reduced rat bladder contraction to topically applied K^+ in a dose-dependent manner (Angelico *et al.,* 1992). Verapamil only produced inhibition at toxic doses. A single intravenous

dose of nitrendipine 15mg Kg^{-1} has been shown to reduce the amplitude of rat detrusor contraction by 50% and higher doses by 90% but two animals died as a result of a sudden fall in blood pressure (Dederichs *et al.,* 1992).

Marti-Cabrera *et al* (1994) examined the effect of calcium antagonists from different sub-groups (dihydropyridine (nifedipine), phenylalkamines (verapamil), benzothiazipines (diltiazem) and others (cinnarizine) on the contractile response of normal and skinned rat urinary bladder. Skinning rat detrusor smooth muscle of its plasmalemma is used to detect a direct action of calcium antagonists on the sensitivity of intracellular contractile mechanisms to calcium (Meisheri *et al.,* 1985). Cinnarizine (100μM) and trifluoperazine (100μM) but not nifedipine, verapamil and diltiazem (all at 100μM) depressed Ca^{2+} (20mM)-evoked contractions of skinned bladder. However, all five of these calcium antagonists produced a concentration-dependent inhibition of responses to calcium chloride, potassium chloride and acetylcholine in normal bladder muscle. It was concluded that the action of nifedipine, verapamil and diltiazem is restricted to the plasmalemma whereas cinnarazine and trifluoperazine act on the intracellular contractile apparatus (Marti-Cabrera *et al.,* 1994).

The evidence to date suggests that acute treatment *in vitro* and *in vivo* with calcium anatgonists has a significant inhibitory effect on detrusor contractile response; however this is not evident in humans treated chronically with these drugs (Levin *et al.,* 1994). In view of findings on acute treatment in animals, the poor clinical response of patients with detrusor instability to calcium channel blockers is surprising. However, daily oral dosage is the normal drug regime in man, and there is little information in the literature regarding such administration on the contractile activity of

detrusor muscle in animals or in man. A single oral dose of 20-40mg nifedipine in 19 patients with urgency and/or urge incontinence did not affect the pressures within the bladder but there was a significant increase in residual urine (Forman *et al.*, 1978). In one study, nifedipine 10-20mg orally reduced the amplitude and frequency of uninhibited detrusor contractions in women with urge incontinence (Rud *et al.*, 1979) but not in another (Laval & Lutzeyer 1980). Multiple oral doses of flunarizine produced no urodynamic improvement in women with idiopathic detrusor instability although their symptoms regressed significantly (Palmer *et al.*, 1981). Contrary to these results treatment with diltiazem for ten days in patients with hyperreflexic detrusor instability has been shown to increase bladder capacity and lower bladder pressure and maximum detrusor pressure significantly (Faustini *et al.*, 1989). This class of detrusor instability has a neurological aetiology unlike idiopathic detrusor instability which is of major interest in this thesis. Diltiazem was not examined in the present study as the lack of efficacy of calcium antagonists used to treat idiopathic detrusor instability was under investigation. However, it would be of interest to determine the effect of diltiazem on the contractile response of isolated detrusor muscle in future experiments.

To investigate the reason why chronic administration of calcium antagonists has little effect on bladder function in man, the effects of chronic oral administration of nimodipine on rat detrusor muscle was evaluated. Rats were treated with either a single dose or a chronic 8 day treatment before bladders were removed for *in vitro* contractile response studies. The present results demonstrated that *in vitro* treatment and *in vivo* treatment with a single dose of nimodipine reduced significantly the contractile response of rat detrusor muscle to EFS, carbachol and K^+. However, *in vivo* treatment with nimodipine for 8 days had no significant effect on rat detrusor contractile response. It is unlikely that 8 days treatment with nimodipine was ineffective because of inadequate absorption, distribution or tissue concentrations in the detrusor muscle because a single dose significantly reduced the contractile

response. Also the serum nimodipine concentrations were within a therapeutic range, being similar to serum concentrations obtained in man (Drug Information Service, Leicester Royal Infirmary). The concentration of nimodipine was found to be significantly higher in the serum of rats treated for 8 days, and therefore the lack of effect on detrusor contractile response in this group was not due to lower serum drug levels, nor to enhanced metabolic breakdown of the drug with repeated dosage.

It is unlikely that nimodipine was washed out of the samples in this study as this would have occurred also after a single oral dose, and the relative stability of inhibition of contractile response in detrusor muscle by nimodipine despite multiple washings has been demonstrated also in this study. Desensitisation was not occurring as nimodipine still had an inhibitory effect on the contractile response of detrusor muscle in rats pre-treated for 8 days when added to the bath chamber, demonstrating the presence of functional calcium channels after the treatment period. Although not reaching statistical significance it is interesting to note that the maximum contractile response to carbachol, EFS and K^+ after 8 days treatment with nimodipine was increased compared to controls, possibly suggesting an increase in the number of calcium channels. This applies particularly to the K^+ response which is solely dependent on the influx of calcium through voltage-sensitive calcium channels. It is therefore possible that repeated administration of nimodipine caused an increase in the number of functional calcium channels to overcome the inhibition of a proportion of the channel population by nimodipine.

Calcium channels are regulated by homologous, heterologous and pathological factors (Ferrante & Triggle 1990). Prolonged or persistent receptor stimulation can cause changes in receptor metabolism but receptor regulation occurring within a short time has different mechanisms (Ferrante & Triggle 1990). These mechanisms include phosphorylation state, receptor distribution, modifications in coupling factors, membrane potential and the membrane lipid environment (Ferrante

& Triggle 1990). Therefore the mechanisms involved in receptor regulation are varied and dependent on the ligand (agonist, antagonist), the receptor and the extent and duration of receptor occupancy (Ferrante & Triggle 1990).

In the human, the density of dihydropyridine binding sites (0.27fmol/mg wet weight) in four pooled bladder strips was found to be small (Shapiro *et al.*, 1991). Regional differences in dihydropyridine binding sites were found in the lower urinary tract of the rabbit with the density of binding sites being higher in the urethra (64.1 ±7.8 fmol/mg protein) than in the bladder dome (21.9 ±3.0 fmol/mg protein) or bladder base (18.8 ± 4.2 fmol/mg protein) (Latifpour *et al.*, 1992).

Chronic exposure of a clonal PC12 cell line to nifedipine for 5 days produced a 29% increase in high affinity 1,4-dihydropyridine binding sites. In contrast, incubation with Bay K8644 reduced 1,4-dihydropyridine binding site density by 24% (Skattebol *et al.*, 1989). These results demonstrate that repeated exposure at a cellular level can influence the number of dihydropyridine binding sites. Up-regulation of dihydropyridine binding sites has also been observed in cardiac membranes from spontaneously hypertensive rats treated with nitrendipine and a high salt diet for 21 days (Garthoff & Bellemann 1987). It is likely therefore that chronic oral administration of nimodipine caused an up-regulation of dihydropyridine-sensitive calcium channels, as a compensatory mechanism.

It was not possible to confirm the hypothesis of up-regulation of calcium channels by ligand binding studies in the present study. The density of calcium channels in a single rat bladder is very low, making it impractical to detect small changes in receptor numbers. Previous studies on bladder smooth muscle have used pooled tissue samples to achieve an adequate density of sites in binding experiments which was not part of the design of this study (Shapiro *et al.*, 1991).

The conclusion of an up-regulation of calcium channels is an important novel hypothesis. There are no reports of similar experiments in the literature. Previous investigation of *in vivo* treatment with drugs has been carried out in anaesthetised animals (Hassouna *et al.*, 1986, Diederichs *et al.*, 1992). It has been demonstrated herein that the inhibitory effect of a calcium antagonist on detrusor muscle is still demonstrable after the bladder has been removed from the animal. The methods used in this thesis can therefore negate the use of live animals for some future experiments.

If the lack of effect of chronic treatment with nimodipine on rat detrusor muscle contractility applies to human detrusor muscle, it may explain why the treatment of patients with urinary incontinence with calcium antagonists is disappointing (Levin *et al.*, 1994). It is possible that calcium antagonists could still be effective when administered in an intermittent manner. Since the intensity of symptoms in urinary incontinence is variable, this may be an appropriate regime for the treatment of some patients.

It would be important to confirm the hypothesis of calcium channel regulation by chronic dosing. Future experiments using radioligands to determine the density of calcium channels in pooled samples of bladders from rats treated with nimodipine for 8 days and a single dose would be desirable. Further clinical investigations comparing the effect of intermittent treatment with calcium antagonists and chronic dosing on urodynamic parameters in patients with detrusor instability would also be most useful.

6.2.1 Conclusion

In conclusion, if the results of this study are applicable to the response of human detrusor muscle to chronic oral treatment with calcium antagonists, it may explain the lack of effect of these drugs when used to treat detrusor instability. An

intermittent treatment regimen may be more effective in inhibiting unstable contractions.

6. 3 Caffeine

Caffeine is consumed by most people during the ingestion of tea, coffee and other drinks. Symptoms of urgency and frequency which soon follow are possibly due to its diuretic effect. However early evidence suggested that caffeine could have a direct effect on the smooth muscle of the lower urinary tract (Ransom 1911).

Urinary bladder smooth muscle contraction is mediated by an increase in the concentration of cytoplasmic free calcium. This is achieved through both an influx of extracellular calcium and the stimulated release of calcium from intracellular stores (Iacovou *et al.,* 1990). Caffeine has been shown to contract various smooth muscles by mobilising an intracellular calcium pool from the sarcoplasmic reticulum (Itoh *et al.,* 1982; 1983, Leijten & VanBreemen 1984). More recently Ganitkevich and Isenberg (1992), investigating calcium movement in myocytes from guinea-pig urinary bladder, concluded that depolarisation-induced influx of calcium through L-type calcium channels induces the release of calcium from intracellular caffeine-sensitive stores. Maggi *et al* (1989) investigated the different sources of calcium for contraction of human bladder muscle. Caffeine (2.5-20mM) was found to induce a procaine-sensitive (procaine inhibits calcium release from internal stores) but nifedipine resistant contraction only at low bath temperatures 25°C while at 37°C a relaxant effect was observed (Maggi *et al .,* 1989). It was postulated that at 25°C the relaxant action was slowed or inhibited unmasking a contractile effect (Maggi *et al.,* 1989). It has been suggested that the transient elevation of intracellular calcium production by caffeine is enhanced at low temperature (Karaki *et al.,* 1987). This possibility has been confirmed recently (Nagai *et al.,* 1992). The relaxant effect of caffeine on smooth muscle contraction via the inhibition of phosphodiesterase has also been noted (Leijten & Van Breeman 1984).

Caffeine therefore has differing pharmacological actions on different smooth muscles depending not only on the concentrations of caffeine used but also on the experimental conditions (Sandow & Brust 1966, Ito & Kuriyama 1971, Ito *et al.*, 1973, Bolton 1979, Huddart *et al.*, 1983, Palermo & Zimskind 1977, Burdyga & Magura 1986, Savineau & Mironneau 1990). Low doses of caffeine induced a transient contraction in smooth muscle (Sandow & Brust 1966, Nagi *et al.*, 1992, Nasu & Urakawa 1974) and caused a significant increase in the rate of pressure generation in whole rabbit bladder preparations *in vitro* in response to 32 Hz field stimulation (Lee *et al.*, 1993). Concentrations of caffeine, higher than used in this thesis, were found to inhibit the K^+-induced tonic contractures of rat bladder muscle strips. This effect was due to a reduction in calcium influx (Huddart *et al.*, 1983). Different concentrations of caffeine and aminophylline have also been found to decrease urethral closure pressure in dogs (Palermo & Zimskind 1977).

There are few studies investigating the effect of caffeine on isolated detrusor muscle contraction. However, the effect of caffeine on the contractile response of isolated whole rabbit urinary bladder has been examined (Lee *et al.*, 1993). Low concentrations of caffeine were found to cause a significant increase in the rate of pressure generation due to an increased rate of release of intracellular calcium. This effect was dependent upon the concentration of caffeine used and the concentration of extracellular calcium in the bathing medium. The concentration of caffeine being inversely proportional to the extracellular calcium concentration. Lee *et al* (1993) concluded that caffeine primarily affects the phasic component of the contractile response to field stimulation and not the tonic component which is responsible for emptying the bladder.

These studies suggest a possible direct effect of caffeine on bladder smooth muscle contractile response. Clinical evidence of such an effect has been demonstrated by Creighton & Stanton (1990). They found that after the

administration of 200mg caffeine to patients with detrusor instability, there was a significant increase in the detrusor pressure rise on bladder filling compared with no caffeine administration.

The results in this thesis demonstrated that caffeine, at the concentration studied, slightly increased the maximum contractile response of human isolated detrusor muscle to acetylcholine, but had no significant effect on rat detrusor contractile response. The dose response curves to carbachol were not affected by caffeine in both human and rat detrusor muscle samples. Overall the effects of caffeine in both human and rat detrusor muscle were small and unlikely to be of clinical significance. The application of a higher concentration of caffeine (1mM) to the organ bath resulted in a small inhibitory effect on the contractile response to acetylcholine compared to control. This difference was not significant.

The slight potentiating effect of caffeine on detrusor muscle contractile response was abolished by the addition of ryanodine to the organ bath and by the reduction of extracellular calcium. Ryanodine prevents the release of calcium from internal stores (Iino *et al.*, 1988) and the contractile response to acetylcholine decreases after repeated stimulation in low calcium medium as intracellular calcium stores are not replenished. The small enhancement of the response of detrusor muscle to acetylcholine was abolished under these conditions. These results suggest that caffeine slightly increased the response to acetylcholine by increasing the release of calcium from internal stores.

The human samples in this study were from patients with benign prostatic hypertrophy and carcinoma of the bladder and could therefore be regarded as abnormal. This may explain the small enhancing effect of caffeine on acetylcholine-evoked response in human detrusor muscle strips compared to rat detrusor samples which were normal and homogeneous. It would be interesting for future research to

investigate whether this is a species difference or possibly why abnormal detrusor muscle appears to be more susceptible to the action of caffeine than normal muscle. It is possible abnormal detrusor muscle may have irregular caffeine-sensitive intracellular calcium release.

6.3.1 Conclusions

In conclusion low concentrations of caffeine had a very small enhancing effect on the contractile response of rat and human detrusor muscle. This effect may be more pronounced in abnormal detrusor muscle, but overall the effect of caffeine was small and may not be clinically significant. On the basis of these results it would not be necessary to advise patients with urinary incontinence to avoid caffeine containing beverages. However, the diuretic effect of caffeine must still be considered.

CHAPTER 7: CONCLUSIONS AND FUTURE EXPERIMENTATION

Many drugs used for the treatment of detrusor instability have not undergone appropriate investigation before clinical use. This situation was highlighted recently by Turner and Brading (1995). Female mini-pigs with partial urethral obstruction were subjected to intravenous administration of drugs, some of which are currently in use for the treatment of detrusor instability. Their results were consistent with clinical impressions that no drug used so far abolishes unstable contractions consistently (Turner & Brading 1995). They suggested that this type of investigation should be standard practice by those developing drugs for the treatment of detrusor instability.

In principle the investigation of drugs prior to clinical administration is desirable. However, in the mini-pig model detrusor instability is secondary to outlet obstruction and would therefore not be a suitable model for idiopathic detrusor instability, the mechanism of which is poorly understood. At present there are no animal models for idiopathic detrusor instability. Preliminary investigation of drugs for the treatment of this condition is dependent upon isolated human bladder biopsy samples and normal animals for *in vitro* and *in vivo* treatment. These experiments, although not an ideal model for preclinical evaluation, are preferable to the clinical use of drugs without laboratory investigation.

Clinical trials for the treatment of detrusor instability in post-menopausal women with oestrogen, will soon be in progress. These trials are a progression from the results obtained in this thesis and previous studies from this laboratory plus other workers. Progesterone will need to be included in future clinical trials because of its anti-oestrogenic action. Before these trials it would be useful to investigate further the mechanism of interaction between oestrogen and progesterone in detrusor smooth muscle, including the effects of different concentrations of hormones.

The inhibition of calcium entry through voltage-sensitive calcium channels is the mechanism of action for *in vitro* application of female hormones in isolated detrusor muscle. This mechanism is thought to inhibit detrusor smooth muscle contraction by the reduction of intracellular free calcium, a rise in which initiates smooth muscle contraction with tone being sustained by the influx of extracellular calcium. On the basis of this mechanism of contraction calcium channel blockers were thought to have a possible role in the treatment of detrusor instability. Unfortunately some preliminary investigations with these drugs in animals did not use drug concentrations applicable to human usage nor was the method of drug administration suitable. Single doses were employed which were mainly administered intravenously not orally. If preliminary animal experiments are to be carried out before clinical trials the concentration of the drug and method of administration must be similar to regimens used in humans. As demonstrated in this thesis the chronic and acute administration of nimodipine had opposing effects on isolated rat detrusor contraction. Clinical trials of this drug should therefore include intermittent as well as a chronic and acute drug regimes.

Apart from the use of drugs which inhibit detrusor contraction it is important to know which drugs enhance detrusor contractile response. It will then be possible to advise patients with urinary incontinence on the avoidance of such agents. Caffeine is an example of such a drug. Although it appears to have little *in vitro* effect on detrusor contractile response it would be of interest to examine its effect after *in vivo* treatment. This would determine if *in vivo* administration results in a greater or lesser effect than *in vitro* application. It is also possible that caffeine could affect abnormal detrusor muscle only. The effect of *in vitro* treatment with caffeine on abnormal human detrusor biopsy samples taken from patients with confirmed detrusor instability would produce valuable information.

The animal and *in vitro* human detrusor muscle experiments carried out for this thesis provide an insight into the mechanisms of action of the drugs studied and determine their possible role in the treatment of detrusor instability. Bearing in mind the possible species difference that can affect results, it is preferable to examine drugs in this way before stepping directly into clinical treatment without having the knowledge of the mechanism of action of such agents on detrusor muscle contraction.

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Effect of treatment with calcium antagonists *in vitro* and *in vivo* on the contractile response of isolated rat and human detrusor muscle

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1. The effect of calcium antagonists on the contractile response of human and rat isolated detrusor muscle *in vitro* was investigated. The effect of treatment with nimodipine on rat detrusor muscle *in vivo* was also examined.

2. Nimodipine 0.1 μmol/l, nifedipine 0.1 μmol/l, nifedipine 0.25 μmol/l and verapamil 1.5 μmol/l reduced the maximum contractile response of isolated human detrusor muscle to carbachol by 42%, 35%, 41% and 28% respectively ($P<0.01$). Verapamil 0.1 μmol/l had no significant effect on contractile response.

3. Nimodipine 0.1 μmol/l reduced the maximum contractile response of isolated rat detrusor muscle *in vitro* to electrical field stimulation and carbachol by 53% and 84% respectively ($P<0.01$).

4. Rats were pretreated with nimodipine for 8 days (5 mg day^{-1} kg^{-1}) or with a single dose. Serum nimodipine concentrations were higher in rats treated for 8 days. In rats treated with nimodipine for 8 days there was no significant difference in detrusor contractile response compared with controls. However, after one dose of nimodipine the maximum contractile response was significantly reduced compared with controls ($P<0.05$).

5. At the concentrations studied, nimodipine had a greater inhibitory effect on the contractile response of isolated human detrusor muscle. Nimodipine significantly reduced the contractile response of rat detrusor muscle *in vitro* and after a single dose *in vivo*, but had no significant effect after 8 days' treatment *in vivo*. It is possible that chronic oral treatment with nimodipine caused an up-regulation of 1,4-dihydropyridine-sensitive calcium channels, which may explain the lack of clinical effect of chronic treatment with calcium antagonists in patients with detrusor instability.

INTRODUCTION

The influx of Ca^{2+} through potential-operated and receptor-operated Ca^{2+} channels is an important trigger for smooth muscle contraction, and there is ample evidence from studies of isolated detrusor muscle preparations *in vitro* that calcium antagonists have a significant inhibitory effect on the contractile response of urinary bladder muscle [1–3]. Dihydropyridine receptors at relatively low density are found in human detrusor muscle [4]. Animal experiments *in vivo* also suggest that calcium antagonists reduce the contractility of detrusor muscle. A single intravenous dose of nitrendipine reduced spontaneous and nerve-induced contraction of the bladder [5], and a similar administration of nifedipine, nicardipine and verapamil inhibited K^+-induced contractions of the rat urinary bladder [6]. A single oral dose of nicardipine, 60 min after administration, reduced the contractile response of rat bladder, whereas verapamil under the same conditions only had such effects at toxic doses.

The majority of investigations to date have therefore been carried out *in vitro*, or have involved single doses in animals *in vivo*. These methods bear little relation to chronic oral drug administration in humans, and do not explain the disappointing clinical experience with calcium antagonists in detrusor instability [7]. Nevertheless, calcium antagonists have not undergone sufficiently rigorous scientific investigation before being introduced and rejected in clinical practice.

The results of clinical studies on the effects of single oral doses of calcium antagonists on the unstable bladder are conflicting. Nifedipine (10–20 mg) has been shown to reduce the amplitude and frequency of uninhibited detrusor contractions in women with urge incontinence [8, 9]. In contrast, similar oral doses of nifedipine had no significant effect on such bladder contractions in 30 patients [10]. A clinical study using chronic oral drug administration could not demonstrate significant urodynamic improvement in women with proven

Key words: calcium antagonist, human, rat, urinary bladder.

Abbreviations: BPH, benign prostatic hyperplasia; EFS, electrical field stimulation.

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idiopathic detrusor instability after treatment with flunarizine for 1 week, although their symptoms improved significantly [11]. The effects of such drugs are different in different tissues [12], and compounds of the same class are also known to have different actions [13]. Furthermore, chronic dosing could cause tachyphylaxis, as has been reported with verapamil [14]. This would result in a diminished response of the tissues to repetitive exposure to the same concentration of the drug.

The purpose of this study was to investigate whether the response of detrusor muscle to calcium antagonists is modified after chronic oral treatment compared with treatment with a single dose.

To establish the sensitivity of human detrusor muscle to calcium channel antagonists, the effect of three calcium blockers *in vitro* on the contractile response of human detrusor muscle was examined. At the concentrations used, the drug producing the most inhibition was used for treatment of rats *in vitro* and *in vivo*. Treatment was administered *in vivo* either for 8 days or as a single dose to determine any difference in effect produced by chronic and acute dosing.

METHODS

In vitro treatment

Male Wistar rats (300–400 g) were killed by a blow to the head followed by dislocation of the neck. Bladders were removed and placed in Krebs solution. Human detrusor samples were taken using cold cup biopsy from the bladder dome of male and female patients undergoing routine cystoscopic procedures. Tissues were dissected free of fat and serosa and strips of muscle (4 mm × 1 mm) were suspended in a 50-ml organ bath chamber. The chamber contained Krebs solution at 37°C aerated with 95% oxygen and 5% carbon dioxide. The base of the muscle strip was attached to a fixed hook in the chamber and passed through two circular parallel electrodes. The apex was attached by a silk suture to an isometric transducer connected to a two-channel Washington oscillograph. The electrodes were connected to a Digitimer stimulator capable of delivering a frequency of 1–80 Hz, voltage 10 V, pulse width 0.5 ms in 10-s trains at 2-min intervals. The tissues were allowed to equilibrate for 1 h under a tension of 10 mN.

Calcium antagonist time–response curves

Time–response curves to calcium antagonists used in this study were obtained to establish the optimum incubation time for each drug.

Rat detrusor strips were stimulated at 2-min intervals at a frequency of 40 Hz until consistent responses were obtained; this was taken as the control response. Nifedipine 0.25 μmol/l, verapamil 1.5 μmol/l or nimodipine 0.1 μmol/l was added to the bath, and at intervals of 2 min the samples were restimulated until no further decrease in response was obtained. Responses were presented as an increase in tension and plotted against time in minutes.

Rat bladder strips

After equilibration, the preparations were stimulated at frequencies of 1, 5, 10, 20, 40, 60 or 80 Hz at 2-min intervals to obtain a frequency–response curve. When consistent responses had been achieved, the tissues were washed and re-equilibrated. Nimodipine was added to the bath at a concentration of 0.1 μmol/l, and after 15 min incubation, at which time the maximum effect was observed as estimated from time–response curves, a second frequency–response curve was obtained. The tissues were washed and stimulated again after 30, 45 and 60 min to establish whether nimodipine could be washed out of the samples. Responses were presented as a percentage of the maximum control response.

Under the conditions used for electrical field stimulation (EFS), the contractile response of rat detrusor muscle was abolished by 1.6 mol/l tetrodotoxin, indicating its neurogenic origin.

Dose–response curves were obtained by the addition of increasing concentrations of carbachol (0.01–100 mol/l). The detrusor samples were exposed to 10-s applications of the agonist and then washed. After 3 min they were restimulated. The dose–response curve was repeated 15 min after the addition of 0.1 μmol/l nimodipine to the bath chamber. It was not necessary to reapply nimodipine after each application of carbachol because of the stability of nimodipine in the tissue samples. Results obtained after reapplication of nimodipine were the same (not shown).

Human bladder strips

Human bladder samples were obtained by cold cup biopsy from men (who had consented to cystoscopy) with benign prostatic hyperplasia (BPH) with acute or chronic retention and from women with frequency. Strips of detrusor muscle were prepared and mounted in the organ bath as described. After equilibration, the samples were stimulated with increasing concentrations of carbachol (0.0–100 mol/l) before and after the addition of either nimodipine (0.1 μmol/l), nifedipine (0.1 μmol/l and 0.25 μmol/l) or verapamil (0.1 μmol/l and 1.5 μmol/l). These concentrations were chosen because they are likely to be similar to plasma levels achieved after chronic oral administration of these drugs in humans (Drug Information Service, Leicester Royal Infirmary). The free, unbound fraction of the drug is

considered to be pharmacologically active, but the concentration of nimodipine in the serum also takes into account the bound fraction.

The response to carbachol was investigated because human detrusor contraction is principally mediated via cholinergic mechanisms [15].

Responses were measured as a percentage of the maximum control response.

In vivo treatment

A group of six male Wistar rats weighing 350–400 g were treated for 8 days with nimodipine 5.14 mg/kg daily, administered orally by gastric intubation. The daily dose was dissolved in 0.5 ml of vehicle, which was prepared by mixing 96.6 g of polyethylene glycol 400, 6.0 g of glycerine and 10 g of water. Two other groups of six rats received either the vehicle only or no treatment, and one group of five rats received only one dose of nimodipine. One hour after the last dose of nimodipine rats were killed by a blow to the head followed by dislocation of the neck. Bladders were removed and placed into 10 ml of Krebs solution. The tissue was dissected free of fat and serosa and strips of muscle from the bladder body (4 mm × 1 mm) were prepared and mounted in the organ bath as described above.

After equilibration, the samples were stimulated as described to obtain response curves to EFS and carbachol. Dose–response curves to K^+ were also obtained because this response is dependent exclusively on calcium influx via voltage-sensitive calcium channels. Response curves were obtained by the cumulative addition of potassium chloride (10–110 mmol/l).

Responses were measured as an increase in tension (mN) and compared with those obtained in rats who received no treatment or vehicle only.

Response curves were also obtained after 8 days' treatment with nimodipine in the bath chamber to establish the presence of functional calcium channels.

Serum nimodipine levels

Serum concentrations of nimodipine in rats in the acute and chronic treatment groups were measured by HPLC, using the method of Ferguson et al. [16]. The free, unbound fraction of nimodipine was not estimated, but from pharmacokinetic studies in animals, including rats, it is known to represent 2–4% of the total drug concentration (drug information from Bayer).

Solutions and chemicals

The Krebs solution had the following composition: NaCl 119 mmol/l, KCl 4.4 mmol/l, $NaHCO_3$ 20 mmol/l, NaH_2PO_4 1.2 mmol/l, $MgCl_2$ 1.2 mmol/l, $CaCl_2$ 2.5 mmol/l, glucose 11 mmol/l, made up in distilled water.

Nimodipine (Bayer) and nifedipine (Sigma) were stored in the dark and made up on the day of the experiment in ethanol. The concentration of ethanol in the organ bath did not exceed 20 mmol/l. High concentrations of ethanol have a slight potentiating effect on the contractile response of detrusor muscle. Verapamil (Sigma) was made up in ethanol on the day of the experiment. Potassium chloride was made up in Krebs solution on the day of the experiment.

For treatment *in vivo*, nimodipine was dissolved in polyethylene glycol (Sigma), glycerine (Fisons) and distilled water before oral administration.

Carbachol (Sigma) was made up in distilled water on the day of the experiment.

Statistical analysis

Statistical analysis was carried out using one-way analysis of variance and Student's *t*-test.

A Bonferroni correction was applied for multiple comparisons. A *P*-value of <0.05 was considered significant.

RESULTS

Human detrusor response *in vitro*

The addition of 0.1 μmol/l nimodipine to the organ bath chamber reduced the maximum contractile response of isolated human detrusor muscle to carbachol by 42% ($P<0.01$, Fig. 1*a*). The addition of 0.1 μmol/l or 0.25 μmol/l nifedipine to the organ bath also reduced the maximum contractile response to carbachol by 35% ($P<0.05$) and 41% ($P<0.01$) respectively (Fig. 1*b*). Increasing the concentration of nifedipine did not produce a further dose-related inhibitory effect.

Verapamil, when added to the organ bath at a concentration of 0.1 μmol/l, did not have a significant inhibitory effect on the contractile response, whereas 1.5 μmol/l verapamil reduced the maximum contractile response to carbachol by 28% ($P<0.01$, Fig. 1*c*).

At 0.1 μmol/l, nimodipine had a greater inhibitory effect than nifedipine on the contractile response of human isolated detrusor muscle *in vitro*.

Time–response curves

Nimodipine was added to the organ bath chamber at a concentration of 0.1 μmol/l and the contractile response of isolated rat detrusor muscle to 40 Hz EFS was recorded after 5, 10, 15, 20, 25 and 30 min incubation. The contractile response to EFS was reduced by 36%, 50%, 55%, 55%, 57% and 56%, respectively, compared with control values. Nifedipine at 0.25 μmol/l reduced the contractile response to 40 Hz EFS by 29%, 47%, 57%, 57%,

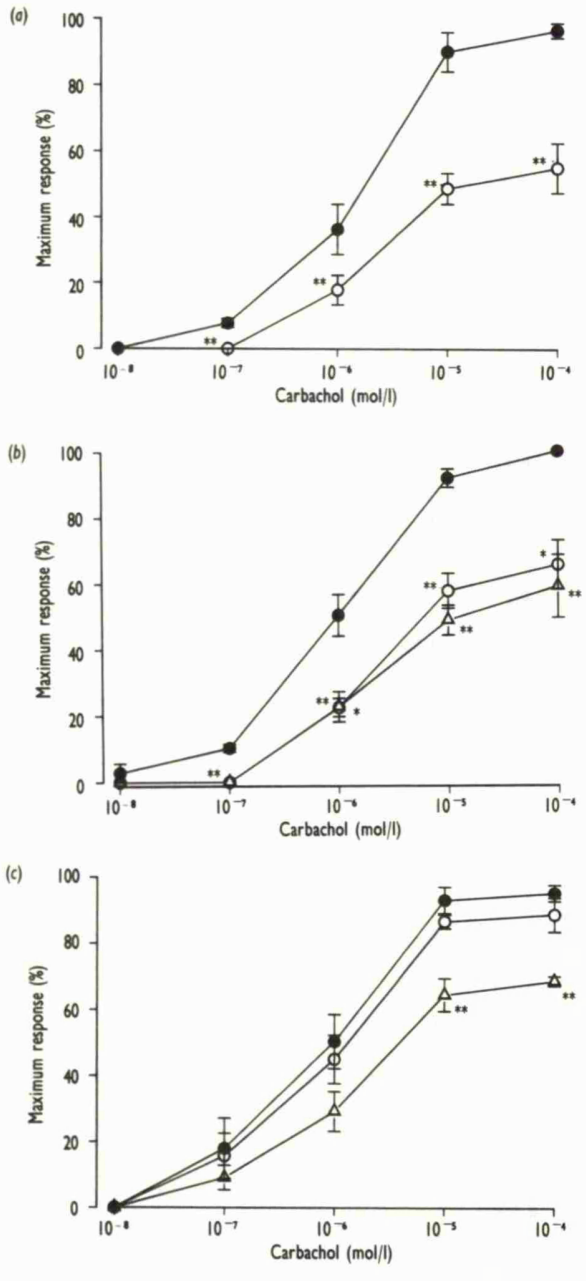

Fig. 1. Effect of (*a*) nimodipine, (*b*) nifedipine and (*c*) verapamil on the contractile response of isolated human detrusor muscle to carbachol stimulation. (*a*) ●, Control; ○, after 0.1 μmol/l nimodipine. (*b*) ●, Control; ○, after 0.1 μmol/l nifedipine; △, after 0.25 μmol/l nifedipine. (*c*) ●, Control; ○, after 0.1 μmol/l verapamil; △, after 1.5 μmol/l verapamil. Vertical bars represent SEM ($n=5$). Statistical significance: $^{*}P<0.05$, $^{**}P<0.01$ compared with controls.

63% and 63% respectively, and verapamil 1.5 μmol/l by 12%, 30%, 38%, 38%, 41% and 41% respectively. An increase in inhibitory effect was observed for each calcium antagonist investigated up to a plateau level after 15 min incubation in the organ bath (not shown).

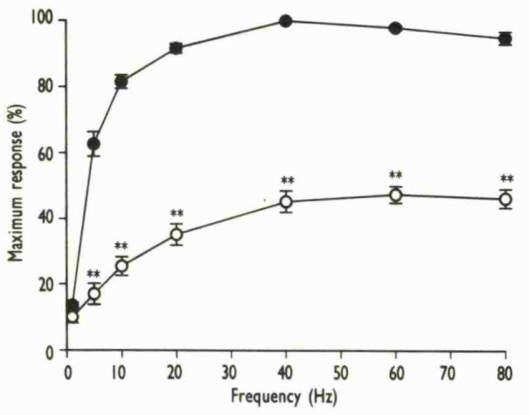

Fig. 2. Effect of nimodipine on the contractile response of isolated rat detrusor muscle to EFS. ●, Control; ○, after 0.1 μmol/l nimodipine. Vertical bars represent SEM ($n=5$). Statistical significance: $^{**}P<0.01$ compared with controls.

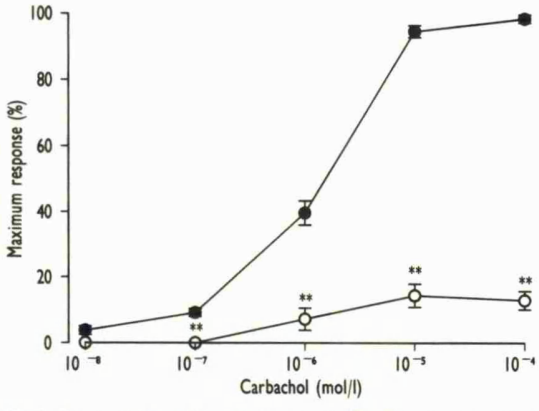

Fig. 3. Effect of nimodipine on the contractile response of isolated rat detrusor muscle to carbachol. ●, Control; ○, after 0.1 μmol/l nimodipine. Vertical bars represent SEM ($n=4$). Statistical significance: $^{**}P<0.01$ compared with controls.

Rat detrusor response *in vitro*

The addition of 0.1 mol/l nimodipine to the organ bath chamber reduced the spontaneous contractions of isolated rat detrusor muscle. Nimodipine reduced the maximum contractile response to EFS by 51% ($P<0.01$, Fig. 2)

After the addition of nimodipine, the tissues were washed and restimulated with 20, 40, 60 and 80 Hz at intervals of 30, 45 and 60 min. The maximum contractile response was reduced by 44%, 34% and 31%, respectively, compared with control values (not shown). Although still significantly different from controls ($P<0.01$), 22% of the original inhibition after nimodipine was lost after 60 min and three washes.

The maximum contractile response to carbachol was reduced by 84% after the addition of 0.1 μmol/l nimodipine to the bath chamber ($P<0.01$, Fig. 3). Nimodipine was added once to the bath and the

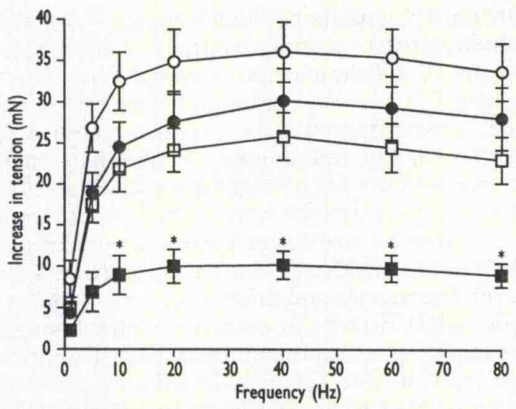

Fig. 4. Effect of nimodipine pretreatment on the contractile response of isolated rat detrusor muscle to EFS. ●, Control; ○, after 8 days' nimodipine treatment; □, after pretreatment with vehicle; ■, after one dose of nimodipine. Vertical bars represent SEM ($n=5$). Statistical significance: $^*P<0.05$ compared with controls.

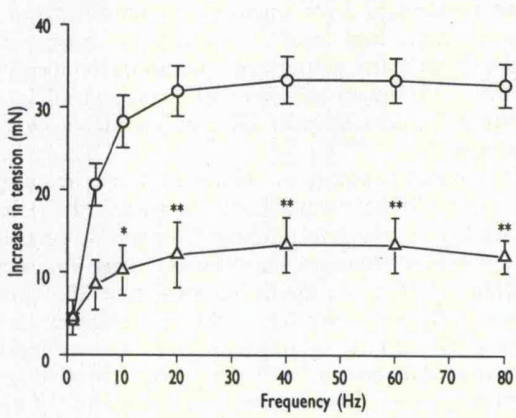

Fig. 5. Effect of nimodipine on the contractile response to EFS of isolated detrusor muscle from rats pretreated with nimodipine. ○, After 8 days' nimodipine treatment; △, after 0.1 μmol/l nimodipine in the bath. Vertical bars represent SEM ($n=4$). Statistical significance: $^*P<0.05$, $^{**}P<0.01$ compared with nimodipine treatment.

samples were washed after each stimulation with carbachol. Nimodipine was not easily washed out of the samples.

In vivo treatment

The maximum contractile response of rat detrusor muscle to EFS *in vitro* after pretreatment with nimodipine or vehicle for 8 days *in vivo* was not significantly different from control values (Fig. 4). However, the contractile response after the rats had received only one dose of nimodipine was reduced significantly by 66% compared with control values ($P<0.05$). When 0.1 μmol/l nimodipine was added to the bath chamber containing detrusor muscle from rats treated for 8 days with nimodipine, the contractile response to EFS was reduced significantly by 60% ($P<0.01$, Fig. 5).

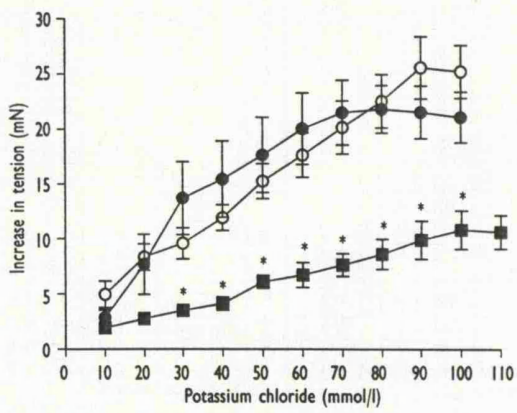

Fig. 6. Effect of nimodipine pretreatment on the contractile response of isolated detrusor muscle to potassium chloride. ●, Control; ○, after 8 days' nimodipine treatment; ■, after one dose of nimodipine. Vertical bars represent SEM ($n=5$). Statistical significance: $^*P<0.05$ compared with controls.

Serum nimodipine levels

The mean serum nimodipine concentration in rats treated with a single dose was 35 ng/ml (SEM ± 2.72; $n=6$) and for those treated for 8 days was 46 ng/ml (SEM 1.84; $n=6$).

Rats treated for 8 days had a significantly higher serum nimodipine concentration than those treated with a single dose ($P<0.01$).

K^+ response

In rats pretreated with nimodipine for 8 days, the contractile response of isolated detrusor muscle to K^+ was not significantly different from control values (untreated); the maximum contractile response was increased, but the increase was not statistically significant (Fig. 6). When the rats had received only a single dose of nimodipine, the contractile response of detrusor muscle was significantly reduced by 51% compared with controls ($P<0.05$).

The addition of 0.1 μmol/l nimodipine to the organ bath containing detrusor muscle from rats pretreated with nimodipine for 8 days abolished the contractile response to K^+ (Fig. 7).

Carbachol response

Detrusor muscle from rats treated for 8 days with nimodipine tended to contract more in response to carbachol than did detrusor from untreated control animals (Fig. 8), but the difference did not reach statistical significance. The addition of 0.1 μmol/l nimodipine to the bath chamber reduced the maximum contractile response by 91% ($P<0.01$). This was similar to the reduction in contractile response seen in control animals after the addition of nimodipine to the bath (Fig. 3).

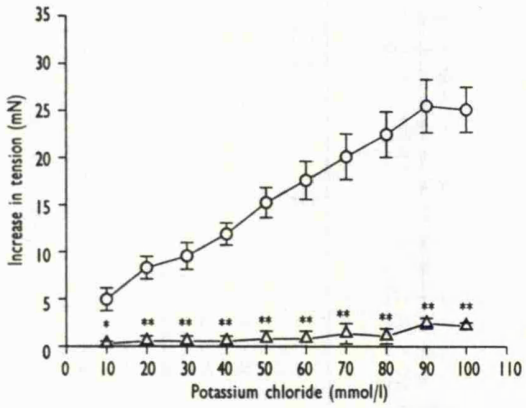

Fig. 7. Effect of 0.1 μmol/l nimodipine on the contractile response to potassium chloride in detrusor muscle from rats treated with nimodipine for 8 days. ○, After 8 days' nimodipine treatment; △, after 0.1 μmol/l nimodipine added to the bath. Vertical bars represent SEM ($n = 5$).

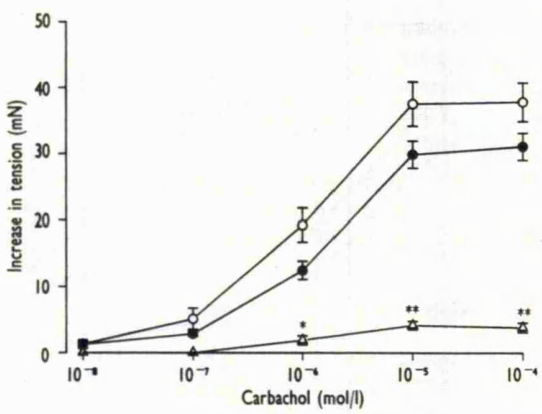

Fig. 8. The effect of nimodipine on the contractile response of isolated detrusor muscle to carbachol in pretreated rats. ●, Control; ○, after 8 days' nimodipine treatment; △, after 0.1 μmol/l nimodipine in the bath. Vertical bars represent SEM ($n = 4$). Statistical significance: $^{*}P < 0.05$, $^{**}P < 0.01$ compared with controls.

DISCUSSION

Multiple sources of calcium are mobilized for human detrusor muscle contraction induced by different stimulants, but the sole source of calcium for contraction induced by potassium chloride is entry of extracellular calcium through dihydropyridine- and voltage-sensitive L-type calcium channels [17]. Calcium channel blockers have been shown to inhibit the non-cholinergic component of the neurogenic response in the rat urinary bladder *in vitro*, whereas acetylcholine-induced contractions are less susceptible to calcium channel blockers [18]. There is much evidence that these drugs have a significant inhibitory effect on both human and animal detrusor muscle contractions *in vitro* [1, 3, 9, 19–21].

In the present study we examined the effect of nifedipine, nimodipine and verapamil on isolated human detrusor muscle contraction to confirm the actions of nifedipine and verapamil reported previously [1] and to establish the action of nimodipine. Time–response curves in the presence of calcium channel antagonists established that the incubation time for a steady-state effect was 15 min. At the concentrations used, which were similar to those achieved in human plasma after chronic oral administration (Drug Information Service, Leicester Royal Infirmary), nimodipine had the greatest inhibitory effect on human detrusor contractile response to carbachol, and verapamil the least. The present *in vitro* experiments therefore confirmed that human detrusor muscle is sensitive to calcium channel antagonists, and nimodipine was chosen for *in vitro* and *in vivo* treatment of rat detrusor muscle in subsequent experiments.

Most *in vivo* animal studies investigating the effects of the calcium antagonists nifedipine, nicardipine and verapamil on bladder contraction have been performed after intravenous administration or after a single high oral dose [5, 6]. In both rat and guinea pig, dihydropyridine antagonists have been shown to be more effective than verapamil in inhibiting K^+ and transmurally stimulated bladder contractions [5, 6, 22].

In view of findings on acute treatment in animals, the poor clinical response of patients with detrusor instability to calcium channel blockers is surprising. Daily oral dosage is the normal drug regimen in humans, but there is little consistent information concerning the effect of such a regimen on the contractile activity of detrusor muscle in animals or humans. Treatment with a single oral dose of nifedipine [8–10] or chronic flunarizine administration [11] to women with various forms of urinary incontinence has produced inconsistent improvement in urodynamic parameters. However, treatment with diltiazem for 10 days in patients with hyper-reflexic detrusor instability has been shown to increase bladder capacity and reduce bladder pressure and maximum detrusor pressure significantly [23].

To allow the effects of chronic oral administration of nimodipine on detrusor contraction to be evaluated, rats were treated with either a single dose or a chronic 8-day treatment before bladders were removed for *in vitro* contractile response studies. Our results show that nimodipine significantly decreases contractile responses after one dose but has no effect on rat detrusor contractile response to EFS, carbachol and potassium chloride after 8 days' oral treatment. It is unlikely that 8 days' treatment with nimodipine was ineffective because of inadequate absorption, distribution or tissue concentrations in the detrusor muscle because a single dose significantly reduced the contractile response. Also, the serum nimodipine concentrations were within a therapeutic range, being similar to serum concentrations obtained in humans (Drug Information

Service, Leicester Royal Infirmary). The concentration of nimodipine was found to be significantly higher in the serum of rats treated for 8 days, and therefore the lack of effect on detrusor contractile response in this group was not due to lower serum drug levels or to enhanced metabolic breakdown of the drug with repeated dosage.

It is unlikely that nimodipine was washed out of our samples as this would also have occurred after a single oral dose, and we have demonstrated that inhibition of the contractile response in detrusor muscle by nimodipine is relatively stable despite multiple washings. Desensitization did not occur as addition of nimodipine to the bath chamber still had an inhibitory effect on the contractile response of detrusor muscle in rats pretreated for 8 days, demonstrating the presence of functional calcium channels after the treatment period. Although not reaching statistical significance, it is interesting to note that the maximum contractile response to carbachol, EFS and K^+ after 8 days' treatment with nimodipine was increased compared with control values, suggesting a possible increase in the number of calcium channels. This is particularly relevant to the K^+ response, which is solely dependent on the influx of calcium through voltage-sensitive calcium channels. It is possible that the repeated administration of nimodipine caused an increase in the number of functional calcium channels to overcome the inhibition of a proportion of the channel population by nimodipine.

Calcium channels are regulated by homologous, heterologous and pathological factors [24]. Chronic exposure of a clonal PC12 cell line to nifedipine for 5 days produced a 29% increase in high-affinity 1,4-dihydropyridine binding sites. In contrast, incubation with Bay K8644 reduced 1,4-dihydropyridine binding site density by 24% [25]. These results demonstrate that repeated exposure at a cellular level can influence the number of dihydropyridine binding sites. Up-regulation of dihydropyridine binding sites has also been observed in cardiac membranes from spontaneously hypertensive rats treated with nitrendipine and a high-salt diet for 21 days [26]. It is likely, therefore, that chronic oral administration of nimodipine caused an up-regulation of dihydropyridine-sensitive calcium channels, as a compensatory mechanism. It has not been possible to confirm this by ligand-binding experiments in the present study because the density of calcium channels in a single bladder is very low, making it impracticable to detect small changes in receptor numbers. Previous studies on bladder smooth muscle have used pooled tissue samples to achieve an adequate density of sites in binding experiments, but this was not part of the design of this study [4].

If the lack of effect of chronic treatment with nimodipine on rat detrusor muscle contractility applies to human detrusor muscle, this may explain why treatment of patients with urinary incontinence with calcium antagonists is disappointing [7]. However, it is possible that calcium antagonists could be effective when administered intermittently. As the intensity of symptoms in urinary incontinence is variable, this may be an appropriate regimen for the treatment of some patients.

In conclusion, nimodipine had a greater inhibitory effect on isolated human detrusor muscle contraction than nifedipine or verapamil, at the concentrations studied. Nimodipine was reasonably stable in rat detrusor muscle samples, and was not easily washed out of the tissues. Rats treated orally with nimodipine for 8 days showed no significant difference in the contractile response of detrusor muscle compared with controls, whereas rats treated with a single oral dose of nimodipine showed a significant inhibition of detrusor contractility. It is possible that chronic oral administration of nimodipine caused an increase in calcium channel density as a compensatory mechanism. This possibility is currently under investigation.

If these results apply to human detrusor muscle response to chronic oral treatment with calcium antagonists, then an intermittent treatment regimen may be more effective in inhibiting unstable contractions.

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Effect of progestogens and oestrogens on the contractile response of rat detrusor muscle to electrical field stimulation

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1. The effect of oestradiol and progesterone pretreatment on the contractile response of isolated rat detrusor muscle to electrical field stimulation was investigated. The response to direct administration of 2 μmol/l progesterone and 2 μmol/l diethylstilboestrol in the organ bath was also examined.
2. Virgin female Wistar rats were injected subcutaneously with oestradiol benzoate (150 μg/kg) for 3 days followed by 1 day of progesterone (160 μg/kg). This cycle was repeated once. Control rats received no injections.
3. In controls, progesterone significantly reduced the maximum contractile response of rat detrusor muscle *in vitro* by 12% ($P<0.01$). The EF_{50} was significantly increased compared with control. When 2 μmol/l diethylstilboestrol, was added, the maximum contractile response was significantly reduced by 42% ($P<0.01$) and the frequency–response curve showed a further increase in EF_{50}.
4. Progesterone had no effect on the atropine-resistant component of electrical field stimulation, but progesterone and diethylstilboestrol reduced the atropine-resistant response by 16% ($P<0.01$).
5. Detrusor muscle from pretreated rats showed a non-significant increase in maximum contractile response compared with untreated controls. The addition of 2 μmol/l progesterone to the bath chamber had no effect on this response, but the further addition of 2 μmol/l diethylstilboestrol reduced the maximum contraction.
6. Pretreatment with oestradiol and progesterone had no effect on the atropine- or tetrodotoxin-sensitive response to electrical field stimulation.
7. In conclusion, the direct effect of progesterone and diethylstilboestrol inhibited the contractile response of detrusor muscle to electrical field stimulation and the effects of each summated. Atropine blocked this effect of progesterone, but not that of diethylstilboestrol. Pretreatment with progesterone and oestradiol had no significant effect on rat detrusor contractile response. Since treatment with oestradiol alone has been shown to have a significant inhibitory action on contractile response, the addition of progesterone would appear to alter this effect of oestradiol.

INTRODUCTION

Urinary incontinence is an increasing and distressing problem in women after the menopause. Sex hormones are known to have a physiological action on the female urinary tract, with stress incontinence being worse in the progesterone-dominated phase of the menstrual cycle and in advancing pregnancy [1]. High-affinity oestradiol receptors have been isolated in human [2, 3], rabbit [4–6] and baboon [7] detrusor muscle. Progesterone receptors have also been isolated in the lower urinary tract of the rabbit [8].

Previous work in this laboratory has shown that the direct administration of diethylstilboestrol (DES) decreased the contractile response of isolated rat and human detrusor muscle by blocking calcium ion entry [9, 10]. Pretreatment with oestradiol in the rat further decreased the contractile response of detrusor muscle, possibly by decreasing muscarinic receptors [11]. Such work provides evidence for a possible beneficial effect from oestrogens for women suffering from motor-urge incontinence. However, it would be unwise to use unopposed oestrogens in women long-term, and thus progestogens would need to be given. These hormones are known to oppose the action of oestrogen in oestrogen-sensitized tissues, probably by decreasing the concentration of oestrogen receptors [12, 13]. Furthermore Benness et al. [14] found that women on hormone replacement therapy reported an exacerbation of their urinary incontinence during the progestogen phase of their treatment and they concluded that oestrogen deficiency might not be as important in the pathogenesis of these symptoms as progestogen concentration.

It is usual practice to include progestogens with oestrogen in the treatment regimen of postmenopausal women. Because of the possible detrimental effects of progesterone on urinary in-

Key words: detrusor muscle, oestrogens, progestogens.
Abbreviations: DES, diethylstilboestrol; EF_{50}, effective frequency at 50% maximum response; EFS, electrical field stimulation; TTX, tetrodotoxin.
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continence, and its interaction with oestrogen, we investigated the effects of progesterone and oestrogen treatment on the contractile response of isolated rat detrusor muscle *in vivo* and *in vitro*.

MATERIALS AND METHODS

Animals and protocol

Virgin female Wistar rats (300–350 g) were injected subcutaneously with oestradiol benzoate (150 μg/kg) for 3 days followed by progesterone (160 μg/kg) for 1 day. This cycle was repeated once. Treatment was commenced when rats were in the dioestrus phase, as judged by vaginal smears. At the end of the treatment period the bladders were removed and placed in Krebs' solution. After the removal of fat and serosa, strips of detrusor muscle were suspended in a 50 ml organ bath chamber containing Krebs' solution at 37°C, aerated with 95% oxygen and 5% carbon dioxide. Control rats received no injections.

The base of the muscle strip was attached by braided silk sutures to a fixed hook in the chamber. The apex was passed through two parallel circular electrodes and attached to an isometric transducer connected to a two-channel Washington oscillograph. The electrodes were connected to a Digitimer stimulator capable of delivering single square wave pulses at 5 V with a pulse width of 1.0 ms.

The tissues were allowed to equilibrate for 1 h under a resting tension of 10 mN before stimulation. Samples were washed at least twice during this stage.

Frequency–response curves were obtained by stimulating the bladder strips with 1, 5, 10, 20, 40, 60 and 80 pulses/s in 10 s trains at 2 min intervals.

Response curves were obtained after the treatment period and were compared with untreated rat detrusor muscle responses. Progesterone (2 μmol/l), alone and with DES (2 μmol/l), was added directly to the organ bath containing either treated or untreated rat detrusor muscle strips. This was to determine any difference between the direct effect of progesterone and DES and the metabolic effect produced after systemic administration, and also to see if there was any interaction between DES and progesterone when applied directly to the muscle tissue. All chemicals were left in the organ bath for 15 min before muscle stimulation.

The responses were presented as an increase in tension (mN) when comparing preteated rats with untreated controls and as a percentage of maximum control response in untreated rats.

The effect of atropine (10 μmol/l) on the frequency–response curve before and after treatment with oestradiol and progesterone was also investigated to establish the action of pretreatment on the cholinergic response to electrical field stimulation (EFS).

Frequency–response curves before and after the addition of tetrodotoxin (TTX) (1.3×10^{-6} mol/l) were provided to determine the effect of oestradiol and progesterone treatment on the TTX sensitivity of the neurogenic response.

Solutions and chemicals

The Krebs' solution had the following composition: NaCl, 119 mmol/l; KCl, 4.4 mmol/l; $NaHCO_3$, 20 mmol/l; NaH_2PO_4, 1.2 mmol/l; $MgCl_2$, 1.2 mmol/l; $CaCl_2$, 2.5 mmol/l; glucose, 11 mmol/l; made up in distilled water. Oestradiol benzoate and progesterone were supplied by Paines and Byrne in 1 ml ampoules for injection. DES (Sigma) and progesterone (Sigma) were dissolved in ethanol and made up on the day of the experiment. The concentration of ethanol in the organ bath chamber did not exceed 3 mmol/l. Atropine (Sigma) was dissolved in distilled water and also made up on the day of the experiment. TTX (Sigma) was made up in distilled water and stored in aliquots of 1 ml at −20°C.

Each point on the curve is the mean of five different bladder samples, unless otherwise stated. Differences between mean values were compared by one-way analysis of variance followed by Dunnett's or Bonferroni's method for multiple comparisons. The EF_{50} (effective frequency at 50% maximum response) was estimated from the median effect plot computated using a Dose Effect Analysis Program (Biosoft).

RESULTS

The responses of detrusor muscle samples to EFS did not significantly alter over the time period of the experiment. Ethanol alone had a slight, but not significant, potentiating effect on contractile response.

Direct effect of progesterone and DES on the contractile response

The mean frequency–response curves obtained from six different bladder muscle preparations are shown in Fig. 1. The control EF_{50} (2.44 ± 0.426 pulses/s) showed a significant difference from the EF_{50} (6.202 ± 1.114 pulses/s) obtained after the addition of 2 μmol/l progesterone ($P < 0.01$). The addition of 2 μmol/l DES to the bath chamber shifted the frequency–response curve further to the right. The mean maximum contractile responses were reduced by 12% compared with controls after the addition of progesterone and by a further 30% after the addition of DES. In two out of six samples, the addition of DES resulted in the maximum contractile response being reduced to less than 50% of controls. The reduction in maximum responses after the addition of progesterone and progesterone plus DES were both significantly different from control ($P < 0.01$ and $P < 0.01$, respectively). There was also a significant difference between the maximum con-

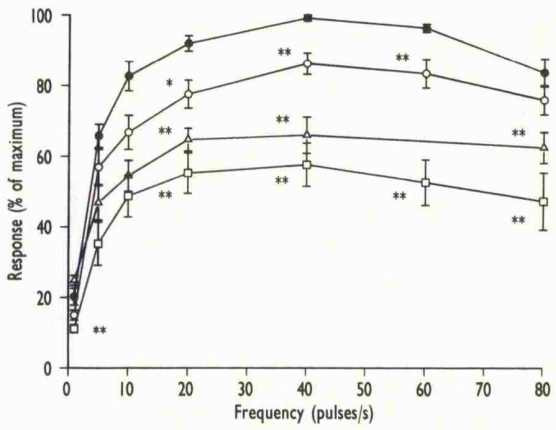


Fig. 1. Frequency–response curves to EFS in isolated rat detrusor muscle (controls). ●, Control; ○, after the addition of 2 μmol/l of progesterone; △, after the addition of 2 μmol/l DES; □, after the addition of 2 μmol/l progesterone and 2 μmol/l DES. Vertical bars represent SEMs ($n=6$). Statistical significance: $^*P<0.05$, $^{**}P<0.01$ compared with control.

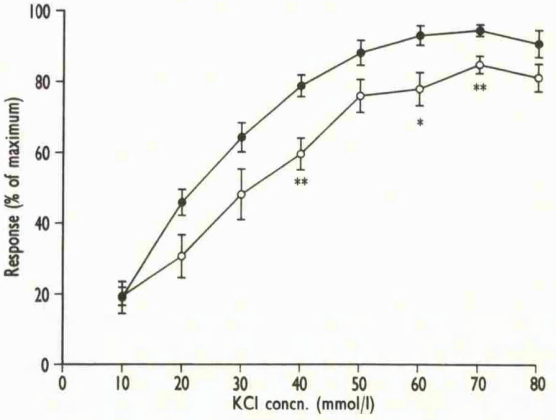
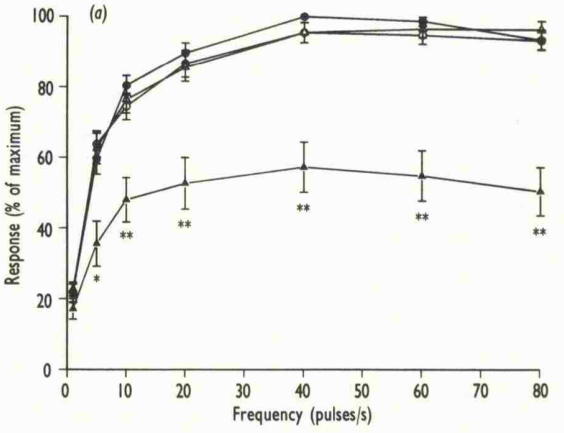


Fig. 2. Dose–response curves to KCl in isolated rat detrusor muscle. ●, Control; ○, after the addition of 2 μmol/l progesterone. Vertical bars represent SEMs ($n=5$). Statistical significance: $^*P<0.05$, $^{**}P<0.01$ compared with control.



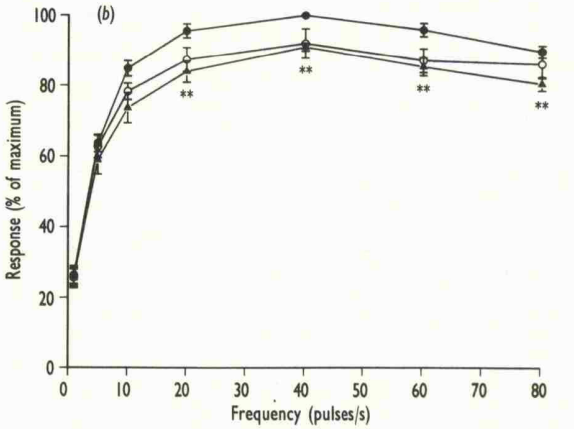


Fig. 3. Effect of DES (*a*) and progesterone (*b*) on frequency–response curves to EFS in isolated rat detrusor muscle. (*a*) ●, Control; ○, after the addition of 0.02 μmol/l DES; △, after the addition of 0.2 μmol/l DES; ▲, after the addition of 20 μmol/l DES. (*b*) ●, Control; ○, after the addition of 0.2 μmol/l progesterone; ▲, after the addition of 20 μmol/l progesterone. Vertical bars represent SEMs ($n=5$). Statistical significance: $^*P<0.05$, $^{**}P<0.01$ compared with control.

tractile responses obtained after the addition of progesterone and progesterone plus DES ($P<0.01$).

Effect of progesterone on the KCl contractile response

The contractile response to KCl is solely dependent on extracellular calcium. The effect of 2 μmol/l progesterone on this response was examined to establish whether the inhibitory effect of progesterone *in vitro* was due to the inhibition of calcium entry.

Progesterone significantly reduced the KCl contractile response, suggesting an effect on calcium movement (Fig. 2).

Direct effect of different concentrations of progesterone and DES on the contractile response

Very low concentrations of DES (0.02 μmol/l and 0.2 μmol/l) did not have a significant effect on the detrusor contractile response, whereas a very high concentration, (20 μmol/l) had a significant inhibitory effect on contraction ($P<0.01$), reducing the maximum response by 43% (Fig. 3*a*).

Progesterone at a low concentration (0.2 μmol/l) did not significantly effect the contractile response. A concentration of 20 μmol/l progesterone did, however, significantly reduce bladder contraction ($P<0.01$) (Fig. 3*b*). The maximum response was reduced by 8%. This inhibition was similar in magnitude to the reduction of contractile response produced by 2 μmol/l progesterone, suggesting that the direct inhibitory action of progesterone was not dose-dependent.

Direct effect of progesterone and DES on the contractile response in the presence of atropine

The addition of atropine (10 μmol/l) to the bath chamber reduced the maximum contractile response by 37% compared with controls ($P<0.01$), thereby

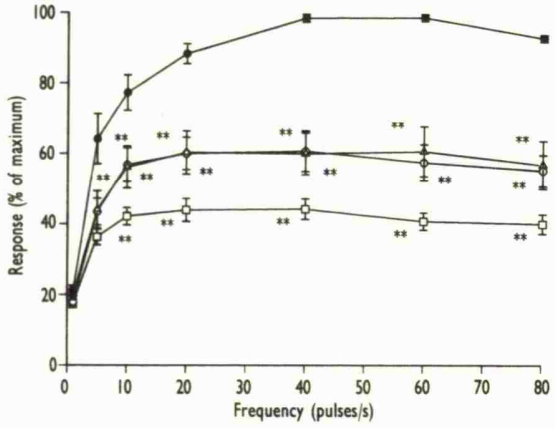


Fig. 4. Frequency–response curves to EFS in isolated rat detrusor muscle (controls). ●, Control response curve; ○, after the addition of 10 μmol/l atropine; △, after the addition of 10 μmol/l atropine and 2 μmol/l progesterone; □, after the addition of 10 μmol/l atropine and 2 μmol/l progesterone and 2 μmol/l DES. Vertical bars represent SEMs ($n=5$). Statistical significance: $^{**}P<0.01$ compared with control.

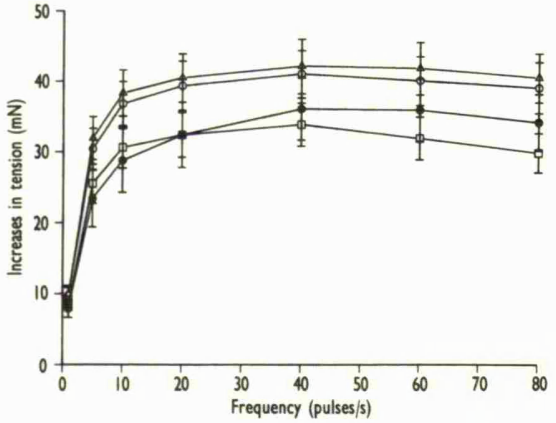


Fig. 5. Frequency–response curves to EFS in detrusor muscle from pretreated rats. ●, Control response curve (untreated); △, after pretreatment with progesterone and oestrogen ($n=10$); ○, after the addition of 2 μmol/l progesterone; □, after the addition of 2 μmol/l progesterone and 2 μmol/l DES. Vertical bars represent SEMs ($n=6$).

blocking the cholinergic response to EFS. The non-cholinergic response was not affected by the further addition of progesterone, but DES significantly reduced the maximum response by a further 16% ($P<0.05$) (Fig. 4).

Effect of oestradiol and progesterone pretreatment on the contractile response

The frequency–response curves in pretreated rats are shown in Fig. 5. There was a non-significant increase in maximum contractile response in the detrusor muscle of pretreated rats compared with that of untreated control rats ($n=10$). The addition of progesterone to the bath chamber resulted in a small, non-significant, decrease in maximum response, and the further addition of 2 μmol/l DES caused an additional reduction in maximum response towards that of controls.

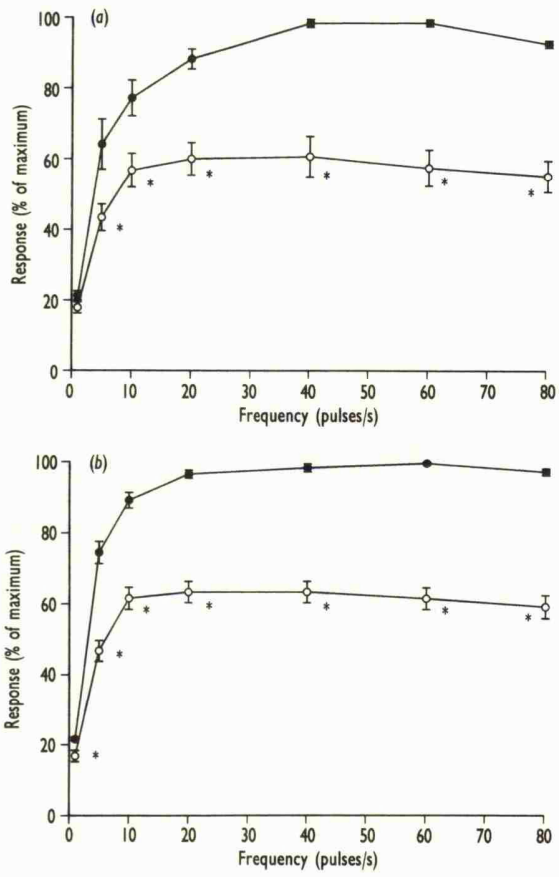


Fig. 6. Effect of atropine on the frequency–response curves in (*a*) control rats and (*b*) pretreated rats. ■, Control response curve; ○, after the addition of 10 μmol/l atropine. Vertical bars represent SEMs ($n=5$). Statistical significance: $^{*}P<0.01$ compared with control.

Effect of oestradiol and progesterone pretreatment on atropine sensitivity to electrical stimulation

In control animals the maximum contractile response of detrusor muscle to electrical stimulation was reduced by 38% after the addition of 10 μmol/l atropine to the bath chamber. The frequency–response curve showed an increase in the mean EF_{50} from 2.966 to 16.173 pulses/s (Fig. 6*a*).

In pretreated animals the atropine sensitivity to electrical stimulation was not significantly different from that in untreated animals. The maximum contractile response was reduced by 35% and the mean EF_{50} increased from 2.311 to 12.265 pulses/s (Fig. 6*b*).

Effect of oestradiol and progesterone pretreatment on the TTX-resistant response

At our parameters TTX blocked 50% of the maximum contractile response to EFS, but pre-

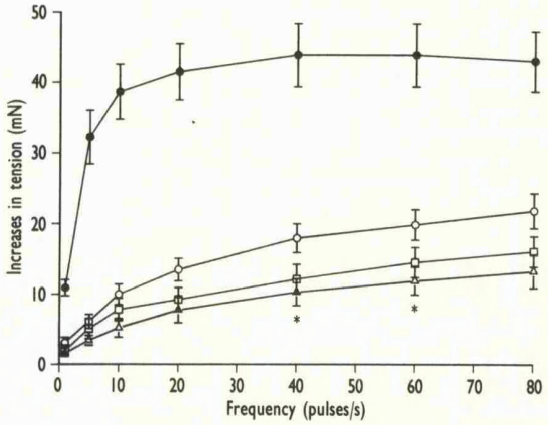


Fig. 7. Frequency–response curves showing the effect of oestradiol and progesterone pretreatment on the TTX-resistant response to EFS in isolated rat detrusor muscle. ●, Control response curve; ○, after TTX; △, after 8 days of oestradiol treatment and TTX; □, after 8 days of oestradiol and progesterone pretreatment plus TTX. Vertical bars represents SEMs ($n=5$). Statistical significance: $^*P<0.05$ compared with control plus TTX.

treatment with oestradiol and progesterone did not affect this response significantly. Pretreatment with oestradiol alone significantly reduced the TTX-resistant response ($P<0.05$) (Fig. 7).

DISCUSSION

In patients with motor-urge incontinence, powerful detrusor muscle contraction overcomes the urethral sphincter and produces incontinence. Motor-urge incontinence increases particularly with age and is more common in women than men.

Previous results from this laboratory have shown that DES added directly to the bath chamber and pretreatment with oestradiol had differing mechanisms of action on detrusor muscle contraction. Both summated to produce a highly significant decrease in contractile response to cholinergic stimulation and EFS in rats and man. It was proposed that the direct effects inhibited calcium uptake [10] and that pretreatment altered muscarinic receptor effectiveness [11].

Before such work could be investigated further in clinical trials it was important to establish what effect progestogens would have on this action of oestrogen. Our present results demonstrate that pretreatment with oestradiol and progesterone has no significant effect on the contractile response of rat detrusor muscle to EFS. Thus progesterone antagonized the inhibitory action of oestrogen on rat detrusor muscle contraction.

The neurogenic response to EFS in the rat involves cholinergic and a non-cholinergic, non-adrenergic neurotransmitter, almost certainly ATP [15]. Pretreatment with oestradiol significantly reduced the cholinergic response to EFS [11], whereas treatment with oestradiol and progesterone had no significant effect on this response. The effect of oestrogen on muscarinic receptor responsiveness would appear to be altered by the presence of progesterone.

Sato et al. [16] showed that both oestradiol and progesterone pretreatment significantly increased the response of the rat uterus to nerve stimulation, whereas Batra and Bengtsson [17] showed that oestrogen and progesterone inhibited the contractile activity of the rat uterus *in vitro*. Sato et al. [16] were also unable to obtain a significant effect *in vivo* of oestradiol and progesterone treatment on the bladder in anaesthetized rats after stimulation of hypogastric and pelvic nerves. However, they pretreated their rats for only 4 days, which may not have been sufficient [4]. A similar problem may explain the results of Batra [18], who found that calcium uptake in the bladder was unaffected after treatment with oestrogen alone or oestrogen and progesterone together, Sorenson et al. [19] could not demonstrate any change in urodynamic characteristics during the menstrual cycle which may have been due to the influence of progesterones on oestrogen action.

Progesterone alone was not investigated in the present study as it is unlikely to be given without oestrogen in clinical practice, except for certain gynaecological abnormalities or in women unable to take oestrogen-containing birth control pills. Nevertheless, Ekstrom et al. [20] demonstrated that progesterone increased the sensitivity of the female rabbit detrusor muscle to parasympathomimetics and also increased the maximal nerve-induced contractile response. They also showed that oestrogen treatment shifted the frequency–response curve of the bladder to the right.

The antagonism between oestrogen and progesterone is not immediate, but is the result of metabolic changes altering receptor numbers. Nevertheless, we also looked at the possibility of an interaction between DES and progesterone *in vitro* by looking at their effect on the contractile response when added to the organ bath. Progesterone alone significantly decreased the contractile response to EFS, an effect also observed in uterine cells [17]. This effect was augmented by the presence of DES, therefore providing no evidence of a direct antagonism between these hormones. Progesterone also inhibited the contractile response to KCl, indicating its action was due to the reduction of calcium uptake.

The direct inhibitory action of DES and progesterone on EFS had differing modes of action. Atropine blocked the effect of progesterone but not that of DES. It is likely that progesterone affected the cholinergic component of EFS, whereas DES also affected the non-cholinergic, non-adrenergic response.

The direct effects of DES, oestradiol and progesterone on detrusor muscle contraction were not significant at very low concentrations, suggesting

their inhibitory action on calcium influx is significant at pharmacological but not physiological concentrations.

The neurogenic response to EFS in the rat can be blocked by TTX. Our parameters for EFS produced a TTX component which was due to direct muscle stimulation. Oestrogen treatment has been shown to significantly increase the TTX sensitivity [11], thereby reducing the direct muscle stimulation. However, progesterone and oestrogen treatment did not significantly alter the TTX sensitivity.

In conclusion our results demonstrate that pretreatment with pharmacological doses of oestradiol and progesterone had no significant effect on rat detrusor muscle contraction in response to EFS. This was in contrast to our previous finding that pretreatment with oestradiol alone had a significant inhibitory action on rat detrusor contractile response. We conclude that progesterone alters oestrogen's functional effect on rat detrusor muscle contraction. In this study and in previous work we have used virgin female rats, which are not representative of the post-menopausal condition.

These results are supported by clinical evidence [14] that the addition of a progestogen may worsen urinary tract symptoms in post-menopausal women previously on oestrogen therapy.

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The effect of *in vivo* oestrogen pretreatment on the contractile response of rat isolated detrusor muscle

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1 The effect of oestradiol pretreatment was investigated on the response of rat isolated detrusor muscle to cholinergic, electrical and 5-hydroxytryptamine (5-HT) stimulation with and without diethystilboestrol (DES) (2 μM) in the organ bath.

2 Virgin female Wistar rats were injected subcutaneously for 8 days with oestradiol benzoate 150 μg kg^{-1}. Control rats received no injections or injection only with the vehicle, ethyl oleate.

3 Detrusor muscle from treated rats showed a decreased sensitivity to acetylcholine (ACh) and carbachol-induced contractile responses. The dose-response curves to these agonists showed a 44% reduction in maximum contractile response for ACh ($P<0.001$), and a 38% reduction in maximum contractile response for carbachol ($P<0.05$). The addition of 2 μM DES to the bathing medium further significantly reduced the maximum contractile response by 56 and 57% of control respectively.

4 Electrically stimulated detrusor muscle from treated rats showed a significant 49% reduction in the maximum contractile response ($P<0.001$). The addition of 2 μM DES to the bathing medium further significantly reduced the maximum contractile response by 66% of control. The tetrodotoxin resistant responses were smaller in pretreated rats, suggesting a reduced sensitivity of the smooth muscle to direct electrical stimulation.

5 The response to 5-HT stimulation by detrusor muscle samples from oestradiol-treated rats showed a non-significant reduction in maximum contractile response, but the addition of 2 μM DES to the bath chamber resulted in a 67% reduction in the response ($P<0.001$).

6 Oestradiol pretreatment did not affect the potassium dose-response curve.

7 Oestradiol pretreatment reduced the rat detrusor muscle sensitivity to the blocking effect of atropine on the response to electrical field stimulation. Pretreatment also reduced the potentiating effect of physostigmine on the same response.

8 These results suggest that oestradiol pretreatment had a modulating effect on cholinergic responses. The addition of oestrogen to the tissue environment enhances this inhibitory effect.

Keywords: Oestrogen; bladder; carbachol; 5-hydroxytryptamine

Introduction

Oestrogens have been used for a number of years to treat urinary symptoms especially those associated with the lower urinary tract such as atrophic urethritis. Their place in the management of motor urge incontinence has never been established (Miodrag *et al.*, 1988) despite the fact that high affinity oestradiol receptors have been isolated in human (Iosif *et al.*, 1981; Batra & Iosif, 1983), rabbit (Urner *et al.*, 1983; Shapiro, 1986; Batra & Andersson, 1989) and baboon detrusor muscle (Weaker *et al.*, 1983). Treatment with oestradiol decreased the muscarinic receptor density in rabbits (Shapiro, 1986) but the muscarinic response to carbachol and the cholinergic neurogenic response following electrical field stimulation was not greatly decreased despite a markedly decreased density in muscarinic receptors (Batra & Andersson, 1989).

Earlier experiments in our laboratories have shown that diethylstilboestrol (DES) added directly to the organ bath had a profound effect on the contractile response following cholinergic, 5-hydroxytryptamine (5-HT), calcium ion, potassium and electrical field stimulation of rat and human detrusor muscle. This was probably due to a reduction in calcium ion uptake by the detrusor muscle cells rather than an effect on intracellular calcium release (Elliott *et al.*, 1992). If *in vivo* treatment with oestradiol had similar effects on muscarinic receptors in rats as in rabbits (Shapiro, 1986) then pretreatment and direct oestrogen would have different mechanisms of action and thus a summation of effects might be seen on cholinergic stimulation. Because there appear to be no reports illustrating the effects of pretreatment and direct oestrogen treatment on the same detrusor muscle, the present experiments were performed.

Methods

Virgin female wistar rats were injected subcutaneously with oestradiol benzoate 150 μg kg^{-1}, twice a day for 8 days. Treatment was initiated when the rats were in the dioestrus phase, as judged by vaginal smears. The 8 day treatment regimen covered two cycles, after which the rats were killed by a blow to the head. The bladders were removed and dissected free of fat and serosa. Strips of bladder muscle 7 mm by 4 mm were suspended in a 50 ml organ bath chamber containing Krebs solution at 37°C and aerated with 95% oxygen and 5% carbon dioxide. The bladder base was attached to a fixed hook in the chamber and the apex by a thread attached to an isometric transducer connected to a two channel Washington oscillograph. The tissues were allowed to equilibrate for 1 h under a tension of 10 mN.

After equilibration, acetylcholine (ACh) (10^{-8} M–2×10^{-4} M), carbachol (10^{-8} M $\times$ 10^{-4} M) or potassium chloride (KCl) (10 mM–60 mM) was injected cumulatively into the bath chamber to obtain dose-response curves. 5-Hydroxytryptamine (5-HT) (10^{-8} M–10^{-5} M) was injected at 5 min intervals and samples were washed between doses to avoid tachyphylaxis. For electrical field stimulation, muscle strips were

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passed through two parallel circular electrodes connected to a Digitimer stimulator. The stimulator delivered 1–80 pulses s^{-1} at 4–6 V and a 1 ms pulse width in 10 s trains at 2 min intervals. Frequency-response curves were obtained by stimulating the tissue with 1, 5, 10, 20, 40, 60, 80 pulses per second. The effect of oestradiol pretreatment plus the presence of 2 μM DES in the external medium was investigated by repeating the dose-response curves after the addition of DES to the bath chamber. Effects of bath-applied DES were not easily reversible. Even after several washes the response did not return to pre-applied levels.

Tetrodotoxin (TTX, 1.6×10^{-6} M) was used to distinguish between nerve-mediated contractile responses, and those due to direct muscle stimulation in controls and pretreated samples.

Different bladder muscle samples were used for each agonist. Control dose-response curves were obtained from rat bladder muscle taken from untreated rats in the dioestrus phase. Samples were also taken from rats injected only with the vehicle ethyl oleate. Dose-response curves for the comparison of bladders from rats pretreated with oestradiol and controls are presented as concentration of agonist against the increase in tension, and not percentage of maximum response. This is to demonstrate the absolute decrease in response obtained after oestradiol treatment which would not be apparent when calculating percentage of maximum response if the same bladder samples were used as its own control.

Solutions and chemicals

Krebs solution contained (mM): NaCl 119, KCl 4.4, $NaHCO_3$ 20, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.5 and glucose 11.

ACh chloride (Sigma), carbamylcholine chloride (Sigma), atropine sulphate (Sigma), physostigmine (Sigma) and 5-HT (Sigma) were all dissolved in distilled water and made up on the day of the experiment. Diethylstilboestrol (DES) (Sigma) was dissolved in ethanol and the concentration of ethanol in the organ bath chamber did not exceed 3 mM. Oestradiol benzoate (Paines & Byrne) was supplied in vials containing 5 mg ml^{-1}. TTX (Sigma) was made up in distilled water and stored at −20°C in 1 ml aliquots.

For each experiment the results were the mean of 5 different bladder muscle samples, unless otherwise stated. Statistical analysis was carried out with Student's t test.

Results

Following 8 days oestradiol treatment the rat uterus showed marked hypertrophy compared to non-treated animals. This was taken as an indication of oestrogenisation. The bladders removed from treated rats were also hypertrophic (mean weight 94.32 ± 8.60 mg) compared to the non-treated animals (mean weight 66.65 ± 2.54 mg) ($P<0.05$). The response of the detrusor muscle in control rats and those treated with vehicle only did not differ.

Effect of oestrogen on electrical field stimulation

The spontaneous contractions normally exhibited by rat detrusor muscle were markedly reduced in frequency and amplitude in samples taken from oestradiol treated rats. The frequency dose-response curve of the detrusor muscle of such rats to electrical field stimulation showed a 49% reduction in maximum response compared to control ($P<0.001$). When 2 μM DES was added to the surrounding medium, the result was a further significant reduction of maximum response (66%, Figure 1). The maximum response obtained by electrical field stimulation was 61.4% of the maximum response obtained with 10^{-4} M carbachol in controls and 61.5% in pretreated rats.

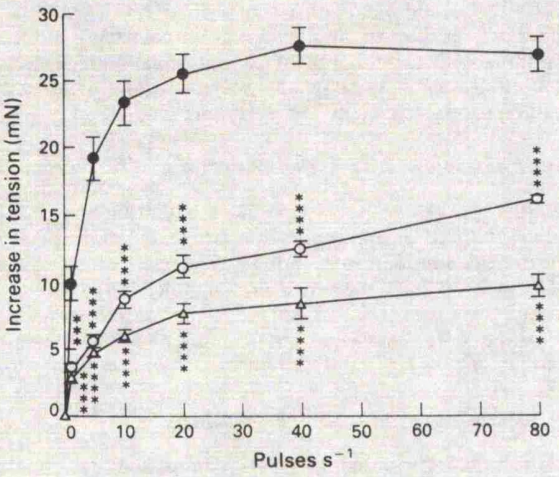

Figure 1 Dose-response curves to electrical field stimulation in rat isolated detrusor muscle: (●) control; (○) after 8 days oestradiol treatment; (△) after 8 days oestradiol treatment plus 2 μM diethylstilboestrol in the organ bath ($n = 5$). Vertical bars represent s.e. mean. ***$P<0.01$; ****$P<0.001$.

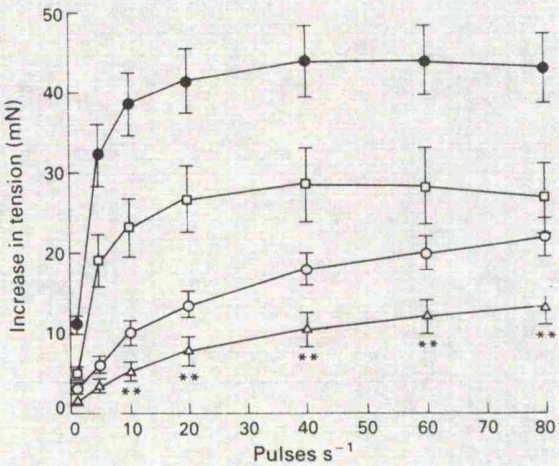

Figure 2 Frequency-response curves showing the effect of oestradiol pretreatment on the tetrodotoxin-resistant response to electrical field stimulation in rat isolated detrusor muscle: (●) control; (○) control after TTX; (□) after 8 days treatment; (△) after 8 days treatment and TTX, ($n = 5$). Vertical bars represent s.e.mean. **$P<0.05$.

Effect of oestrogen on tetrodotoxin sensitivity

Tetrodotoxin blocked about 50% of the maximum contractile response to electrical field stimulation at our parameters. At lower frequencies (10 pulses s^{-1}) about 75% of the contractile response was blocked. The TTX-resistant responses were significantly smaller in rats pretreated with oestradiol than in control rats ($P<0.05$, Figure 2).

Effect of oestrogen on acetylcholine and carbachol response

The contractile response of detrusor muscle to ACh stimulation was reduced in amplitude in oestradiol-treated rats compared to controls. The dose-response curve showed a 44% reduction in maximum response ($P<0.001$). The addition of 2 μM DES to the water bath resulted in a further significant

reduction in maximum response (56%, Figure 3a). Oestradiol pretreatment resulted in similar effects on carbachol-induced contractions with a reduction of 38% in maximum response being obtained ($P<0.05$), which was reduced further with the addition of 2 μM DES (57%, Figure 3b).

Effect of oestrogen on 5-hydroxytryptamine response

Rat detrusor muscle response to 5-HT stimulation was phasic and much reduced in amplitude compared to cholinergic and electrical field stimulation responses. Detrusor muscle from oestradiol-treated rats showed a non significant reduction in maximum response of 22%, but with the addition of 2 μM DES to the bath chamber this response was significantly reduced by 67% ($P<0.001$), Figure 4.

Effect of oestrogen on atropine sensitivity

Detrusor muscle from control rats, stimulated electrically, showed a 19% reduction in maximum contractile response in the presence of 10 μM atropine (Figure 5a). Atropine exerted its inhibitory effect mainly on high frequency induced contractile responses.

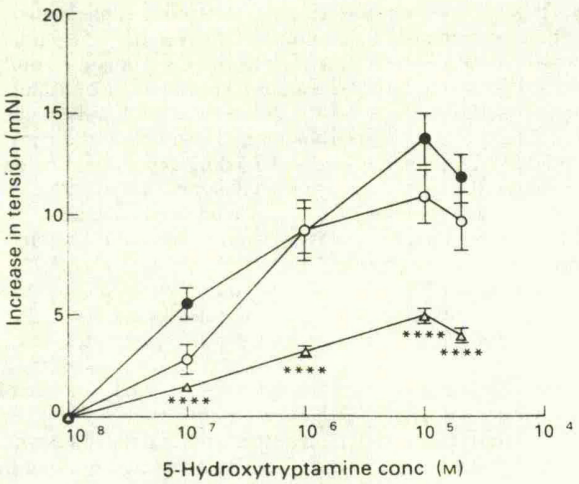

Figure 4 Dose-response curves to 5-hydroxytryptamine (5-HT) stimulation of rat isolated detrusor muscle: (●) control; (○) after 8 days oestradiol treatment; (△) after 8 days oestradiol treatment plus 2 μM diethylstilboestrol in the organ bath ($n=5$). Vertical bars represent s.e.mean. ****$P<0.001$.

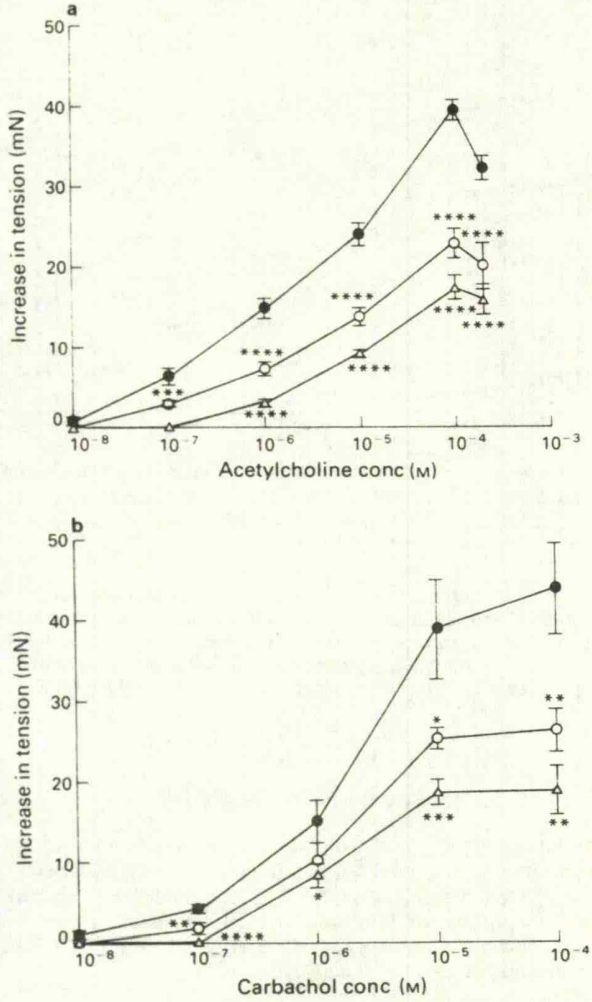

Figure 3 (a) Dose-response curves to acetylcholine stimulation in rat isolated detrusor muscle: (●) control ($n=8$); (○) after 8 days oestradiol treatment; (△) after 8 days oestradiol treatment plus 2 μM diethylstilboestrol (DES) in the organ bath ($n=9$). (b) Dose-response curves to carbachol stimulation in rat isolated detrusor muscle: (●) control; (○) after 8 days oestradiol treatment; (△) after 8 days oestradiol treatment plus 2 μM DES in the organ bath ($n=5$). Vertical bars represent s.e.mean. *$P<0.10$; **$P<0.05$; ***$P<0.01$; ****$P<0.001$.

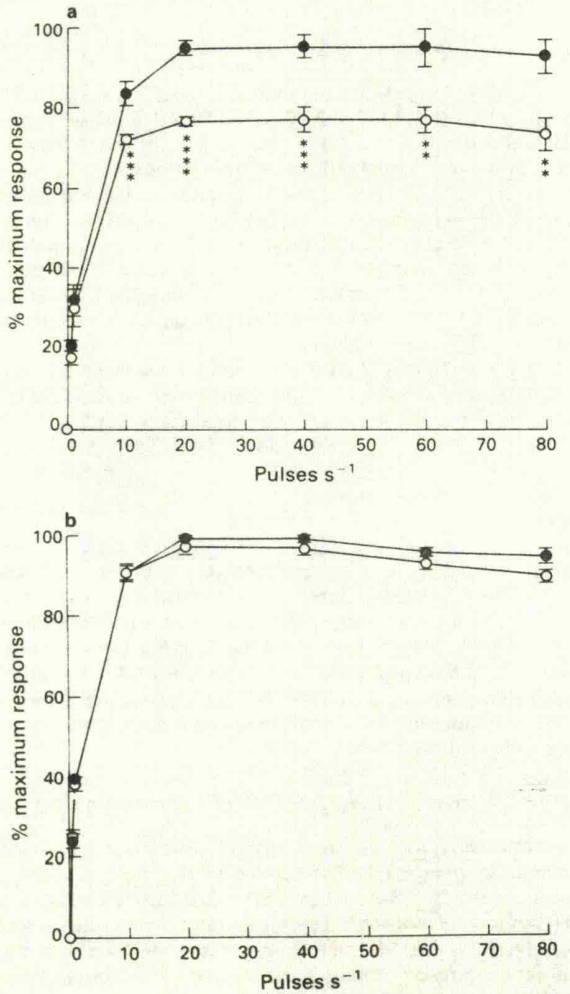

Figure 5 (a) Dose-response curves to electrical field stimulation in rat isolated detrusor muscle from untreated rats ($n=4$). (b) Dose-response curves to electrical field stimulation in rat isolated detrusor muscle from rats pretreated with oestradiol for 8 days: (●) control; (○) after 10 μM atropine ($n=5$). Vertical bars represent s.e.mean. **$P<0.50$; ***$P<0.01$; ****$P<0.001$.

The sensitivity of the detrusor muscle to blockade of the cholinergic component of electrical stimulation was almost totally abolished in bladder muscle samples taken from oestradiol-treated rats. The dose-response curve showed no significant difference before and after the addition of atropine 10 μM (Figure 5b).

Effect of oestrogen on physostigmine potentiation

Control detrusor muscle samples showed potentiation of electrically induced contractile responses in the presence of 0.01 μM physostigmine. The maximum contractile response was increased by 30% ($P<0.001$) (Figure 6a).

Bladder muscle from oestradiol pretreated rats had lost its sensitivity to physostigmine. The electrically induced contractile responses were not significantly different in the presence or absence of the drug (Figure 6b).

Effect of pretreatment on KCl response

Oestradiol pretreatment did not affect the KCl dose-response curve.

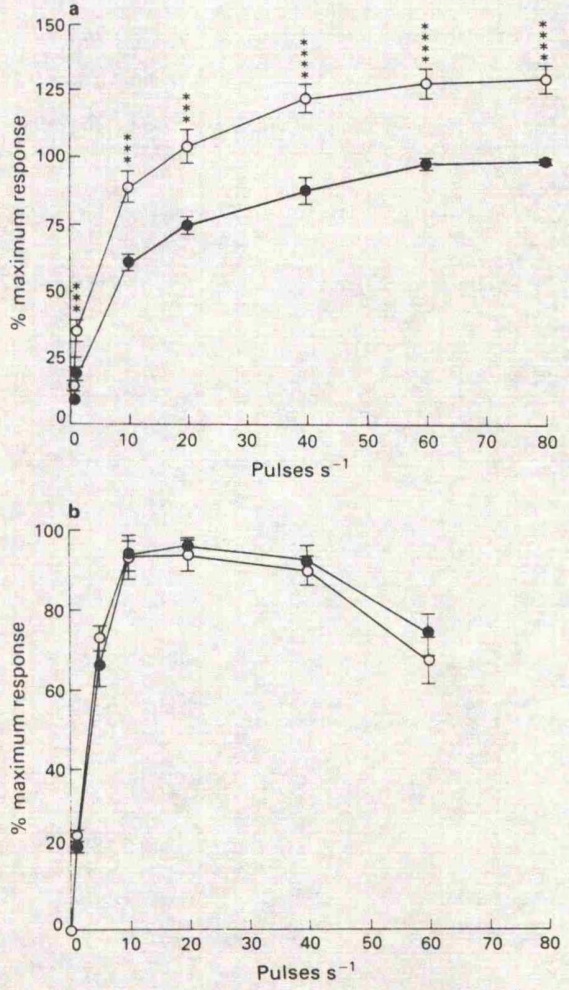

Figure 6 (a) Dose-response curves to electrical field stimulation in rat isolated detrusor muscle from untreated rats ($n=5$). (b) Dose-response curves to electrical field stimulation in rat isolated detrusor muscle from rats pretreated with oestradiol for 8 days ($n=5$): (●) control; (○) after the addition of 0.01 μM physostigmine. Vertical bars represent s.e.mean. ***$P<0.01$; ****$P<0.001$.

Discussion

Previous results from our laboratory have shown that the addition of DES to the organ bath had a profound effect on the contractile response of rat and human detrusor muscle. The results suggested that DES affected the movement of extracellular calcium ions into the muscle cells (Elliott *et al.*, 1992). If previous studies were plotted on the present dose- and frequency-response curves, the resultant curve would be situated between the control and pretreatment curves.

The present results showed that there was a significant decrease in the contractile response to muscarinic receptor stimulation after pretreatment with oestradiol and that this reduction in contractility could be further enhanced by the direct addition of DES to the tissue environment. Since there was no difference between the effect of oestradiol on the responses to ACh and carbachol, pretreatment is unlikely to have affected cholinesterase activity. The decrease in contraction following electrical stimulation was due predominantly to an effect on the cholinergic component since pretreatment with oestradiol almost abolished the atropine sensitive component of electrical field stimulation. The enhancing effect of physostigmine on this response was also greatly reduced after oestradiol pretreatment. In man it is likely that the effect of oestradiol pretreatment will be even greater as the contraction following electrical field stimulation is mainly cholinergic (Sjögren *et al.*, 1982; Sibley, 1984; Kinder & Mundy, 1985). Pretreatment did not affect the KCl response and it is unlikely therefore that pretreatment is affecting calcium ion permeability. This is in contrast to the direct effect of DES (Elliott *et al.*, 1992). The results with TTX show that, with our parameters of stimulation, the nerve-mediated response was 50% of the contractile response to electrical field stimulation at high frequencies and that 50% was direct muscle action. This is true for both control and pretreated rats. The TTX-sensitive response was reduced after pretreatment with oestradiol, suggesting that oestradiol decreases the sensitivity of detrusor muscle to direct electrical stimulation. Brading & Williams (1990) have clearly shown that the predominant mechanical response to intrinsic nerve stimulation of rat and guinea-pig detrusor was through non-muscarinic receptors and that contractile responses resistant to atropine are most clearly seen in the early response to electrical field stimulation. Conversely neostigmine (Brading & Mostwin, 1989) and physostigmine potentiated electrical field stimulation at low although less than at high frequencies. There is now good evidence that the non-cholinergic transmitter is adenosine 5′-triphosphate (ATP) (Brading & Mostwin, 1989; Brading & Williams, 1990; Parija *et al.*, 1991), but whether pretreatment affects this mechanism is unknown at present.

Pretreatment with oestradiol in the rat also had no effect on the contractile response to 5-HT. Chen (1990) has shown that there is a cholinergic component to 5-HT stimulation in the rabbit in addition to non-adrenergic, non-cholinergic excitatory neurotransmission. The present results would suggest that this cholinergic component was relatively unimportant following 5-HT stimulation in the rat. The addition of DES to the tissue environment caused a significant reduction in contractile response to 5-HT possibly due to changes in cell membrane permeability to calcium ions (Elliott *et al.*, 1992).

The most likely explanation for the inhibitory effect of pretreatment with oestradiol on the cholinergic response was that there was down-regulation of muscarinic receptors, although there may have been a minor effect on the sensitivty to direct electrical stimulation. There was no overall decrease in contractility of the pretreated detrusor muscles in the present study, despite considerable hypertrophy of the bladder. As early as 1977, Roberts *et al.* showed that pretreatment with oestradiol could increase α-adrenoceptor densities in the rabbit uterus. Larsson *et al.* (1984) reported that this increase in α-adrenoceptors could also be induced in the female rabbit urethra. They clearly showed that the increase

in receptors was not proportional to the increase in the weight of the tissue as it was in the uterus. Shapiro (1986) found that pretreatment with oestradiol decreased the muscarinic cholinoceptor density in the rabbit bladder; this was despite a marked increase in the weight of the bladder body. Batra & Andersson (1989) also using the rabbit confirmed previous work that muscarinic receptor density was reduced following oestradiol treatment but were unable to show that contractile responses to electrical field stimulation and carbachol were significantly decreased. The difference between their results and the present ones could represent species differences, but is unlikely to be due to inadequate oestrogen-treatment since Batra & Andersson (1989) produced a reduction in muscarinic receptor density of 90% after 4 weeks. Anderson & Marks (1982) showed that only a small fraction of the cholinoceptors needed to be occupied to produce contractile responses, and thus there was a large receptor reserve. They further argued that the rate limiting step for regulation of the contractile response to carbachol was neither muscarinic receptor occupation nor membrane calcium channel opening but the intracellular mechanisms which regulate the responsiveness of the myofibrils to calcium ions.

In conclusion, pretreatment with oestradiol in rats significantly reduced muscarinic receptor-stimulated contractions of detrusor muscle although the exact mechanism by which this was brought about is uncertain. The addition of DES directly to the organ bath in pretreated animals caused a further decrease in contractile response. This combined effect of oestradiol has not been shown before but if confirmed in man would clearly mimic the situation in women given oestradiol therapy long-term for over-activity of the detrusor muscle. In such women detrusor contractions are associated with urge incontinence and thus pretreatment with oestradiol may have a very significant clinical role in the control of urinary incontinence.

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The direct effects of diethylstilboestrol and nifedipine on the contractile responses of isolated human and rat detrusor muscles

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Summary. We have studied the direct effect of 2 $\mu mol \cdot l^{-1}$ diethylstilboestrol on isolated rat and human detrusor muscles. Diethylstilboestrol significantly reduced the amplitude of the contractile response of rat detrusor muscle to stimulation with acetylcholine, carbachol, electrical field stimulation, and 5-hydroxytryptamine. In isolated human bladder it also significantly reduced contractions stimulated with acetylcholine, carbachol, and electrical field stimulation. In depolarized rat detrusor muscle stimulated with different concentrations of calcium ions, the contractile responses were significantly reduced by the addition of diethylstilboestrol. Diethylstilboestrol also significantly reduced the amplitude of contractile response to potassium chloride. The inhibitory action of diethylstilboestrol was enhanced by the reduction of extracellular calcium ions, the maximum contractile response to acetylcholine, carbachol, and electrical field stimulation being reduced by a further 32%, 23%, and 45% respectively. Diethylstilboestrol did not have a significant effect on carbachol-induced contractions in depolarized rat detrusor muscle suspended in a calcium-free environment. Diethylstilboestrol was effective in blocking rat and human detrusor muscle contraction. The likely mechanism is a reduction of the influx of calcium ions into the cell during contraction rather than an effect on intracellular calcium release. These results give support for treating incontinent patients with drugs that block calcium ion uptake, and may suggest a further beneficial effect of oestrogen therapy in postmenopausal women.

Key words: Oestrogen, Nifedipine, Muscle contraction; bladder; acetylcholine; carbachol; 5-hydroxytryptamine

Urinary incontinence affects between 5 and 10% of old people in the community and up to 50% in institutions. The prevalence is considerably higher in women than in men [1]. Most patients with urinary incontinence have detrusor instability, in which powerful detrusor muscle contractions overcome urethral sphincter closure pressure [2]. Current pharmacological intervention is therefore aimed at blocking or suppressing these contractions with anticholinergic drugs, antispasmodics, and calcium antagonists [3].

A line of therapy hitherto unexplored scientifically, although widely used in some countries, is oestrogen therapy [4]. Such treatment is commonly used for atrophic urethritis and stress incontinence [5], but there is little information on its potential use in detrusor instability. Studies in rabbits have shown that there are oestrogen receptors in detrusor muscle, and that treatment with oestrogens can reduce muscarinic receptor density [6, 7]. It has also been shown in these animals that oestrogens shift the carbachol dose-response curve to the right. Preliminary studies in man have confirmed that there are oestrogen receptors in detrusor muscle [8, 9], but such studies clearly needed to be extended before this potentially beneficial treatment could be used rationally in man. We have therefore compared the direct effects of an oestrogen, diethylstilboestrol, on detrusor muscles in rat and man.

Materials and methods

Rat experiments

Virgin female Wistar rats (150–200 g) in the dioestrus phase, as judged from vaginal smears, were killed by a blow on the head and exsanguinated. The bladders were removed and two strips per bladder were placed in Krebs solution (see below). After the removal of fat and serosa, strips of muscle (7 mm × 4 mm) were suspended in a 50 ml organ-bath chamber containing Krebs solution at 37°C, aerated with 95% oxygen and 5% carbon dioxide.

The base of the muscle strip was fixed to a hook in the chamber and the apex was attached by a thread to an isometric transducer connected to a two-channel Washington oscillograph. The tissues were allowed to equilibrate for 1 h under a tension of 10 mN before the addition of any drugs. Acetylcholine (10^{-8} to 2×10^{-4} $mol \cdot l^{-1}$), carbachol (10^{-8} to 10^{-4} $mol \cdot l^{-1}$) or potassium chloride (KCl) (10 $mmol \cdot l^{-1}$ to 60 $mmol \cdot l^{-1}$) were each injected into the bath in a cumulative manner to obtain dose-response curves. 5-hydroxytryptamine (10^{-8} to 10^{-5} $mol \cdot l$) was injected at 5 min intervals and the preparation was washed between doses to avoid tachyphylaxis.

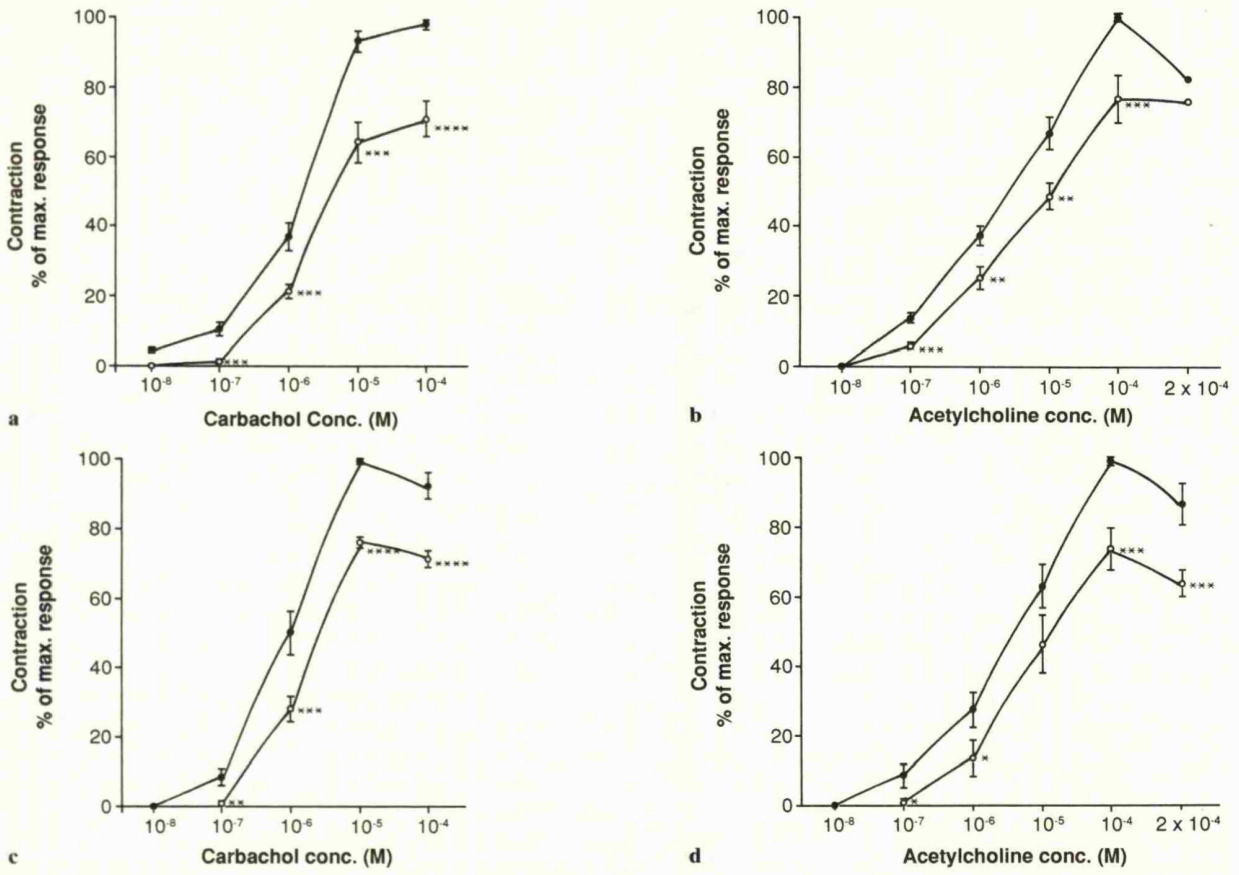

Fig. 1 a–d. The effect of 2 μmol·l^{-1} of diethylstilboestrol on the carbachol (**a**) and acetylcholine (**b**) dose-response curves of isolated rat detrusor muscle, and on the carbachol (**c**) and acetylcholine (**d**) dose-response curves of isolated human detrusor muscle. ● = control, ○ = after the addition of diethylstilboestrol. Vertical bars represent the standard error of the mean ($n = 5$). **** = $P < 0.001$, *** = $P < 0.01$, ** = $P < 0.05$; * = $P < 0.10$

When consistent dose-response curves had been obtained, diethylstilboestrol 2 μmol·l^{-1} was added to the bath. This concentration was used because the concentrations produced in the organ-bath chamber could have been produced pharmacologically in women. When dose-response curves were repeated an incubation time of 10 min was allowed between them in every case.

Human detrusor muscle

Bladder muscle biopsies were obtained from women with healthy bladders undergoing routine gynaecological operations. Muscle strips (7 mm × 4 mm) were removed from the fundus of the bladder at the time of abdominal hysterectomy. These women had not taken any oestrogens before surgery. Full informed consent was obtained. The local ethics committee gave permission for the study.

Biopsy samples were immediately placed into Krebs solution, and taken to the laboratory. They were mounted in the organ-bath chamber and treated in the same way as the rat samples.

Electrical field stimulation

For these experiments the bladder strips were passed through two parallel circular electrodes connected to a Digitimer stimulator. The stimulator delivered 1–80 pulses per second at 4–6 volts with a 1 msec pulse width in 10 s trains at 2 min intervals. A frequency response curve was obtained by stimulating the tissue with 1, 5, 10, 20, 40, 60, and 80 pulses per second. When consistent curves were obtained, diethylstilboestrol 2 μmol·l^{-1} was injected into the bath and the frequency-response curves were repeated.

Depolarized preparations

After equilibration the samples were stimulatetd with acetylcholine 10^{-4} mol·l^{-1} at 10 min intervals until consecutive responses were almost the same. This was taken to be the maximum contractile response. The tissues were depolarized by placing them into calcium-free potassium-rich Krebs solution containing 127 mmol·l^{-1} KCl and 1.2 mmol·l^{-1} EGTA to reduce the concentration of free calcium ions in the external medium. This resulted in an initial contraction followed by relaxation.

After 90 min of equilibration, during which the preparation was washed twice, the strips were stimulated with increasing concentrations of calcium ions (0.1 mmol·l^{-1} to 1.5 mmol·l^{-1}), to obtain a dose-response curve. The preparations were then washed in calcium-free potassium-rich Krebs solution for a further 15 min and the procedure was repeated 10 min after the addition of diethylstilboestrol 2 μmol·l^{-1}.

For preparations suspended in a low-calcium medium, the concentration of calcium chloride in Krebs solution was reduced to 0.3 mmol·l^{-1}. In depolarized and low-calcium experiments, the ef-

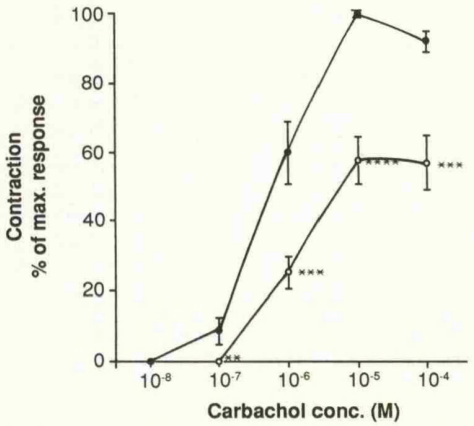

Fig. 2. The effect of 0.03 μmol·l^{-1} nifedipine on the carbachol dose-response curve of isolated human detrusor muscle. ● = control, ○ = after the addition of nifedipine. Vertical bars represent the standard error of the mean ($n = 5$). **** = $P < 0.001$, *** = $P < 0.01$, ** = $P < 0.05$

fects of the calcium antagonist nifedipine (0.03 μmol·l^{-1}) were also investigated and compared with those of diethylstilboestrol. This concentration reflected those in the plasma after therapeutic doses.

Solutions and chemicals

Krebs solution contained (mmol·l^{-1}): NaCl 119, KCl 4.4, $NaHCO_3$ 20, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.5, glucose 11.

Calcium-free potassium-rich Krebs solution contained (mmol·l^{-1}): KCl 127, $NaHCO_3$ 20, NaH_2PO_4 1.2, $MgCl_2$ 1.2, EGTA 0.01, glucose 11.

Low-calcium Krebs solution contained (mmol·l^{-1}): NaCl 119, KCl 4.6, $NaHCO_3$ 20, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 0.3, glucose 11.

Acetylcholine chloride (Sigma), carbamylcholine chloride (Sigma), and 5-hydroxytryptamine (Sigma) were all dissolved in distilled water and made up on the day of the experiment. Diethylstilboestrol (Sigma) and nifedipine (Sigma) were dissolved in ethanol. The concentration of ethanol in the organ bath chamber did not exceed 3 mmol·l^{-1}.

Nifedipine was kept in a darkened container.

For each experiment the results are the means of five different bladder samples. Different bladders were used for each agonist. Statistical analyses were carried out using Student's *t*-test. The individual dose-response curves for acetylcholine and carbachol were drawn by hand and the EC_{50} values were calculated graphically by determining the concentration of agonist required to produce a 50% response for each dose-response curve. These concentrations were read from the graphs as log concentrations. Individual values were meaned and the SEM established statistically. Comparisons of the EC_{50} values were carried out using Student's *t*-test. In the presence of diethylstilboestrol the values were 50% of the maximum response; all curves were scaled on their own maxima.

Results

Effects of diethylstilboestrol and nifedipine on carbachol- and acetylcholine-induced responses

Rat detrusor muscle showed rhythmic spontaneous contractions when set up in Krebs solution. This activity was maintained for up to 7 h. Diethylstilboestrol 2 μmol·l^{-1} did not alter these contractions, but 20 μmol·l^{-1} totally abolished them. Carbachol and acetylcholine produced a rapidly developing contraction which was dose-dependent. Diethylstilboestrol 2 μmol·l^{-1} caused a 30% reduction in the maximum contractile response for carbachol ($P < 0.001$) and a 25% reduction for acetylcholine ($P < 0.01$). The carbachol dose-response curve, but not that for acetylcholine, showed a significant shift to the right, with an increase in the mean log EC_{50} from 1.8 (SD 0.5) × 10^{-6} mol·l^{-1} to 5.4 (1.6) × 10^{-6} mol·l^{-1} ($P < 0.01$) (Fig. 1 a, b).

Human bladder muscle did not exhibit spontaneous contractile activity, and its response to stimulation with carbachol and acetylcholine was less rapid than that of rat detrusor muscle. However, 2 μmol·l^{-1} of diethylstilboestrol inhibited the human detrusor contractile response. The dose-response curve for carbachol, but not for acetylcholine, showed a significant shift to the right, with an increase in the mean log EC_{50} from 1.3 (0.5) × 10^{-6} mol·l^{-1} to 4.8 (0.6) × 10^{-6} mol·l^{-1} ($P < 0.001$). The maximum contractile response was reduced by 26% to acetylcholine and by 23% to carbachol ($P < 0.001$) (Fig. 1 c, d).

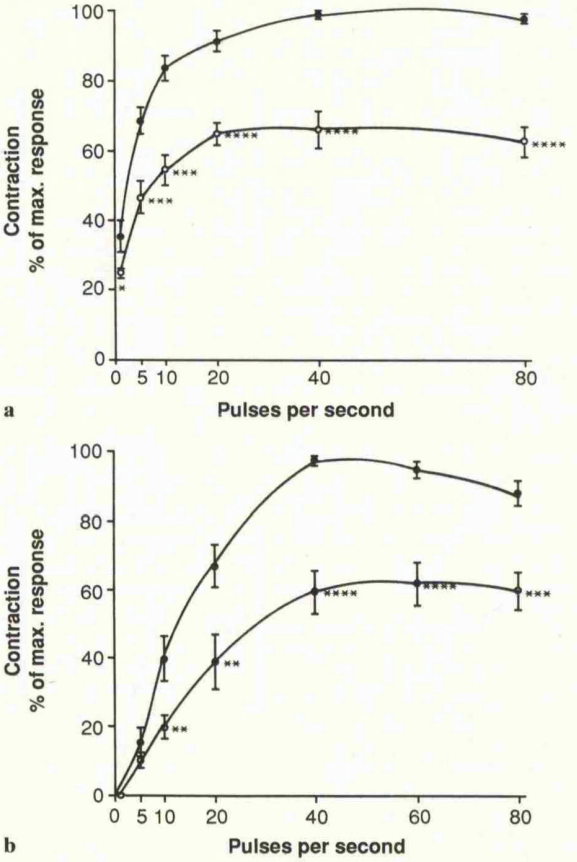

Fig. 3 a, b. The effect of 2 μmol·l^{-1} of diethylstilboestrol on the electrically-induced frequency dose-response curve of isolated rat (**a**) and human (**b**) detrusor muscle. ● = control, ○ = after the addition of diethylstilboestrol. Vertical bars represent the standard error of the mean ($n = 5$). **** = $P < 0.001$, *** = $P < 0.01$, ** = $P < 0.05$; * = $P < 0.10$

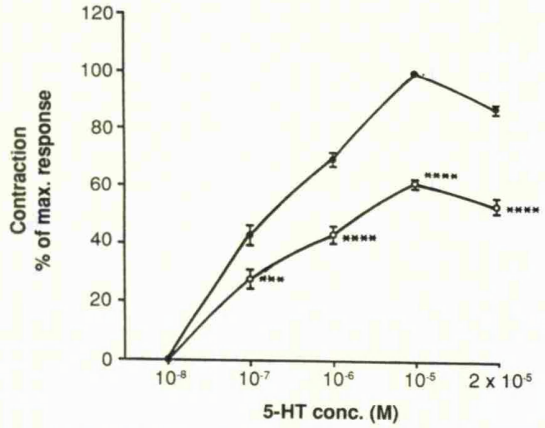

Fig. 4. The effect of 2 μmol·l^{-1} of diethylstilboestrol on the 5-HT-induced dose-response curve of isolated rat detrusor muscle. ● = control, ○ = after the addition of 2 μmol·l^{-1} of diethylstilboestrol. Vertical bars represent the standard error of the mean ($n = 5$). **** = $P < 0.001$, *** = $P < 0.01$

The effect of nifedipine, 0.03 μmol·l^{-1}, on carbachol-induced responses in human detrusor samples was similar to that of diethylstilboestrol, with a 39% reduction in the maximum response ($P < 0.001$) (Fig. 2).

Effects of diethylstilboestrol on contraction in response to electrical field stimulation

Rat detrusor muscle showed frequency-dependent contractile responses to electrical stimulation. The responses were rapid and relaxation was immediate on withdrawal of stimulation. Diethylstilboestrol 2 μmol·l^{-1} significantly reduced rat detrusor muscle contraction in response to electrical field stimulation ($P < 0.001$). The maximum contractile response was reduced by 33% (Fig. 3a).

Human bladder muscle showed similar inhibition, with a reduction in the maximum contractile response of 32% ($P < 0.001$) (Fig. 3b).

Effect of diethylstilboestrol on 5HT stimulation

Rat detrusor muscle had a slowly developing phasic response to stimulation with 5-hydroxytryptamine. The maximum response was much less than with cholinergic or electrical stimulation. The inhibitory action of diethylstilboestrol was more potent on 5HT-induced contractions than on cholinergic or neuronally-evoked responses, with a reduction in the maximum contractile response of 40% ($P < 0.001$) (Fig. 4).

Effect of diethylstilboestrol and nifedipine on calcium-induced contractions

When rat detrusor muscle was placed in calcium-free potassium-rich Krebs solution there was an immediate contraction followed by relaxation. The rhythmic spontaneous contractions normally seen in rat detrusor preparations were also abolished. The contractile response to the readdition of calcium reached a maximum at a concentration of 1.5 mmol·l^{-1}. The addition of 2 μmol·l^{-1} of diethylstilboestrol to the external medium resulted in inhibition of these contractions. The maximum response was reduced by 40% ($P < 0.001$) (Fig. 5a). Nifedipine 0.03 μmol·l^{-1} completely abolished the rat detrusor response to calcium stimulation and a concentration of 0.01 μmol·l^{-1} reduced the maximum response by 72% (Fig. 5b).

Effect of diethylstilboestrol and nifedipine on carbachol- and acetylcholine-induced contractions in a low-calcium medium

Rhythmic spontaneous contractions in the rat detrusor muscle were maintained in a low-calcium (0.3 mmol·l^{-1}) Krebs solution. However, the frequency and magnitude of this activity were slightly reduced when compared with tissue exposed to an external medium of normal calcium content (2.5 mmol·l^{-1}). The inhibitory effect of 2 μmol·l^{-1} of diethylstilboestrol on carbachol- and ace-

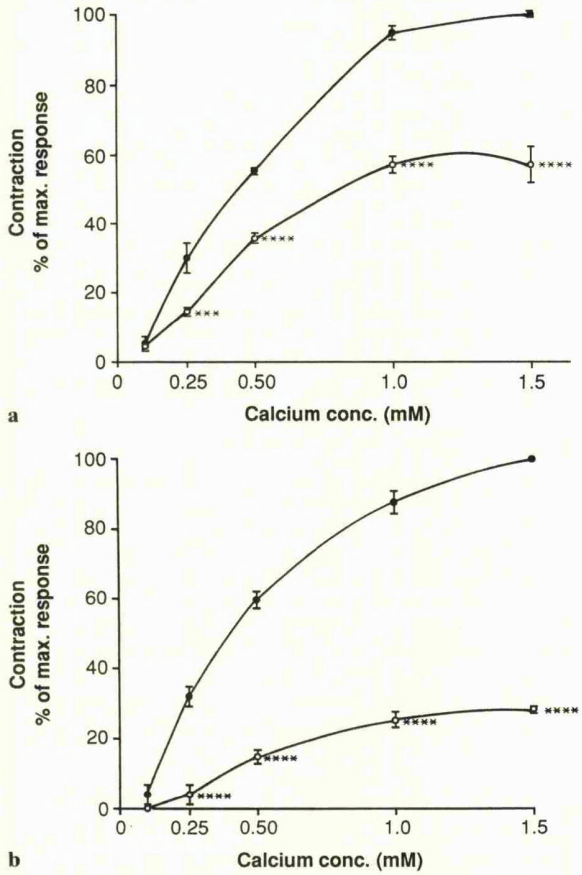

Fig. 5a, b. The effect of 2 μmol·l^{-1} of diethylstilboestrol (**a**) and 0.01 μmol·l^{-1} of nifedipine (**b**) on the calcium-induced dose-response curve of depolarized rat detrusor muscle. ● = control, ○ = after the addition of diethylstilboestrol or nifedipine. Vertical bars represent the standard error of the mean ($n = 5$). **** = $P < 0.001$, *** = $P < 0.01$

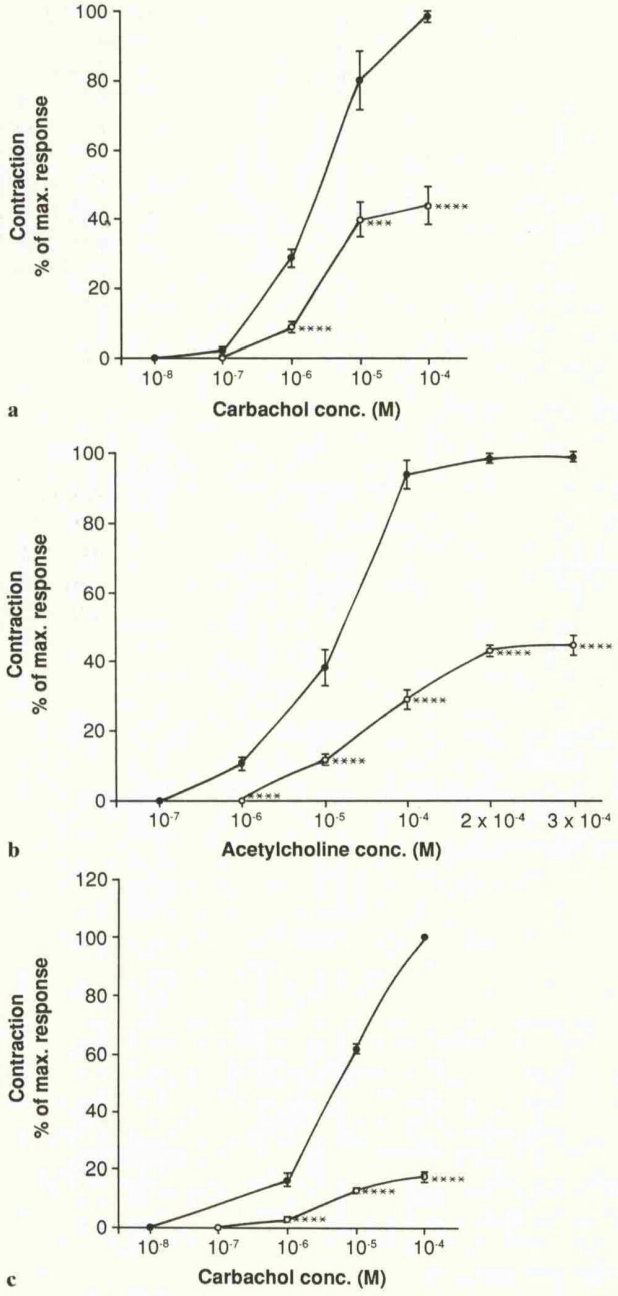

Fig. 6a–c. The effect of 2 μmol·l^{-1} of diethylstilboestrol on (**a**) carbachol and (**b**) acetylcholine dose-response curves, and (**c**) the effect of 0.03 μmol·l^{-1} nifedipine on the carbachol-induced dose-response curve of isolated rat detrusor muscle suspended in a low-calcium medium. ● = control, ○ = after the addition of 2 μmol·l^{-1} of diethylstilboestrol or nifedipine. Vertical bars represent the standard error of the mean, ($n = 5$). **** = $P < 0.001$, *** = $P < 0.01$

tylcholine-induced contractions was increased when the calcium content of the external medium was reduced. The dose-response curves for these agonists also showed a 53% reduction in the maximum response for carbachol ($P < 0.01$) (Fig. 6a) and a 55% reduction for acetylcholine ($P < 0.001$) (Fig. 6b).

For comparison, the effect of a calcium antagnist, nifedipine 0.03 μmol·l^{-1}, on the carbachol dose-response curve in low-calcium medium was investigated. The results were similar to those of the diethylstilboestrol experiments. However, nifedipine had a more potent inhibitory effect, with an 80% reduction in the maximum response (Fig. 6c).

Effect of diethylstilboestrol on electrical field induced contraction in a low calcium ion medium

Diethylstilboestrol 2 μmol·l^{-1} had a striking inhibitory effect on electrical field-induced contractions in a low-calcium environment. The maximum response was reduced by 78% ($P < 0.001$) (Fig. 7). The control contractile responses to electrical stimulation were also reduced in a low-calcium medium, but only by 17%.

Effect of diethylstilboestrol on KCl-induced contractions

The cumulative dose-response curves to KCl showed a significant reduction in the maximum response after the addition of 2 μmol·l^{-1} of diethylstilboestrol ($P < 0.01$). The maximum response was reduced by 30%. This inhibitory effect was observed at low and high KCl concentrations (Fig. 8).

Effect of diethylstilboestrol on carbachol-induced contractions in depolarized rat detrusor muscle

Before the addition of each dose of carbachol, the bladder tissues were primed with a low concentration of calcium (0.3 mmol·l^{-1}) for 10 min to replace released calcium and then washed with calcium-free Krebs. After 5 min carbachol (10^{-4} mol·l^{-1}) was injected into the bath and the response was recorded. The response before and after the addition of diethylstilboestrol were not significantly different.

Discussion

These preliminary in vitro studies support a possible role of oestrogens in the treatment of female patients with motor-urge incontinence secondary to detrusor instability. The results also corroborate previous work on the likely beneficial use of nifedipine in this condition [3]. It is clear that diethylstilboestrol had a significant inhibitory effect on rat and human detrusor muscle contractions when added directly to the bathing solution.

Our results suggest that diethylstilboestrol affected the movement of extracellular calcium ions into detrusor muscle cells. Nifedipine is known to inhibit calcium influx into smooth muscle cells from the surrounding medium [10]. Our results strongly suggest that diethylstilboestrol had a similar, although less potent, effect. In the presence of a high external potassium concentration, to open potential-dependent calcium channels, diethylstilboestrol re-

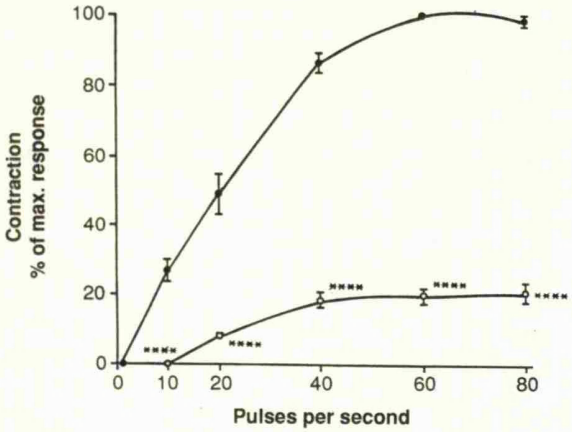

Fig. 7. The effect of 2 μmol·l^{-1} of diethylstilboestrol on the electrically-induced frequency-response curve of isolated rat detrusor muscle suspended in a low-calcium medium. ● = control, ○ = after the addition of diethylstilboestrol. Vertical bars represent the standard error of the mean ($n = 5$). **** = $P < 0.001$

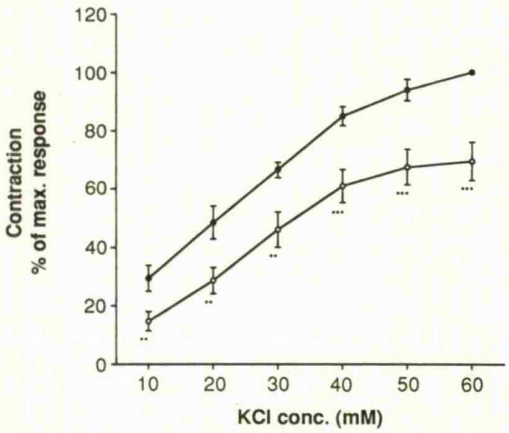

Fig. 8. The effect of 2 μmol·l^{-1} of diethylstilboestrol on the KCl dose-response curve of isolated rat detrusor muscle. ● = control, ○ = after the addition of diethylstilboestrol. Vertical bars represent the standard error of the mean ($n = 5$). *** = $P < 0.01$, ** = $P < 0.05$

duced the maximum response to increasing concentrations of calcium. Furthermore, the effects on carbachol, acetylcholine, 5-HT, and electrical field stimulation were more marked if the calcium concentration of the surrounding medium was kept low. Finally, the inhibitory effect of diethylstilboestrol on KCl-induced contractions suggests a selective effect on calcium influx, since extracellular calcium is the sole source of calcium ions for these contractions in human bladder muscle [11]. Batra & Bengtsson [12] came to similar conclusions on the action of diethylstilboestrol on rat uterine muscle, although they used concentrations which were ten times higher. It is possible that a higher concentration of diethylstilboestrol might have had a greater inhibitory effect on detrusor muscle stimulation, since 20 μmol·l^{-1} of diethylstilboestrol abolished spontaneous activity in the rat detrusor muscle.

Although all the stimulatory mechanisms used in these experiments might have involved cholinergic receptors [13, 14, 15, 16], it is unlikely that diethylstilboestrol had its effect on them. Electrical field stimulation produces its effect through both cholinergic and non-cholinergic neurotransmission in the rat, the latter almost certainly being via ATP [13]. However, in man there appears to be little non-cholinergic effect of electrical field stimulation [14, 15], and so a far greater effect would have been seen in man than in rat if diethylstilboestrol influenced muscarinic receptors. However, the effects were similar in the two species. We found no evidence that diethylstilboestrol altered the activity of cholinesterase, since its effects on acetylcholine and carbachol were similar.

We did not find evidence of inhibition by diethylstilboestrol of calcium release from intracellular stores, because when the detrusor muscle samples were depolarized and primed with a low concentration of calcium, diethylstilboestrol did not affect the contractile response to carbachol. Mostwin [17] has previously shown that muscarinic receptor activation by carbachol can release calcium ions from the intracellular calcium ion store in detrusor smooth muscle. Thus, carabchol can contract the detrusor muscle despite the inactivation of external calcium ion transport mechanisms [11].

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