University of Leicester

Characterisation of P2Y receptor-mediated contractile signalling and its regulation by

G protein coupled receptor kinases and arrestin proteins in a rat bladder smooth muscle

Thesis Submitted for the Degree of

Doctor of Philosophy (PhD)

By

Dr Amal H I Bengreed

Department of Cancer Studies, University of Leicester

September 2017

Supervisors

Dr Jonathon Willets,

Professor Douglas Tincello

I.   Abstract

ATP released from parasympathetic nerves can mediated bladder contraction, can activate purinergic $P2Y/G_{q/11}$-coupled G protein-coupled receptors (GPCR) expressed on detrusor (bladder) smooth muscle cells (DSMC). $P2Y/G_{q/11}$ signalling activates phospholipase C (PLC) and increases intracellular calcium concentrations to induce contraction. DSMC contractile GPCR activity is tightly regulated to prevent inappropriate contraction/incontinence. Additionally, GPCRs activity is regulated by G protein-coupled receptor kinase (GRK) and arrestin proteins, it is likely that they play a similar role in DSMC, and may help to maintain continence.

Combining confocal imaging, calcium-sensitive dyes and selective P2X and P2Y receptor agonists/antagonists, showed that after 3-4 days in culture DSMC calcium signals were mediated by $P2Y_1$ and $P2Y_2$, but not P2X, $P2Y_4$ or $P2Y_6$ receptors. Repeated agonist additions indicated a desensitization of $P2Y_1$ and $P2Y_2$ activated phospholipase C (PLC)/Ca^{2+} signals, which was restored when the washout period between agonist challenges was increased. Transfection of DSMC with dominant-negative, catalytically inactive GRK mutants, which block endogenous GRK function, showed that $P2Y_1$ and $P2Y_2$ receptor stimulated calcium signalling was selectively regulated by GRK3 and GRK2, respectively. Furthermore, desensitization of $P2Y_1$ and $P2Y_2$ receptor PLC/Ca^{2+} was attenuated following RNAi-mediated knockdown of arrestin2 or arrestin3, suggesting both arrestins were able to regulate $P2Y_{1/2}$ receptor signalling. To mimic the effects of obstructive bladder, DSMC were mechanically stretched which resulted in increased GRK2, and decreased GRK3/5/6 expression.

These data show that DSMC express functional $P2Y_1/P2Y_2$ receptors which mediate purinergic agonist PLC/Ca^{2+} signalling, implying roles for $P2Y_1$ and $P2Y_2$ in bladder contraction and voiding. Furthermore, $P2Y_1$ and $P2Y_2$ receptor are selectively regulated by GRK and arrestin proteins, which suggests that GRK and arrestin proteins play an important role in the regulation of bladder tone. Furthermore, since GRK expression following mechanical stretch this may in turn affect GPCR signalling and produce dysregulation of DSMC contraction observed during incontinence.

II.   Acknowledgements

III.   Dedication

To **Dr Amar Eltweri** for his patience and support which made this hard work come to fruition. Thank you for supporting me and for everything. Special thanks go to my father **Mr Hussein Jrid** and my mother **Mrs Amal Bintaboun** for making me smile when things got tough. Thank you for supporting me and thanks for my brothers and sisters for supporting me. My sincerest appreciation goes to my husband **Dr Amar Eltweri**, without him it would be difficult to reach this point, thanks for believing me and supporting me, thanks for putting up with me working countless late nights. To my lovely daughter **Afnan** and my son **Abdulhadi** for brightening my days and always managing to put a smile on my face, which gave me the determination to complete this work, thanks for your love and understanding. And I would also like to extend my warmest thanks go to my father-in-law **Mr Mohamed Eltweri** and mother-in-law **Mrs Baia Shams** for encouraging me to do my best and supporting me through my PhD.
I am thankful to my lovely friends **Torea, Kaltom** and **Hoda** for looking after my kids all through my PhD without them I couldn't finish this work,

IV.   Presentations

Identification of which P2 receptor subtypes mediating purinergic Ca^{2+} signals in isolated rat detrusor smooth muscle sells
Presented at

1. Midlands Academy of Medical Sciences Research Festival, Leicester, UK, (April 2015)
2. UK, Purine club 6th annual meeting, Leicester, UK, (September 2015)
3. British Pharmacology society meeting, London,UK, (December2016)

The following abstract presentations will be submitted for presentation at the next British Pharmacology Society:

1. **Characterisation of purinergic receptor subtype expression and activity in rat detrusor smooth muscle**

2. **Desensitization and resensitization of purinergic receptors and Expressions of GRKs protein in detrusor Smooth muscle of rat bladder**

3. **Characterising the roles that arrestin proteins play in the regulation P2Y-purinoceptor PLC signalling in detrusor smooth muscle cells**

4. **Characterising the effects of mechanical stretch on GRK and arrestin protein expression in isolated DSMC**

V. Table of contents

VI.   List of figures

VII.   List of Tables

VIII.   List of abbreviation

ADP	Adenosine diphosphate
Ach	Acetylcholine
ATP	Adenosine Triphosphate
A1CT	Antiarrestin 2 antibody
ASMC	Aortic smooth muscle cells
ANOVA	Analysis of variance
BMI	Body Mass Index
BPH	Benign prostate hyperplasia
BSA	Bovine serum albumin
βAR	Beta arrestin
Ca^{2+}	Calcium ions
Ca^{2+}_i	Intracellular calcium
cAMP	Cyclic adenosine monophosphate
$CaCl_2$	Calcium Chloride
CICR	Calcium induced calcium release
CaM	Calmodulin
CO_2	Carbone Dioxide
CLSM	Confocal laser scanning microscopy
DSM	Detrusor smooth muscles
DSMC	Detrusor smooth muscle cell
DMSO	Dimethyl sulphoxide
DAG	Diacylglycerol
DAB	3, 3'-diaminobenzidine
DDT	Dichlorodiphenyltrichloroethane
D-PBS	Dulbecco`s phosphate buffer saline
DS	Dissection Solution
DNM	Dominant negative
DNM GRKs	Dominant-negative mutant GRKs
Em	Emission
ECL	Enhanced chemiluminescent
ETA	Endothelin type A
EDTA	Ethylenediaminetetraacetic acid
Fluo 4-AM	Fluo-4-acetoxymethyl ester
EC50	Half maximal effective concentration
eGFP	Enhanced green fluorescent protein
eGFP-PH	Enhanced green fluorescent protein- Pleckstrin homology
F	Fluorescence
F0	Basal fluorescence
FBS	Foetal bovine serum
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
Fluo4-AM	Fluo-4-acetoxymethyl ester
GEFs	Guanine nucleotide exchange factors
GPCR	G protein-coupled receptors
GRK	G protein-coupled receptors Kinases
H_2O_2	Hydrogen peroxide
H	Hour
H_1	Histamine receptor

HEPES	Hydroxyethyl-1-piperazine ethanesulfonic acid
ICS	International continence society
IU	International Unit
IC	Interstitial Cystitis
IC50	Half maximal inhibitory concentration
IP3	1,4,5-triphosphate
JNK	c-Jun N-terminal kinase
KCL	Potassium Chloride
kDa	Kilo delta
LUTS	Lower urinary tract symptoms
mM	Millimolar
ML	Milli Litre
μL	Micro litre
MLCK	Myosin light chain kinase
MLCP	myosin light chain phosphatase
MCP-1	Monocyte chemo-attractant protein -1
M	Muscarinic receptors
MRI	Magnetic resonance imaging
MLC	Myosin light chain
Mgcl2	Magnesium Chloride
mAChR	Muscarinic acetyl choline receptor
mRNA	Messenger Ribonucleic acid
mm^2	Millimetre square
μm	Micro molar
μg	Micro gram
nM	Nano meter
NaH_2PO_4	Sodium Dihydrogen Phosphate
NC	Negative control
NaCl	Sodium Chloride
NICE	National institute for health and care excellence
NGF	Nerve growth factor
NOBLE	National Overactive Bladder Evaluation
OAB	Overactive bladder
P2	Purinergic receptors
PBS-T	Phosphate Buffered Saline–Triton
pcDNA	plasmid control DNA
PIP2	Phospholipids
PKC	Protein Kinase C
PLC	phospholipase C
PMT	Photomultiplier detector
PKA	Protein Kinase A
P value	Probability value
pcDNA3	Plasmide control DNA3
R	Response
R1	First response
R2	Second response
RFP	Red florescent protein
R_{max}	Maximum response
RGS	Regulator of G protein signalling
RNA	Ribonucleic acid

RNAi	RNA interference
RPM	Revolution per minute
RTX	Resiniferatoxin
siRNA	Small/short interfering RNA
Sec	Seconds
SR	Sarcoplasmic Reticulum
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS- Polyacrylamide gel electrophoresis
SSRI	Selective Serotonin reuptake inhibitor
SIGN	Scottish intercollegiate guidelines network
SEM	Standard error of mean
TBS-T	Tric buffered saline with Tween 20
TNP-ATP	2', 3'-O-trinitrophenyl-Adenosine triphosphate
UK	United Kingdom
UTI	Urinary Tract Infection
USS	Ultrasound scan
UTP	Uridine triphosphate
USA	United states America
UTP	Uridine Triphosphate
ULTR	Uterine smooth muscle cell lines
v/v	Volume/volume
w/v	Weight/volume

Chapter 1

1. **General Introduction**

1. General Introduction:

1.1. Overactive Urinary Bladder

Overactive urinary bladder (OAB) also known as overactive bladder syndrome (OBS) as defined by the international continence society (ICS) is a condition that is characterised by abrupt, involuntary contraction of the detrusor smooth muscles of the urinary bladder which leads to a strong, sudden desire to urinate which is difficult to suppress even if the bladder contains small volume of urine (1). It is usually accompanied by urinary frequency and nocturia without signs of urinary tract infection or other obvious pathology (2,3). The unpredictability of the urge to urinate can be disruptive to people activities and as a result it affects the patient's quality of life (3). OAB is often described as a subcategory of urge urinary incontinence (4).

1.2. Different types of urinary incontinence

The most common types of incontinence (1) are as follow:

1. Stress incontinence; it is characterised by leakage of urine during increase in the intra-abdominal pressure such as in coughing, laughing, sneezing, and straining.

2. Urge incontinence; it is the involuntary urine leakage following the sudden compelling need to micturate. If it is associated with urinary frequency and nocturia can be described as overactive urinary bladder.

3. Overactive urinary bladder; it is an abrupt, involuntary contraction of the detrusor smooth muscles of the urinary bladder which leads to a strong and sudden desire to urinate which is difficult to suppress even if the bladder contains a small volume of urine and associated with frequency and nocturia.

4. Overflow incontinence; it is when the urinary bladder is full but the patient are only able to release small amount of urine

5. Mixed incontinence; it is a combination of several or all of the above.

1.3. Prevalence and burdens of OAB

1.3.1. Prevalence

The overactive urinary bladder is a commonly prevalent condition that affects the lives of millions of people around the world (5). As many people do not seek medical advice and are not aware that OAB is a treatable condition, it is difficult to estimate the accurate prevalence of OAB (5).

In 2001, Milsom et al, in a population based prevalence study in six European countries reported the overall prevalence of OAB was approximately 17% among female and 16% among male patients (6). In United Kingdom, the prevalence of OAB was reported as 20% in female and 19% in male patients (6), women were more likely to report OAB symptoms than men and the presence of OAB symptoms increased with age (6).

In the EPIC study one of the largest population based surveys that estimated the prevalence of OAB, conducted in Canada, Germany, Sweden, Italy and the UK, the overall prevalence of OAB was approximately 12%, women were more likely to report symptoms than men and the OAB prevalence increased with age (7).

In the National Overactive Bladder Evaluation (NOBLE) study, the overall prevalence of OAB was approximately 16% with no significant difference between both genders, the NOBLE study showed that specific OAB symptoms were less in men than women but with increasing age these symptoms became more predominant in men, especially over the age of 60 years (8). The prevalence of OAB with or without urge incontinence is considerably different between both genders. In male, the prevalence of OAB with urge incontinence was significantly less than those without urge incontinence (2.6% and 13.4%, respectively). Whereas, in female it was 9.3% and 7.6% for OAB with and without urge incontinence, respectively (8). The prevalence of OAB with urge incontinence increases with age and it was reported to significantly increase from 2% to 19% after the age of 44 years in female and from 0.3% to 9% in male after the age of 64 years (8).

In the NOBLE study, Stewart et al discovered an association between OAB with urge incontinence and body mass index (BMI) more than 30 in women, these patients were approximately two times more likely to develop OAB with urge incontinence than women with BMI less than 24 (8).

1.3.2. Burdens related to OAB and its cost

Overactive urinary bladder has a significant impact on quality of life, higher depression rate, and poor quality of sleep (8). Wagner et al in 2002 reported that patients who suffered from OAB had 20% more visits to their physician and 138% more urinary tract infection (UTI) in a year than people without OAB (4). Additionally, there was an increase in the risk of injuries such as bone fractures from fall (4).

The cost of UTIs associated with OAB in United States was about $1.37 billion in year 2000 and the cost of falls with bone fracture accounts for approximately $386 million in the same year (4). In the USA, Onukwugha et al reported the disease specific total expenditure of the economic costs of managing OAB syndrome in 2009 were approximately 25 billion united state dollars (9).

1.1. Diagnosis of overactive bladder

There are different guidelines which can be approached in making a diagnosis of overactive urinary bladder (1,2,10). Simply, these guidelines using a multistep approach for detailed assessment as follow:

1.1.1. History

This is usually the first step, which can be achieved in the first consultation with the patient looking for the overactive bladder symptoms as detailed above. It is very important to differentiate between symptoms of OAB and other causes of nocturia, frequency and incontinence (11). For example; the nocturia can be associated with certain medical conditions such as diabetes mellitus, cardiovascular diseases. Urinary frequency can be the result of polydipsia which causes polyuria that may resemble OAB, this can be differentiated using the frequency/volume charts as described in the literature (3,10). History taking should include any previous history of pelvic surgery, obstetric and gynaecological procedures as well as co-morbidities.

1.1.2. Examination

The examination should focus on abdominal and pelvic organs; this should include rectal examination in both male and female and vaginal examination for assessment of the pelvic organs to exclude any pelvic masses, or malignancy. Neurological examination should also be performed (10,11).

1.1.3. Investigation
1.1.3.1. Urine analysis

Urine dipstick is an initial bedside test that should be performed in all patients, simply to diagnose or exclude certain conditions such as urinary tract infection and diabetes mellitus as microscopic haematuria which could be a sign of malignancy or other bladder pathology (10). In certain patients urinalysis is not always reliable, hence urine culture should be performed when in doubt (2,11).

1.1.3.2. Bladder diaries

Urinary bladder diaries can be useful tool in monitoring daily intake and bladder emptying, particularly in documenting the baseline symptoms and assessing the progress of the treatment (1,2,11).

1.1.3.3. Urodynamic studies

Urodynamic studies should be performed to exclude other urinary bladder pathology if conservative or medical management of OAB was failed (10) and this includes the following:

1.1.3.3.1. Uroflowmetry

This have a high degree of accuracy (+/- 5%) in assessment of urine flow and the flow rate as well as the voidal volume (1).

1.1.3.3.2. Post-void residual urine volume

It is the volume of urine left in the bladder completion of micturition (1). This can be measured using Ultrasound scan, and it was quoted the upper limit of normal of 30 ml and using urethral catheterization with an upper limit of approximately 50 ml (1).

1.1.3.4. Ultrasound scan (USS)

Bladder wall thickness assessment using ultrasound scan has been suggested as a diagnostic measure for detrusor overactivity (10). Lekskulchai et al in 2008 reported that there is a statistical significant association between bladder wall thickness and detrusor overactivity but it should not replace the urodynamic studies due to low

specificity of the test in women (10,12). In men, the urinary bladder wall thickness can be a marker of bladder outlet obstruction rather than OAB (13). Hence, this test is unreliable diagnostic tool for detrusor overactivity. Nevertheless, the USS can be used to assess the post void residual volume, and other pelvic organs pathology (1).

1.1.3.5. Magnetic resonance (MRI)

MRI is a non-invasive test, good tool to assess the pelvic organs, soft tissues and pelvic floor structures in different urodynamic conditions as in increased intra-abdominal pressure during valsalva manoeuvre (1). It is useful to exclude bladder abnormalities (such as tumour or diverticulum), others e.g. urethral, ovarian and uterine pathology (1).

1.1.3.6. Biomarkers
1.1.3.6.1. Nerve growth factors (NGF)

NGF is produced by urothelium, smooth muscles and mast cells. It can be measured in the urinary bladder tissue (Invasive) or in the urine (non-invasive). As it can rise in several conditions including OAB, DO, bladder outlet obstruction and interstitial cystitis, it has limited specificity to detrusor overactivity (10).

1.1.3.6.2. Cytokines

Certain pro-inflammatory cytokines were found to be raised in the overactive bladder such as monocyte chemo-attractant protein -1 (MCP-1) when compared to UTI and control cases (14).

1.2. Anatomy of the urinary bladder

The urinary bladder is a smooth muscle urine collecting compartment consisting of two parts; the main part is called the body which is formed mainly of smooth muscles called detrusor muscles and the second part is funnel shaped extension of the body passing into the urogenital triangle called the neck of the urinary bladder which continues as the urethra.

The posterior wall of the urinary bladder body forms the Trigone, the latter can be identified by its mucosa and has a smooth surface compared to the rest of the urinary bladder which is folded into rugae. The ureters enter both uppermost angles (base) of this triangle and at the lower most angle (apex) of this triangle is where the urinary bladder neck opens into the urethra. The anatomy of the urinary bladder is demonstrated in (15,16) (Figure 1-1). The urethra has two sphincters; internal (involuntary) and external (voluntary) sphincters. The internal sphincter is 2 to 3 cm long, lies above the urogenital diaphragm and it is composed of smooth muscles (detrusor) as well as some elastic tissue. The external sphincter of the urethra lies below the urogenital diaphragm and it is composed of skeletal muscles. The latter is under voluntary control of the nervous system and can be employed to stop micturition (15).
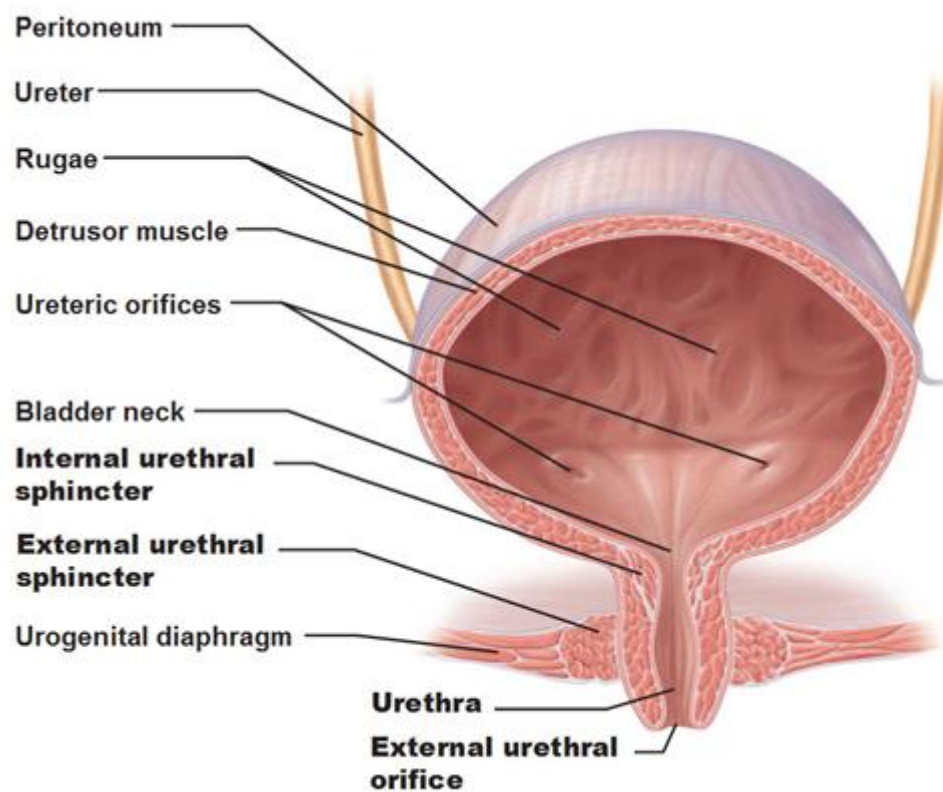
Figure 1-1: Anatomy of a female urinary bladder

Diagram shows the different structures of the urinary bladder, ureter, trigon, urethra and urethral sphincter. Image modified from www.wikipedia.com.

1.3. Innervation of the urinary bladder

The urinary bladder is supplied by sympathetic and parasympathetic nerves systems (17). Sympathetic input is mediated by the hypogastric nerve and originates from T10 to L2 in the spinal cord (Figure 1-2). Parasympathetic nervous input is mediated by pelvic splanchnic nerve that originates from S2 to S4 in the spinal cord. While somatic nerve to the distal urethral sphincter originates from the anterior horn of S2 to S4 in the Onuf nucleus and is mediated via the pudendel nerve. The frequency of these impulses is increased as the intravesicular volume and pressure increases. At small volume, the impulse of micturition can be ignored but once the afferent sensory impulses reach a threshold, micturition becomes essential. On the other hand the internal sphincter remains under sympathetic input during bladder filling, and the internal sphincter is allowed to relax under control of parasympathetic at the micturition. Relaxation of the detrusor muscle is mediated via sympathetic input during the filling. While the micturition under the parasympathetic input control. The detrusor muscle and the internal sphincter react in opposite directions to the input by the sympathetic and parasympathetic systems.

The bladder body contains a high density of β-adrenergic catecholamine receptors which results in detrusor muscle relaxation and urine storage. In contrast, the bladder base and urethra contains a high density of α-adrenergic receptors whose stimulation results in muscle contraction. Thus, the sympathetic nervous system plays an important role in bladder filling. When norepinephrine is released from the sympathetic nerves, it interacts with α-adrenergic receptors on the urethra and the neck of the urinary bladder, which subsequently causes contraction of the smooth muscle sphincter resulting to urine storage.
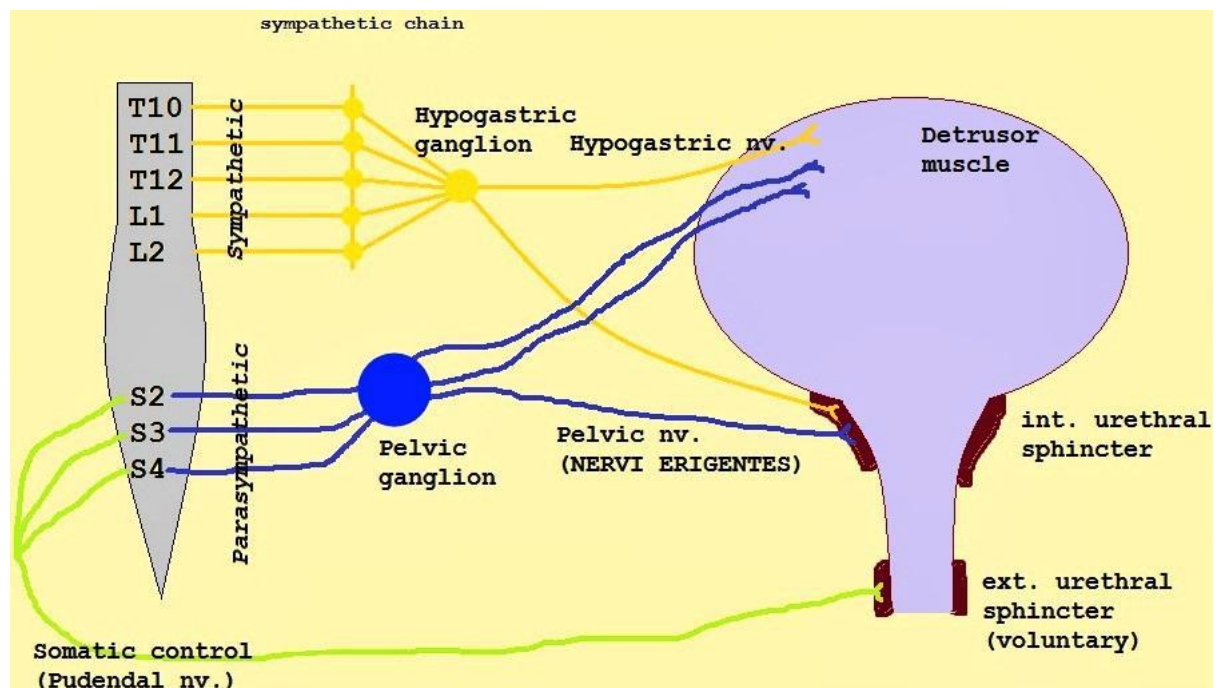
Figure 1-2: Nerve supply of urinary bladder modified from www.wikipedia.com.

1.4. Histology of the urinary bladder

The urinary bladder consists of three layers: an inner urothelium layer, a middle muscular layer and an outer layer (15). The inner layer is formed of transitional cells supported by lamina propria called mucosa, the middle layer consist of bundles of a smooth muscle layers (detrusor muscles) called muscular layer and the outer layer is loose connective tissue called adventitia or serosa and its formed of collagen and elastin fibres (15,16). The internal layer is longitudinal and its fasciculi have a reticular arrangement, and the fibres of the outer layer are arranged longitudinal and obliquely (16). The middle muscular layer arrangement is subdivided into three further layers as follow; inner longitudinal, middle circular and outer longitudinal (Figure 1-3). The circular layer of the detrusor muscles forms the internal urethral sphincter (15,16). The positioning and interaction between the different smooth muscle cells in the bladder regulate changes in the bladder wall and affects bladder shape and intraluminal pressure (15).
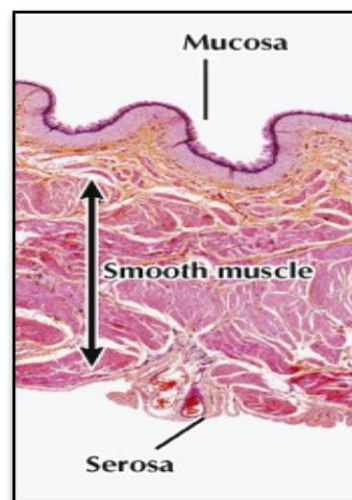
Figure 1-3: Histology of Urinary Bladder wall.

Images adapted from William K Ovalle et al. Netter's essential Histology, 16, 357-380 second edition 2013 (16).

1.5. Physiology of the urinary bladder

The urinary bladder is an elastic and spherical shape smooth muscle chamber and is located behind the pubic bone in the lower abdomen, relaxes to store urine (filling phase) and contracts to empty (voiding phase). The voiding phase requires synchronization between contraction of the urinary bladder and relaxation of the urethra, any disturbance in this phase will lead to hesitancy, dribbling and incomplete emptying of the urinary bladder. Whereas, any disturbances in the filling phase may lead to urgency, frequency and even urge incontinence. The latter symptoms are the main components of an overactive bladder syndrome.

The physiology of the detrusor smooth muscle has been the mainstay for understanding the micturition function and hence, it can help understand several conditions such as overactive urinary bladder (18). The smooth muscles contraction and relaxation is mediated by sympathetic, parasympathetic and somatic nerves, as well as circulating hormones and paracrine factors such as nitric oxide (18,19).

1.6. Symptoms of overactive bladder

The symptoms of an OAB as defined in the definition section above are urinary urgency with or without urinary urge incontinence, frequency and nocturia. Urgency is the symptom of a sudden, compelling feeling to urinate which is difficult to delay, and is the main symptom of OAB (1). Urinary frequency can be measured with a voiding diary and it has been reported that up to seven urinations during waking hours is considered normal (2,3) and any voids more than 8 times per 24 hours is considered as urinary frequency (6). Nocturia is the disturbance of sleep once or more due to the need for micturition and each episode is preceded and followed by sleep (1,3). The consequences of overactive bladder symptoms may lead to numerous other significant conditions such as social, psychological, occupational, physical, sexual and financial burdens (3). Although the prevalence of OAB tends to increase in advanced age, these symptoms should not be considered as normal part of aging process (3).

1.7. Causes and risk factors for overactive bladder

Overactive urinary bladder is generally caused by early, inappropriate and uncontrolled contraction of the detrusor smooth muscles of the urinary bladder causing an urge to micturate (10). The exact cause is unclear but it is generally a problem of the nervous system (20) or any disturbance to the micturition cycle pathways (21) (Figure 1-4).
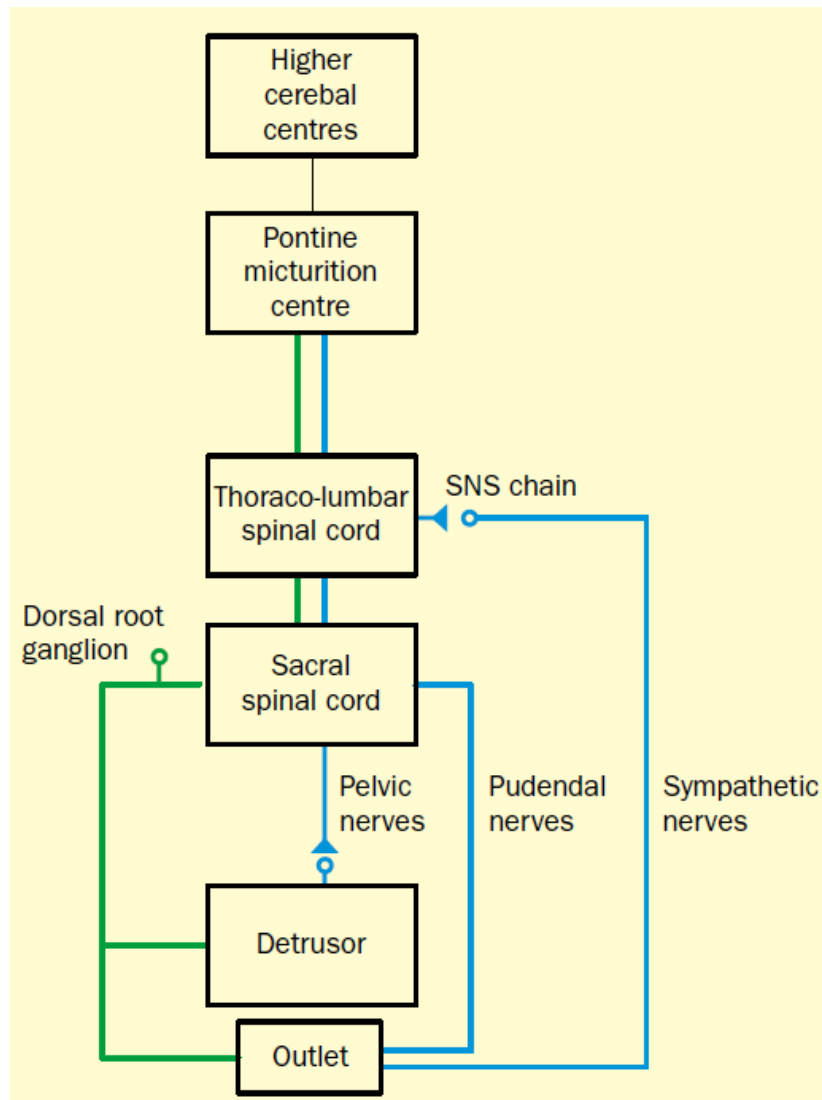
Figure 1-4: Micturition cycle control pathways:

The pontine micturition centre receives the signals through afferent pathways (green) when urinary bladder reaches voiding capacity and the urinary bladder will switches from storage phase to voiding phase. This will activates the efferent pathways (blue) and send signals down to the detrusor muscles to contract and to the urinary outlet to relax for the urinary bladder to empty, this graph adapted from Drake et al (21).

The common conditions of the nervous system that can cause OAB are spinal cord injury, stroke, Parkinson's disease, multiple sclerosis and diabetic neuropathy. Other causes are recurrent UTI, and prostate enlargement or bladder tumours.

There are certain risk factors which are associated with overactive urinary bladder for example condition that causes urinary bladder outlet obstruction such as prostate hyperplasia, and urethral strictures. Conditions that might lead to neuropathic changes such as diabetes mellitus, and other conditions such as prostate surgery, pelvic surgery, multiple pregnancies, and postmenopausal women were risk factors for OAB.

There are different theories which might explain the pathophysiology of the OAB;

The neurogenic theory: the lower urinary tract functions are controlled by the central nervous system (brain and spinal cord) as an on and off circuits under voluntary control. Any injury or damage to the either the central inhibitory (brain) pathway or axonal afferent pathways (Spinal) that transmits the sensitization signals of the urinary bladder can mask voiding reflexes and lead to detrusor overactivity (22).

The myogenic theory: it suggests that partial denervation of urinary bladder detrusor smooth muscle as a result of ischemia, ageing process or bladder outlet obstruction will lead to increase in the spontaneous contractibility and hence detrusor overactivity (10).

The urothelial transmitter theory: the urothelium is multi-layered and divided into three layers of transitional cells; apical cells (umbrella), intermediate and basal cells (18). The urinary bladder urothelium is impermeable to urine and forms a urine-blood barrier (18), the barrier function occurs mainly through the apical urothelial transitional cells (18). The urothelium also act as a barrier to protect the underlying bladder structures, it is the main defence against bacterial infection, and is involved in ionic transport, signalling and modulation of the smooth muscle contraction (18,23). Disruption to this urothelial barrier integrity is associated with alteration in the levels of chemical neurotransmitters such as nitric oxide and ATP which in turn lead to alteration in the epithelial and smooth muscle function. Nevertheless, it will lead to water, urea and toxic substances passing into the underlying tissue and result in urgency symptoms with pain during filling and voiding phases (24). The roles of different receptors and neurotransmitters in urothelial and urinary smooth muscle function will be explored in more details in the next section.

1.8. Contractility of the bladder detrusor smooth muscles

Smooth muscle contraction occurs by interaction between myosin (thick filaments) and actin (thin filaments) which is dependent on an increase in the intracellular calcium ions (15,18,25,26). Several receptors and ion channels associated with neurones have already been identified in the urothelium which includes bradykinin receptors (27), purinergic (P2X and P2Y) receptors (28-33) and acetylcholine (nicotinic and muscarinic) receptors (34-36) (Figure 1-5).

Contraction of the urinary bladder starts when the parasympathetic nerves release acetylcholine which interacts with M_3-receptors that are coupled to $G\alpha_{q/11}$, a member of the G-protein superfamily. Recruitment of $G\alpha_{q/11}$ to the agonist-bound M_3-receptor activates phospholipase C (PLC), leading to the generation of inositol 1, 4, 5-triphosphate (IP_3) and diacylglycerol (DAG). Liberation of intracellular Ca^{2+} from stores is triggered by IP_3 interacting with its IP_3 receptor in the sarcoplasmic reticulum. Increasing intracellular $[Ca^{2+}]_i$ and DAG levels which stimulates protein kinase C (PKC) activity (37). M_3 receptor activation may also activates the RhoA-kinase pathway, which phosphorylates and reduces the activity of myosin light chain phosphatase (26). Detrusor smooth muscle contractility is also generated by the action of ATP which is released from parasympathetic nerves (26). Furthermore, desensitization mechanisms following frequent or repeated stimulation of receptors by agonists, leads to decrease responsiveness to further agonist challenge. This process protects cells from the adverse effects of over stimulation or inappropriate signalling. Desensitization of G protein-coupled receptors (GPCR) is can be mediated by the action of G protein-coupled receptor kinases (GRKs) that phosphorylate key serine and/or threonine residues within the third intracellular loop and/or C-terminal tail of G protein-coupled receptors (38). GPCR phosphorylation by GRKs promotes receptor affinity for arrestins proteins, which inhibit any further interaction between the GPCR and G proteins.
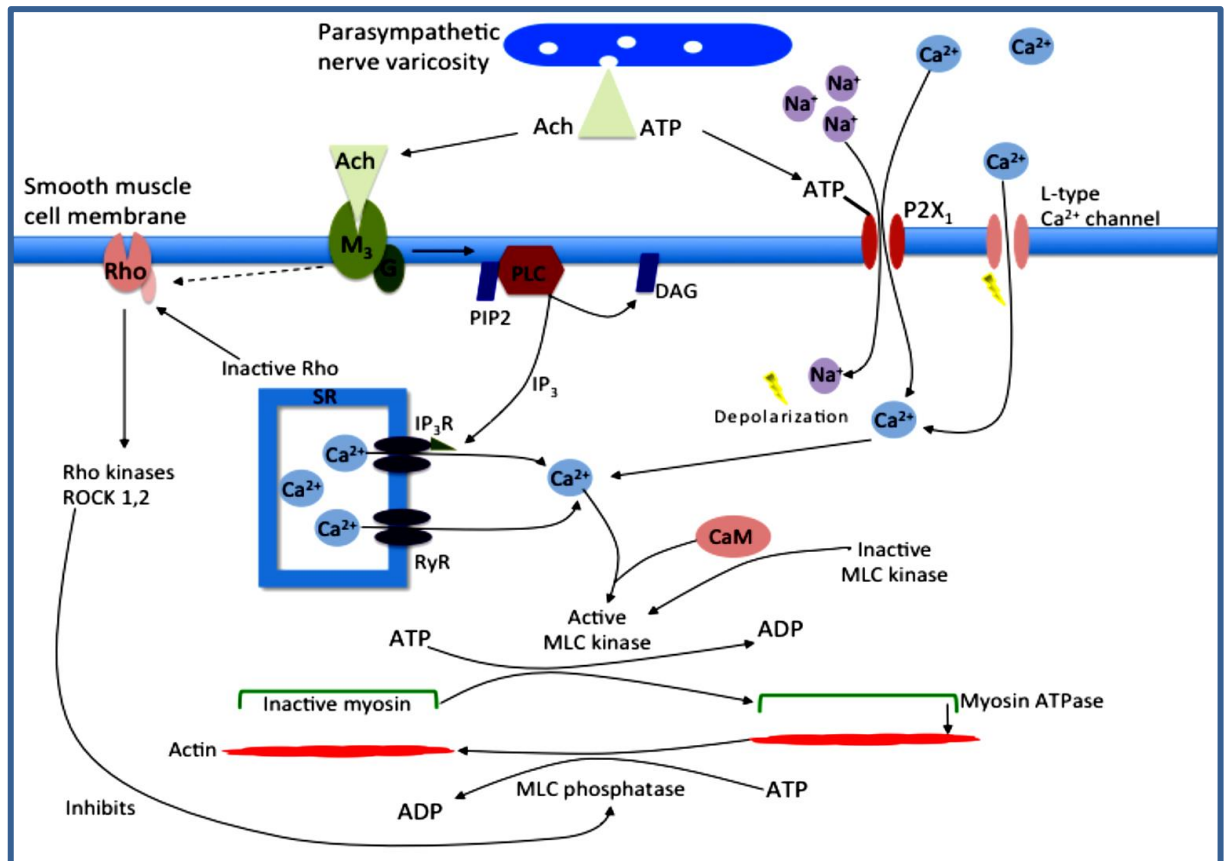
Figure 1-5: Signalling pathways in detrusor muscle cells and the contraction of urinary bladder (26).

Intracellular signalling pathways in the detrusor muscle cell. Shown are two surface receptors: acetylcholine (ACh) M_3 receptor and the $P2X_1$-ATP receptor. The M_3 receptor initiates the formation of inositol triphosphate (IP_3) and diacylglycerol (DAG) from membrane phospholipids (PIP_2) by the action of phospholipase-C (PLC). IP_3 initiates Ca^{2+} release from intracellular stores (SR). Ca^{2+} binds to calmodulin (CaM) to activate myosin light chain (MLC) kinase and phosphorylate and activate myosin to bind to actin. M_3 receptor activation may also activate the Rho-kinase pathway, which phosphorylates and reduces the activity of myosin light chain phosphatase. Also shown there is an influx of cations through the $P2X_1$ receptor ion channel and the L-type Ca^{2+} channel.

Detruser smooth muscle cells have to relax, and extend the bladder wall over a large interval during the period of bladder filling. In contrast, during micturation muscle shortening must be quick to rapidly decrease bladder size. Each part of urinary tract muscle has to have precise distribution of receptors to respond to the nervous and hormonal control systems. Contraction of the smooth muscle is started by escalation of intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ (39). Ca^{2+} enters the cytoplasm through the cell membrane, through Ca^{2+} channels or released from the sacroplasmic reticulum (SR). The source of Ca^{2+} can be extracellular or can be from intracellular supplies (40-42). Openning of Ca^{2+} channels is a vital event in depolarization-mediated changes in $[Ca^{2+}]_i$, and many studies on animal detrusor muscle have confirmed the importance of extracellular Ca^{2+} entry via dihydropyridine-sensitive Ca^{2+} channels to activate the detrusor through the main transmitters, acetylcholine and ATP (15). The bladder consists of smooth muscle sandwiched between an inner surface of urothelium/suburothelium, and on the outer surface by serosa. Detrusor muscle cells are spindle shaped with single nucleus separated by connective tissue, thin filments of α- and β-actin are attached to the cell membrane on the dense bodies, to arrange for binding locations for the myosin thick filments. The contractile proteins are activated by Ca^{2+}, which phosphorylates myosin through myosin light chain kinase activity (MLCK). In contrast, dephosphorylation of myosine light chain phosphatase (MLCP) induces relaxation, and the contractile system can be altered by modifying the activites of MLCK or MLCP (26).

1.8.1. Hormonal regulation of the detrusor smooth muscles contraction

Bladder smooth muscle cell contraction is mediated by cholinergic and non-cholinergic (43) mechanisms. The distributions of adrenergic and muscarinic receptors in the urinary bladder are presented in Figure 1-6.
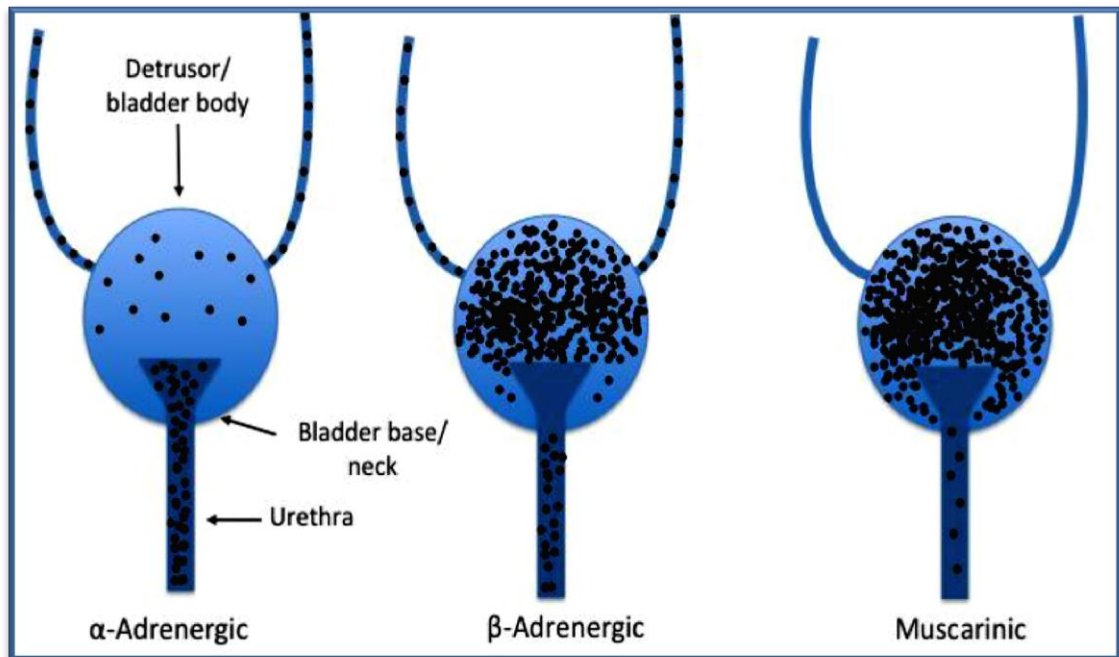
Figure 1-6: Diagram illustrations the distribution of α-adrenergic, β-adrenergic and muscarinic cholinergic receptors in urinary bladder (This Adapted from Caine, 1984).

The body of the bladder contains high density of β-adrenergic receptors for bladder relaxation and urine storage, but has a few α-adrenergic receptors. In contrast, the bladder base and urethra contain a high density of α-adrenergic receptors, which results in contraction of urinary bladder and a small number of β-adrenergic receptors. Cholinergic muscarinic receptors are found through the bladder; however, the concentration of the muscarinic receptors is greater in the bladder body than the base. This figure was adapted from Caine (1984).

1.8.2. Adrenergic receptors in regulation of the detrusor smooth muscles contraction

The stimulation of β-adrenoreceptors results in relaxation of smooth muscle cells (44,45), and activation of α-adrenoceptors leads to contraction of smooth muscle (44,46). The expression of α- and β-adrenoceptors differs in the bladder wall; β-adrenoceptors have been shown to dominate over α-adrenoceptors (47). There are three forms of β-adrenoceptors present in the detrusor smooth muscle cells; β_1, β_2, and β_3 with β3 being the most abundant (48). The β-adrenoceptors are attached to the Gs-protein, which leads to increase in cAMP (49), when stimulated by norepinephrine, producing a DSMC relaxation (47). Alternatively, the bladder base and urethra contain great numbers of α-adrenoceptors, and little number of β-adrenoceptors (50).

1.8.3. Purinergic receptors in regulation of the detrusor smooth muscles contraction

Adenosine triphosphate (ATP) is a co-transmitter with acetylcholine released from parasympathetic nerves system which supply the urinary bladder detrusor smooth muscle (51,52) and there are two types of purinergic receptors known in urinary bladder that respond to ATP. In 1985, Burnstock classified P2 receptors into P2X and P2Y receptors families, P2X ion channels and P2Y G-protein coupled receptors (53,54). P2X receptors have been found in the urinary bladder, and there are seven mammalian P2X-receptors subtypes ($P2X_{1-7}$) (55). $P2X_1$ was identified in detrusor muscle of human bladder (56) and evidence suggests that the $P2X_1$ subtype mediates smooth muscle contraction. In addition the $P2X_3$ subtype is reported to mediate excitation of afferents sensory bladder fibres (57). P2X receptors are ligand-gated cation channels which when activated can produce depolarising Na^+ and Ca^{2+} current, that activates L-type Ca^{2+} channels to produce an action potential and consequently cause an increase in intracellular calcium $[Ca^{2+}]_i$ to induce smooth muscle contraction (54). There are eight subtypes of P2Y receptors ($P2Y_{1, 2, 4, 6, 11, 12, 13, and 14}$), the structure of P2Y receptors includes an extracellular N-terminus and intracellular C-terminus; the extracellular N-terminus have several potential N-linked glycosylation sites, 7-TM spanning domains contribute in creating the ligand binding, three extracellular loops, and three intracellular loops that contribute in G protein coupling. The intracellular C-terminus

contains several consensus binding or phosphorylation sites for protein kinases. P2Y receptors coupling to G- protein heterotrimeric G proteins consist of a Gα subunit that is tightly associated with G$\beta\gamma$ subunits and binds to G protein-coupled receptors (GPCRs) at the internal surface of the cell. P2Y receptors similar to the other members of the GPCR superfamily, act as guanine nucleotide exchange factors (GEFs) for heterotrimeric G proteins, promoting the exchange of GTP for GDP on the Gα subunit. Once GTP bound the Gα subunit is able to dissociate from the G$\beta\gamma$ complex, and is thus able to interact with downstream effectors such as phospholipase C or adenylyl cyclase. P2Y receptors are not specific subtype, which have been involved in relaxation of smooth muscle, possibly by cAMP-dependent PKA activity (58).
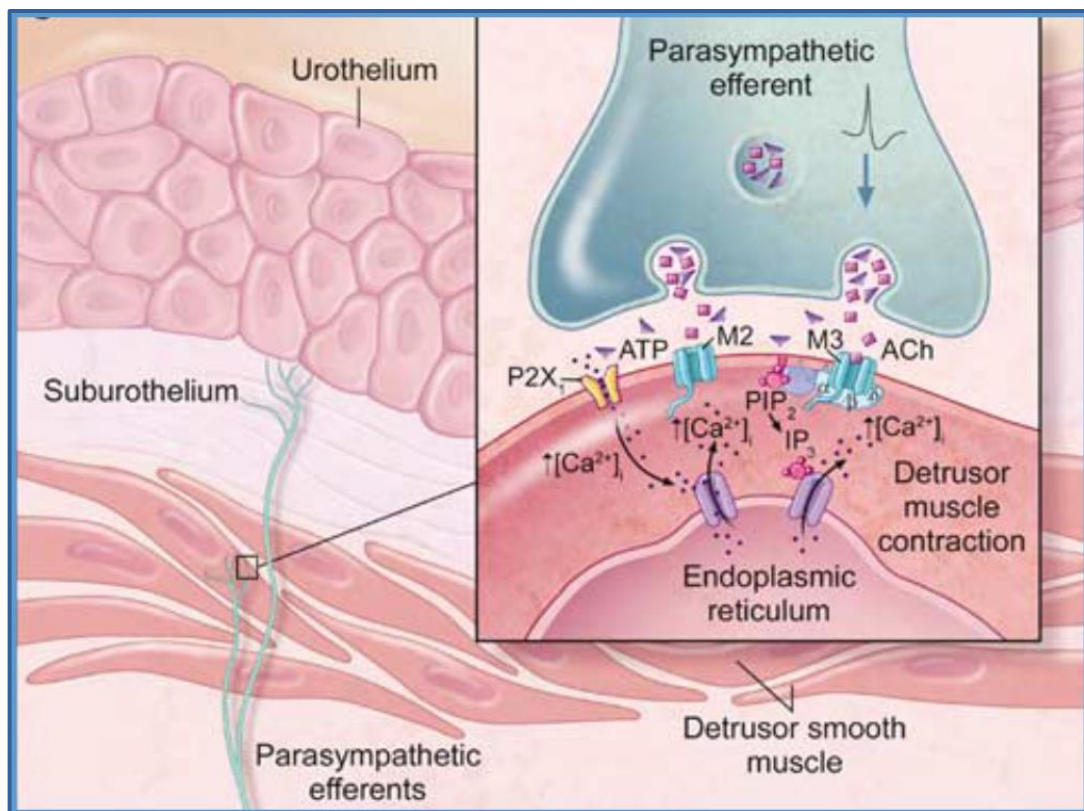
Figure 1-7: The roles of ATP and purinergic receptors in the micturition process (This figure adapted from Ford et al 2006 (59))

Any mechanical expansion or injury to the urothelium can causes release of ATP. In disease conditions for example interstitial cystitis (IC), benign prostate hyperplasia, or spinal cord injury, that can stimulate of parasympathetic efferent to produce a co-release of ATP and acetylcholine, these neurotransmitters act on $P2X_1$ and muscarinic (M_3) receptors, correspondingly to cause detrusor smooth muscle contraction (59).

The presence of P2Y$_2$ immunoreactivity has been reported in the rat urinary bladder including detrusor smooth muscle (32). Current speculation suggests that P2Y receptor subtypes may be expressed in detrusor smooth muscle cells of urinary bladder of mouse and rat (58). The purinergic component of parasympathetic co-transmission is less than 3% in normal conditions, while under pathological conditions associated with obstructive bladder, idiopathic detrusor instability and in most types of neurological bladder the P2Y component increased to 40% (60). Moreover, P2Y$_1$ receptor subtype expression is increased in obstructed bladder (61). Previous data indicate that ATP acts on P2X$_2$ and P2X$_3$ receptors to modulate the afferent pathway to the central nervous system, indicated that when pressure is changes on the rabbit bladder wall can release of ATP, this ATP is produced by urothelial and epithelium cells during stretching of the bladder during the filling phase (62), and activates the afferent nerves fibres to initiate bladder contraction (63) (Figure 1-7). The ATP release can be stimulated by the increase in the bladder volume. In this study suggesting that the regulation of GPCR could provide potential targets for treatment bladder pathologies, such as overactive urinary bladder. In the human bladder evidence suggests that P2X receptors mediate excitation sensory neurons and induce the detrusor muscle contraction in response to release of ATP (Figure 1-8). Furthermore, previous work has established that there were at least 3 subtypes of purinoceptors, P1 receptors which induce relaxation and P2X mediate contraction in rat urinary bladder smooth muscle.
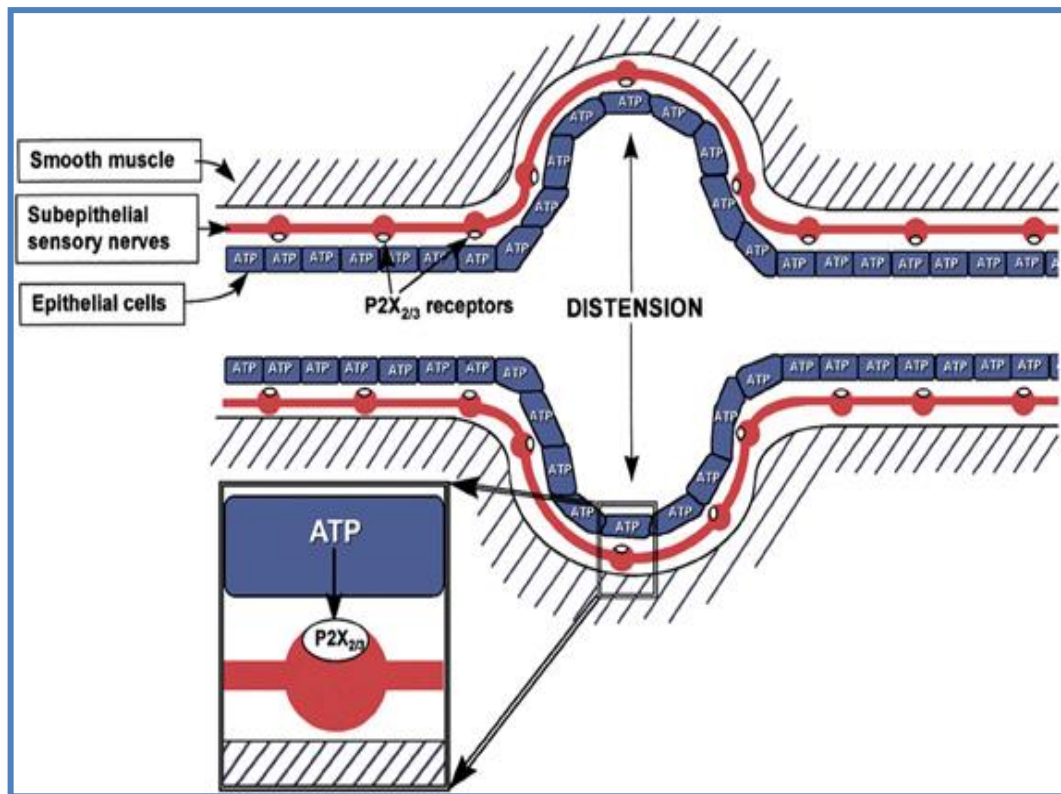
Figure 1-8: The hypothesis for purinergic mechanosensory transduction in tubes and sacs (ureter and urinary bladder).

It is proposed that distension and stretching leads to release of ATP from epithelium lining the tube or sac, which then acts on purinergic receptors such as $P2X_3$ and/or $P2X_{2/3}$ on subepithelial sensory nerves (60).

Commonly the treatment possibilities for patients with lower urinary tract symptoms (LUTS), consist of non-selective muscarinic antagonists such as (tolterodine, oxybutynin, trospium), they are used mainly in patients with over active bladder (OAB) and urinary incontinence (64) Selective α_1-adrenoceptor antagonists such as (tamsulosin, doxazosin, alfuzosin) that are commonly used in males with obstructive bladder caused by benign prostatic hyperplasia (BPH) (65). While these treatments provide some progress to the patients with lower urinary tract symptom, the benefits from the treatment seems to be partial with some sensory symptoms such as noctria, urgency and pain. In addition, muscarinic antagonists produce many systemic side effects such as blurred vision, dry mouth, constipation, dry eyes and drowsiness (59). Moreover, selective α_1-adrenoceptor antagonists such as tamsulosin, due to its lower cardiovascular side effects (66,67), are chosen as a first line of treatment for patients with LUTS or BPH (65). Nevertheless, their effect on storage symptoms is not impressive and most likely limited to use in urinary obstruction (59). More recently, Botox and intravesical treatment with neurotoxic agents have been utilised as a novel therapeutics for the treatment of intra-detrusor botulinum toxin such as capsaicin or resiniferatoxin give some symptom improvement in some conditions; on the other hand, the progress is different and the way of administration unpleasant (68,69).

Several studies have widely covered the role of P2Y and P2X receptors subtypes in lower urinary tract function (70,71). In this study, we focus on ATP signalling within the urinary bladder, with a particular focus on selected P2X and P2Y receptors where recent biological and chemical advances suggest that medicinal exploitation can be achieved. Specifically, these receptors that likely play a significant role in regulation of detrusor smooth muscle excitability and contraction.

1.8.4. Cholinergic receptors in the regulation of smooth muscles contraction

The cholinergic nervous system is mostly responsible for voiding (50,72). Cholinergic (muscarinic) receptors are found throughout the smooth muscle of the bladder (50). Experiments using cholinesterase inhibitors and muscarinic antagonists, for instance the atropine effect. In normal human detrusor smooth muscle, the emptying (contraction) resulting from electrical field stimulation which was facilitated mainly by activation of the muscarinic receptors, and these responses can be totally obstructed by atropine (72-

74). The muscarinic receptors mainly found in the human bladder are of the M_2 (80%) and M_3 (20%) subtypes (75) but M_1, M_4 and M_5 subtypes have also been identified, all of which are coupled to G proteins (76). In humans, the M_3 receptors are assumed to be the most important for inducing detrusor contraction (34). The function of M_2 receptors has not yet been clarified and their action may increase in some pathological conditions (77). M_1, M_3, and M_5 receptors couple with heterotrimeric $G_{\alpha q/11}$, activating phospholipase-C (PLC) (71) Activation of this enzyme generates the second messengers IP_3 and diacylglycerol (DAG) from membrane phosphoinositides (PIP_2). IP_3 binds to receptors on the sarcoplasmic reticulum and releases intracellular Ca^{2+} into the sarcoplasm (78) (Figure 7). The M_2 and M_4 receptors are coupled to a pertussis toxin sensitive $G_{i/o}$, leading to inhibition of adenylate cyclase (AC), resulting in a reduction of cAMP (71). Contraction of the urinary bladder occurs when the parasympathetic nerves release acetylcholine which interacts with M_3-receptors that are coupled to $G\alpha_{q/11,}$ a member of the G-protein superfamily. Recruitment of $G\alpha_{q/11}$ to the agonist-bound M_3-receptor activates phospholipase C (PLC), leading to the generation of inositol 1, 4, 5-triphosphate (IP_3) and diacylglycerol (DAG). Liberation of intracellular Ca^{2+} from stores is trigged by IP_3 interacting with its IP_3 receptor in the sarcoplasmic reticulum. Increasing intracellular $[Ca^{2+}]_i$ and DAG levels stimulates protein kinase C (PKC) activity (37) (Figure 1-11). The M_3 receptor activation may also activate the RhoA-kinase pathway, which phosphorylates and reduces the activity of myosin light chain phosphatase (26). Detrusor smooth muscle contractility is also generated by the action of ATP which is released from parasympathetic nerves and stimulates purinergic receptors (26). Furthermore, the desensitization mechanisms is known as frequent or repeated stimulation of receptors by agonists, leads to decrease responsiveness to further agonist challenge. This process protects cells from the adverse effects of over stimulation or inappropriate signalling.

1.9. Regulation of G-protein coupled receptor (GPCR) signalling

GPCRs are superfamily of cell surface receptors and also known as seven transmembrane receptors (7-TMRs) (79). GPCRs are considered as the biggest protein receptor superfamily in the body (79), there are >800 known GPCRs in the human genome (80). GPCR signalling and its regulation is a multi-step process which is initiated by the binding of extracellular signalling molecules to the receptor leading to receptor-dependant activation of intracellular signalling pathways. Agonist stimulation of a GPCR leads to conformational change in the receptor with the active receptor subsequently associating with heterotrimeric G proteins within the plasma membrane, catalysing the substitution of GDP of GTP at the Gα and Gβγ subunits then dissociate from each other (81). Through activation of their G proteins GPCRs can regulate multiple intracellular interactions, such as ion channels, enzyme activity and gene expression. Low spontaneous hydrolysis reactions from GTP to GDP promote a reversion to the inactive state of the α-subunit leading to G protein subunit regeneration via combination with the Gβγ dimer. GPCRs have been confirmed to produce signals via stimulation with the scaffolding proteins, β-arrestin1 and β-arrestin2, and independent of heterotrimeric G-protein coupling (79). They are involved in several diseases and they are possible therapeutic targets (82). GPCRs play an important role in regulates cellular functions including motility; morphology and metabolism in response to hormones, ions and neurotransmitters (83) GPCRs consist of 7 transmembrane α helices with an extracellular N-terminal domain and intracellular C-terminal connected by three (ECLs) extracellular loops and three (ICLs) intracellular loops (79,84). GPCRs start various signalling pathways through joining to different heterotrimeric G proteins (38,85).

The regulation of GPCR signalling in bladder smooth muscle have focused on alteration in receptor and G protein expression and second messenger generation in the cell. No studies to date have considered the effects of desensitization or re-sensitization of GPCR signalling on GPCR mediated function in DSMC. Detrusor muscle activity is regulated by different inputs of neuronal and hormonal sources; these signals are decoded by a plethora of G protein-coupled receptors (GPCRs). The activity of detrusor is regulated by the balance of signals through these alternative relaxation and contractile

GPCRs. Consequently, to ensure correct bladder function it is vital that the magnitude and the duration of GPCRs signalling are tightly controlled. Like most GPCRs P2Y receptors undergo desensitization, which reduces GPCR receptiveness to stimulus and leads to receptor internalization.

Agonist-dependent GPCR desensitization is generally mediated by a family of GPCR kinases (GRK 1-7) (86)**.** G-protein coupled receptor kinases (GRKs, Figure 1-8) are a family of seven mammalian serine/threonine protein kinases (79). GRKs play an important role in phosphorylating and desensitizing G protein- coupled receptors (GPCRs) (87). There are 7 kinases in the family, separated into 3 subgroups, GRK 1 and 7 which presents in the retina and GRK 1 additionally exists in pineal gland (38). GRKs 2-6 are sharing similar structures as shown in the (Figure 1-9) from the diagram, it is clear that GRK 2 and 3 are similar. In addition the RGS domains are important in direct regulation of $G\alpha_{\delta/11}$ signalling, and catalytic domain, which is highly conserved (38). GRK 4 is expressed in the testis, kidney and cerebellum (87). Previous studies have shown that GRKs can inhibit the interactions between GPCRs and G proteins in a phosphorylation independent way (38,88). GRK2 also binds to activated GTP bound form of $G\alpha_{q/11}$ but not to $G\alpha_S$, $G\alpha_i$ or $G\alpha_{12/13}$ to inhibit PLC-β activation (38). Furthermore, GRK2 expression is decreased in detrusor smooth muscle in the presence of obstructed bladder, which could suggest an altered ability to regulate GPCR signalling in the bladder, and potentially contribute to detrusor over-activity (26,89).
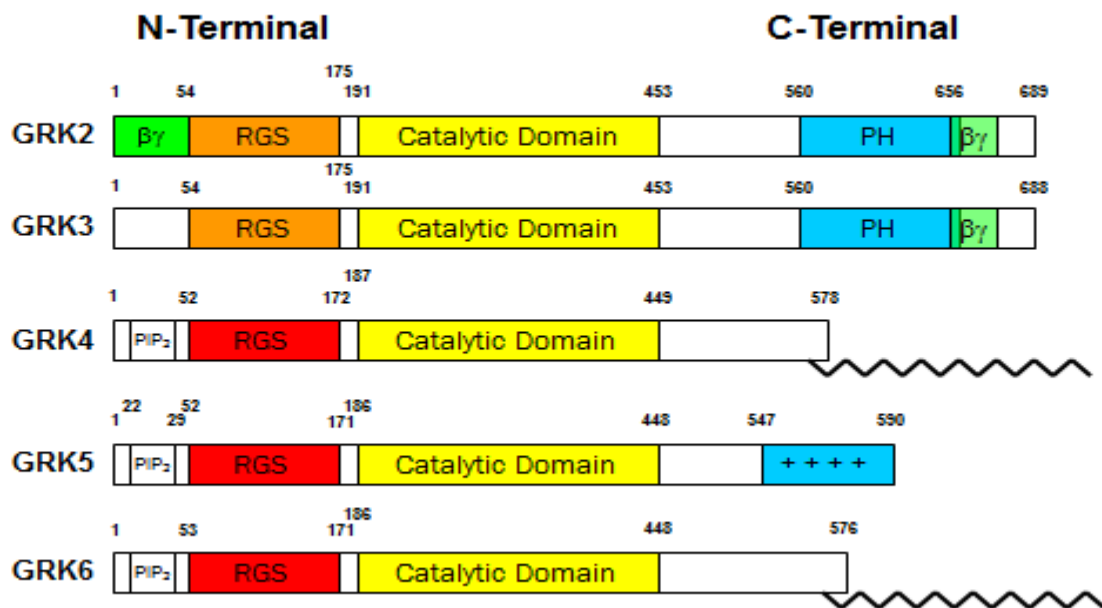
Figure 1-9: G-Protein coupled receptor kinase family (figure adapted from (38)).

GRKs have N-terminal regulator of G-protein signalling (RGS)-domain (90), GRKs have also a fundamental serine/threonine kinase catalytic domain. The main changes between isoform of the GRK subfamilies arise in the N- and C-terminal domains. The GRK2 and GRK3 are mainly cytosolic proteins that are recruited to the plasma membrane subsequent stimulation of receptors by agonists; this procedure is helped by two particular domains at the ending of protein. The C-terminal pleckstrin homology (PH) domain includes sites for $G\beta\gamma$ and phosphatidylinositol 4,5-bisphosphate (PIP_2) binding (91). In addition, the N-terminal RGS-like domain has been shown to bind to stimulated GTP-bound $G\alpha_{q/11}$ (90), so not merely inhibit the interact of $G\alpha_{q/11}$ with phospholipase C (PLC), however may perhaps help the correct GPCR targeting of GRK2 and GRK3 prior to receptor phosphorylation (92). GRK6 is palmitoylation at cysteine residues within the C-terminal site (91), which enables it membrane localization. GRK5 consists of a polybasic region of about 43 amino acids (93), which interacts with negatively stimulating plasma membrane lipids. Amino acid quantity numbering is particular for the human GRK group members the image taken from (38).

GPCR activation drives the intiation of multiple intracellular signalling routes, including second messenger pathways controlled by adenyl cyclyses phospholipases and ion channels. Stimulation of GPCRs triggers their phosphorylation by GRKs (G protein-coupled receptor kinases). Non-visual arrestins bind to the phosphorylated receptors and block any interactions with G proteins, which leads to the loss of receptor responsiveness, termed desensitization (94) (Figure 1-11). Previous evidence suggests that the human bladder expresses GRK2, GRK3 and GRK4. Interestingly, GRK2 expression decreases with bladder obstruction (89), suggesting that the failure of GPCR desensitization might be one of the mechanisms by which an overactive bladder is induced (89).
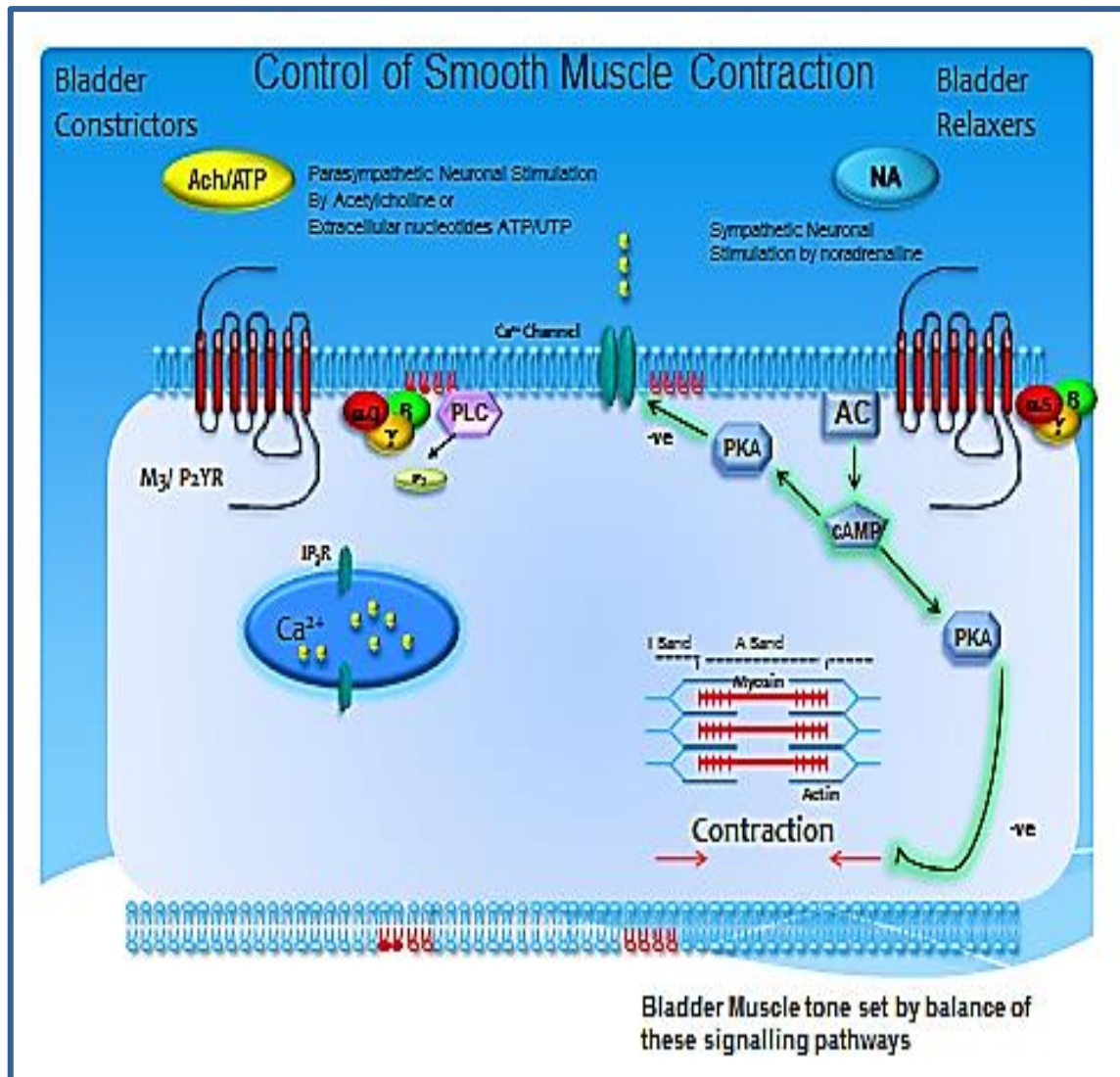
Figure 1-10: Intracellular signalling of GPCR in the smooth muscle

Neurotransmitters (e.g. ACh, ATP) are released from parasympathetic neurones, to stimulate M_3/ P2Y receptors to associate with heterotrimeric $G\alpha_q$-mediated activation of phospholipase C (PLC) and the production of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol, leading to intracellular Ca^{2+} release and activation of protein kinase C, respectively and promoting bladder contraction. While the smooth muscle relaxation occur when the agonist associate with heterotrimeric $G_{i/o}$, leading to inhibition of adenylate cyclase (AC), resulting in a reduction of cAMP (71).

1.9.1. GPCR regulation by arrestin proteins

To date there are four functional members of the arrestin family which have been cloned (95,96). There are two types of arrestins; Firstly cone (97,98), and visual arrestins (99,100), which are expressed in the retina, regulate photoreceptor function. Secondly, β-arrestin1 (101) and β-arrestin 2 (102) are universally expressed proteins with highest level of expression in both the brain and the spleen (102). All of the family members can bind to light stimulated or agonist-occupied heptahelical G-protein-coupled receptors (GPCRs) following their phosphorylated by GRKs (38). Arrestin binding sterically blocks the receptor–G-protein interaction and thus plays a critical role in the process of homologous desensitization (38,79). β-arrestins can also act as adapter proteins that target GPCRs to clathrin-coated pits for endocytosis (79,103). These mechanisms of GPCR sequestration are important not only in decreasing GPCR signalling but also for receptor re-sensitization as well downregulation (79) (Figure 1-10). GPCR/GRK/arrestin interaction is mainly a two-step mechanism. GRKs first phosphorylate the agonist occupied GPCRs (38). GPCR phosphorylation allows activated receptors to be bound to arrestin proteins (94). Arrestins also serve as scaffold proteins that form the agonist-GPCR-GRK complex (88,96,104). Arrestins binding can hinder further G-protein dependent signalling pathway activation by subsequent vesicle trafficking (94). Vesicle trafficking begins with the conformation change of arrestins, followed by endocytosis of the (clathrin-coated agonist-GPCR-GRK-arrestins pit) (105,106). In addition to the core function of GPCR desensitisation and G protein signalling termination, arrestins simultaneously serves as a protein adaptor that brings several signal components together (106). Arrestin scaffold protein complexes are able to activate mitogen-activated protein kinases (MAPK) pathways, known as G protein-independent arrestin-mediated signalling pathway (107).

1.9.2. β-arrestins and GPCR desensitization

Desensitization is initiated by phosphorylation of the receptor. Second-messenger-dependent protein kinases, including cyclic-AMP-dependent protein kinase (PKA) and protein kinase C (PKC), usually agonist occupied GPCRs by recruitment of one or more members of a family of seven serine/threonine kinases, termed G protein-coupled receptor kinases (GRKs). GRKs mediate the phosphorylation of key serine and/or threonine residues within the third intracellular loop and/or C-terminal tail of the GPCR. GPCR phosphorylation by GRKs increases receptor affinity for arrestin 2 and or arrestin 3, which can physically bind to GPCRs and suppress interaction between receptor and G proteins to inhibit signalling (94,108,109) (Figure 1-11).

1
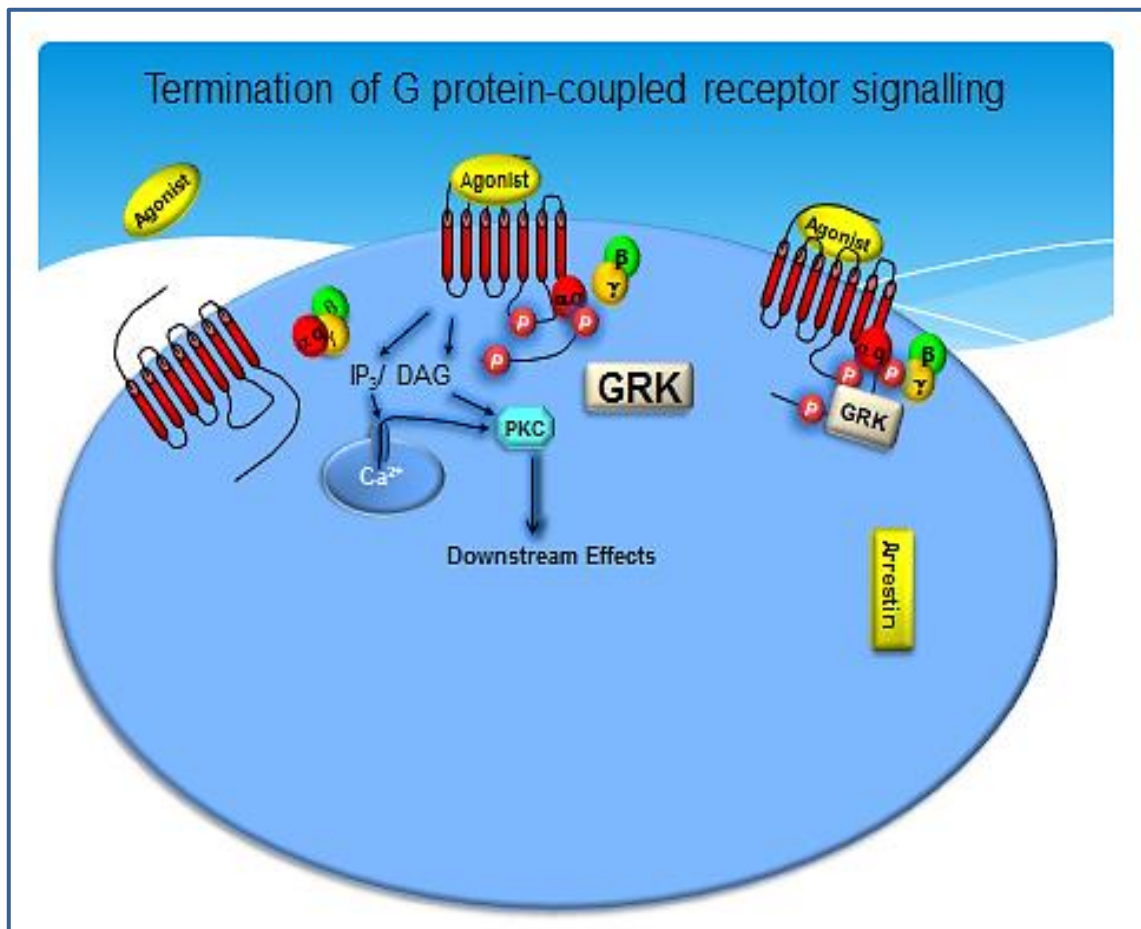
Figure 1-11: Roles of β-arrestins in receptor desensitization

Agonist occupied GPCRs undergo desensitization by recruitment of one or more members of a family of seven serine/threonine kinases. Ligand stimulate $G\alpha_q$-mediated activation of phospholipase C (PLC) and the production of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol, leading to intracellular Ca^{2+} release and activation of protein kinase C, respectively. GRKs mediate the phosphorylation of key serine and/or threonine residues within the third intracellular loop and/or C-terminal tail of the GPCR. GPCR phosphorylation by GRKs increases receptor affinity for arrestin 2 and or arrestin 3, which can physically bind to GPCRs and suppress interaction between receptor and G proteins to inhibit signalling (94,108,109).

1.10. Management of overactive bladder

Most patients with OAB symptoms can be managed with behavioural and life style changes, without the need for specialist consultation or medical treatments. There are several guidelines (2,110,111) including NICE (112) and SIGN (113) that can help guiding the general practitioner in the management of OAB symptoms. This can be subdivided into three subcategories; first, second and third line treatment. I will explore this briefly in the following section.

1.10.1. First line treatment
1.10.1.1. Behavioural therapy

This forms the mainstay of initial OAB management, it is non-invasive, less adverse effects, and once failed can be combined with medical therapy to achieve a good result (110).The behavioural therapy includes lifestyle changes, toileting assistance, bladder training, and pelvic floor exercise.

1.10.1.1.1. Lifestyle changes

There are several reports on the relation between excessive fluid intake and the number episodes of micturition. It is logically anticipated to achieve improvement in the urinary frequency and urgency if water and caffeine intake is reduced (114,115). Certain authors recommended at least 25% decrease in fluid intake to achieve improvement in all overactive bladder symptoms, providing they drink at least one litre per 24 hours and approximately 300 ml of fluid is obtained from food (116). To prevent urge incontinence, it is advisable to use the toilet at an early stages and checking the location of the toilet to avoid or minimise any incontinence episodes (110).

Obesity is another independent factor associated with a negative impact on the urinary frequency and urge incontinence symptoms (8), it has been reported that there was a marked decrease in these symptoms following weight loss (117).

1.10.1.1.2. Toileting assistance

This involves absorbent products, hand held urinals, urinary catheters either intermittent self-catheterisation or long term catheters. This could be an appropriate measure for frail elderly patients (110), especially where skin irritation, excoriation or skin wounds, and pressure ulcers are being contaminated by the urine (112). This should not be used as a treatment option but used as a coping measure until definitive treatment received or as an adjunct to an ongoing treatment and only to be used as a long term management if all management options had failed to be effective (112).

1.10.1.1.3. Bladder training

It is a reliable method for increasing the bladder filling volume through increasing of the micturition intervals (110). This should be offered to patients with OAB symptoms for at least six consecutive weeks before considering medical treatment (112). It is usually started at slow intervals as frequent as 15 minutes intervals and slowly increased to achieve a minimum of 2-3 hours intervals (110). In regards to the urge or mixed incontinence; bladder training was associated with up to 90% and 16% improvements in evaluations based on subjective symptoms and bladder diaries respectively (110). If the bladder training failed to achieve a satisfactory benefits in managing the OAB symptoms, it was recommended to combine bladder training with medical treatment (112).

1.10.1.1.4. Pelvic floor muscles exercise

This helps strengthen the pelvic floor muscles and helps occlude the urethra when involuntary micturition is imminent, but exact mechanism on how does this exercise improves the OAB symptoms is unclear yet, but it has shown clinically that the urinary incontinence is improved by intentional pelvic floor contraction (110). This clinical effect has been reported in a prospective randomised clinical trial of three month course of pelvic floor exercise in women with stress, urge or mixed urinary incontinence, and they found at least one third of participants achieved improvement in urinary

incontinence to the patient's satisfaction for up to 6 months after completion of treatment (118) and others reported a clinician supervised pelvic floor training is much effective and this effect was durable for up to 16 months if patients continued with the exercise (119).

This can be used as a preventative measure for urge incontinence. According to the NICE guidelines published in September 2013, pelvic floor muscles training should be offered to all women during their first pregnancy to minimise the symptoms of incontinence (112).

1.10.2. Second line treatment
1.10.2.1.   Medical management

The medical treatment of overactive urinary bladder is mainly used to interfere with the bladder contractility by blocking the acetylcholine activity at the muscarinic receptors site, urothelial neural inhibition, and suppression of urinary bladder smooth muscles contraction (113).

The following pharmacological agents are in use for the management of overactive bladder symptoms (120). However, it remains a clinical challenge for physicians because of its limitation secondary to the systematic adverse effects;

1.10.2.1.1. Anti-muscarinic drugs

The anti-muscarinic drugs are the mainstay of the medical treatment for overactive bladder symptoms (120). The following three anti-muscarinic pharmacological agents (oxybutynin, tolterodine, and trospium) have been investigated in at least five or more randomized clinical trials and compared to placebo control (120). They were found to be effective in the management of overactive bladder symptoms including urge incontinence but with the burden of frequent systemic side effects and the fact that complete cure cannot be entirely expected. They reported side effects such as dry mouth, blurred vision, nausea and vomiting (120-122).

1.10.2.1.2. β-adrenergic agonists

The urinary bladder has at least three β-adrenergic receptors (β_1, β_2 and β_3 receptors), stimulation of these receptors leads to detrusor smooth muscle relaxation (10). Mirabegron is β_3 agonist and animal studies have shown association with reduction in the pressure inside the urinary bladder and reduce the detrusor smooth muscles contraction during the filling phase (123). It is associated with less dry mouth side effects but increases the cardiovascular adverse effects such hypertension and increase in the heart rate (123).

1.10.2.1.3. Others

Flavoxate is also used in management of urge urinary incontinence, it acts as a muscle relaxant by inhibiting the L-type Ca^{2+} channels in human detrusor smooth muscles and also centrally to suppress the micturition reflex (124-126). Others reported the use of tricyclic antidepressant and oestrogen as an alternative in the management of overactive bladder.

1.10.3. Third line treatment

When OAB symptoms fail to respond to first and second line treatment, they are called refractory OAB. Available treatment for refractory OAB can be achieved either through chemical denervation of the detrusor smooth muscles (using Botulinum toxin A) or via nerve stimulation (11).

1.10.3.1. Botulinum toxins A (Botox®) injection

Botulinum toxin is derived from clostridium botulinum and acts at the level of presynaptic membrane of the neuromuscular junction to inhibit release of acetylcholine and as a consequences prevent contraction, this is a temporary measure as it leads to paralysis of the injected muscle fibres until new fibres can grow (11). The Botox® is injected in at least 20 sites along the posterior wall of the urinary bladder above the trigone, the procedure is done during the cystoscopy procedure to localise the trigone and avoid extreme paralysis which can lead to urinary retention (11). The effect can be achieved in approximately 7-10 days after treatment and lasts for up to one year (11).

This effect was reported in at least two randomised clinical trials comparing Botox injection different doses to placebo control (127,128).

Brubaker et al used Botox® 200 IU compared to placebo equivalent and found approximately 60% of women who received Botox® injection had clinical response and this lasted approximately for a year (127), but this was associated with temporary increase in the residual volume in approximately 40% of participants who received Botox® injection (127). The second randomised clinical trial published this year (2017), the authors used Botox® 100 IU versus placebo control and they found smaller dose of 100 IU of Botox® had significant clinical improvement in all OAB symptoms as well as improvement in the participant quality of life with much less urinary retention symptoms of only 5.4% compared to the previous trial of approximately 40% in patients who received Botox® injection (128). Hence, the smaller dose is the preferred starting dose and all patients should be informed about urinary retention and requirement for self-urethral catheterization (112).

1.10.3.2.   Neuro-stimulation

This is used as a third line therapy if conservative or medical treatment failed to achieve a favourable outcome (110). This can be achieved through pelvic floor muscle nerve stimulation and this can be either non-invasive or invasive techniques which include transcutaneous sacral nerve stimulation, transcutaneous posterior tibial nerve stimulation, and percutaneous posterior tibial nerve stimulation (112).

1.10.4. Surgical treatment

This includes augmentation cystoplasty and urinary diversion. However, these are the last resource of treatment and being reserved for refractory cases (2,111).

1.11.   Summary of over active bladder treatment

Oral anti-muscarinic drugs such as (oxybutynin, tolterodine, and trospium), were found to be effective in the management of overactive bladder symptoms including urge incontinence, but the burden of frequent systemic side effects (i.e. dry mouth, blurred vision, nausea and vomiting (120-122)), and the fact that complete cure cannot be entirely expected, means that compliance is not always 100%. Alternatively, β-adrenoceptor agonists such as Mirabegron as a second-line therapy, effectively reduces detrusor smooth muscle contraction during the filling phase (123). However, this therapy is associated with reduced reports of dry mouth, but do have off target effects on the cardiovascular system, including hypertension and increase in the heart rate (123), which effectively restricts use of β-adrenoceptor agonists as treatment of over active bladder. In addition, the effects of botulinum toxin can be observed in approximately 7-10 days after treatment and last for up to one year (11). However, this therapy still has many said effects such as urinary retention, requirement for self-urethral catheterization and recurrent urinary tract infection (112). As part of this PhD one aim was to characterise novel regulation pathways of GPCRs linked to bladder contraction with the idea of highlighting new approaches for treatment to avoid the side effects associated with existing therapies.

1.12.   Hypothesis and Aim

The regulation and control or lower urinary tract is partially mediated by purinergic signalling. Purinergic receptor actviated signalling pathways need to be tightly controlled, in order to maintain correct bladder function. Since G protein-coupled receptor kinases (GRK) and arrestin proteins play integral roles in regulating GPCR activity we believe these proteins will play a similar role in detrusor smooth muscle muscle (DSMC). The overall aim of this PhD was to determine the role of GRK and arrestin proteins play in the regulation of GPCR signalling in the detrusor of urinary bladder. Therefore, the main aims were as follows:

1. To utilise a pharmacological approach using selective P2 receptor agonist and antagonists to characterise which P2X and or P2Y receptors mediate calcium signalling and thus contractile function of DSMC of the rat urinary bladder.

2. To characterise the profiles of P2Y or P2X receptor desensitization and re-sensitization in isolated DSMC.

3. To characterise the expression of GRK and arrestin subtypes in cultured DSMC and whole bladder.

4. To identify whether endogenously expressed GRK or arrestin proteins regulate P2Y receptor calcium signalling in cultured DSMC.

To mimic the physiological stress that DSMC are exposed to during pathophysiological changes such as obstructive bladder, DSMC will be exposed to mechanical stress/stretch, and the effect on proteins that regulate P2Y receptor calcium/contractile signalling (i.e. GRKs and arrestin proteins) will be assessed.

Chapter 2

2. **General Methods**

2.1. Materials & Methods

2.1.1. Source of materials and instruments

Sigma-Aldrich, Poole, Dorset, UK: Collagenase type IA, papain and dithioerythritol (DDT).

Bio-Rad Ltd. (Hemel Hempstead, Hertfordshire, UK): Bradford assay kit.

General reagents from, Fisher Scientific (Loughborough, Leicestershire, UK): - Potassium chloride, Potassium dihydrogen phosphate, Sodium chloride, Tris-HCl, and HEPES.

Invitrogen (Paisley, UK) (Cell culture): Foetal bovine serum (FBS), albumin, Fungizone, L-Glutamine, Penicillin/streptomycin, Trypsin-EDTA, and Tissue culture media (199), Dulbecco`s phosphate buffer saline (D-PBS).

Sigma Aldrich (Poole, Dorset, UK): **General reagents**- H_2O_2, bovine serum albumin, Calcium chloride, EDTA, Glucose, HEPES, Hydrochloric acid, Magnesium sulphate , and Triton X-100.

Tocris Bioscience (Abingdon, Oxfordshire, UK): Purinergic receptor agonists

(MRS2365), (MRS4062), (MRS2693), (MRS 2279), Adenine triphosphate (ATP),

UTP and ADP**,** Purinergic receptor antagonists 2', 3'-O-trinitrophenyl-ATP (TNP-ATP), (AR-C118925XX), and α,β-methylene adenosine 5'-triphosphate. Selective serotonin reuptake inhibitors (Paroxetine and fluoxetine).

Vector Laboratories (Peterborough, Cambridgeshire, UK): 3, 3'-diaminobenzidine substrate (DAB), normal goat serum (NGS), and Vectashield® Mounting Media

Santa Cruz Biotechnology, Santa Cruz, CA: Anti-GRK antibodies;

Anti-arrestin2 antibody (A1CT): kind gift from Prof. R.J. Lefkowitz, Duke University, Durham, NC, to Dr Willets

Lonza: Nucleofection 2000

GE Healthcare: Enhanced chemiluminescent reagent ECL detected reagent, Amersham Hyperfilm**,** Hyper Processer film developer for western blotting

Invitrogen, Life technologies, Paisley, UK: Fluo-4-acetoxymethyl ester (Fluo 4-AM) and Lipofectamine RNAmaxi for siRNA transfection

New England Bio labs, Hitchin, UK: pre-stained protein molecular size marker

Lonza, Cologne, Germany: siRNA constructs

VWR International, Lutterworth, UK: Tissue culture plastic and cover-slips

2.1.2.   Bladder smooth muscle cells isolation and culture

Adult male Wistar rats were killed by cervical dislocation, this method approved under the United Kingdom Animals (Scientific Procedures) Act 1986. Bladder Smooth muscle cells Isolation and culture was performed according to the protocol approved by Davies, *et al*, 2010.   Immediately after collecting the rat urinary bladder from Dr Richard Rainbow laboratory, the outer membrane covering the bladder was removed, and prior to digest the bladder, 50 mL of dissection solution (NaCl 137 mM, KCl 5.4 mM, Na_2HPO_4 0.44 mM, NaH_2PO_4 0.42 mM, Glucose 10 mM, HEPES 10 mM, $MgCl_2$ 1 mM) was prepared without $CaCl_2$. The DS was filtered using a low protein binding sterile 0.2 μm membrane Acrodisc filter (Life Sciences). The bladder was minced into about 2-4 mm small pieces (Figure 2-1 A). The isolation of detrusor smooth muscle cells was performed by enzymatic dissociation as follow:

Minced tissue was placed into the first digestive solution containing dithioerythritol (DDT) 2 mg (Sigma Aldrich, UK) and papain 2 mg (Sigma Aldrich, UK) in 4 mL of low $CaCl_2$ buffer containing (18 mg of bovine serum albumin and 100 μM $CaCl_2$ in 20 mL of sterile DS), bladder fragments were incubated in the papain/DTT mixture for 15 min at 37°C in water bath.   Next the bladder pieces were transferred into second digestion mixture which consists of 4 mL of low $CaCl_2$ buffer (as outlined above) and 2 mg of collagenase type IA (sigma), which incubated for 20 min at 37°C in water bath. The digested tissue was washed with BSA containing low Ca^{2+} solution and washed twice with 5 mL of low Ca^{2+} solution without BSA to remove the BSA. Finally, the minced tissue was washed with 5 mL of dissection solution without $CaCl_2$.

Bladder pieces were vigorous pipetted using a wide bore plastic Pasteur pipette (VWR International, Lutterworth, UK) to dissociate single detrusor smooth muscle cells and further dissociate cells mixture were pipetted using a fire-polished glass pipette. Following that the mixture was strained through a sieve sterile cell strainer 40 μm nylon meshes (fisher brand) into a fresh 50 mL Falcon tube (VWR International, Lutterworth, UK) to separate isolated cells from the remaining lumps of tissue.

Finally, the sieve was rinsed with 5 mL of dissection solution (no $CaCl_2$) and cells collected by centrifugation at 4°C for 5 min at 800 rpm and detrusor smooth muscle

cells DSMC were separated by gentle trituration in 199 media (Life Technologies, UK), supplemented with 20% foetal calf serum (FCS) and 100 IU/mL penicillin, 100 µg/mL amphotericin B (antifungal) and 100µg/ml streptomycin were maintained at 37°C and under 5% CO_2 in humidified conditions. For the imaging experiments, the cells were re-suspended in 300 µL of cell culture media and 50 µL was spotted onto each coverslip in a 6 well culture plate. Culture plates were then placed in the incubator for 1 h before carefully addition of a further 2 mL culture media to allow cells to adhere to the glass.
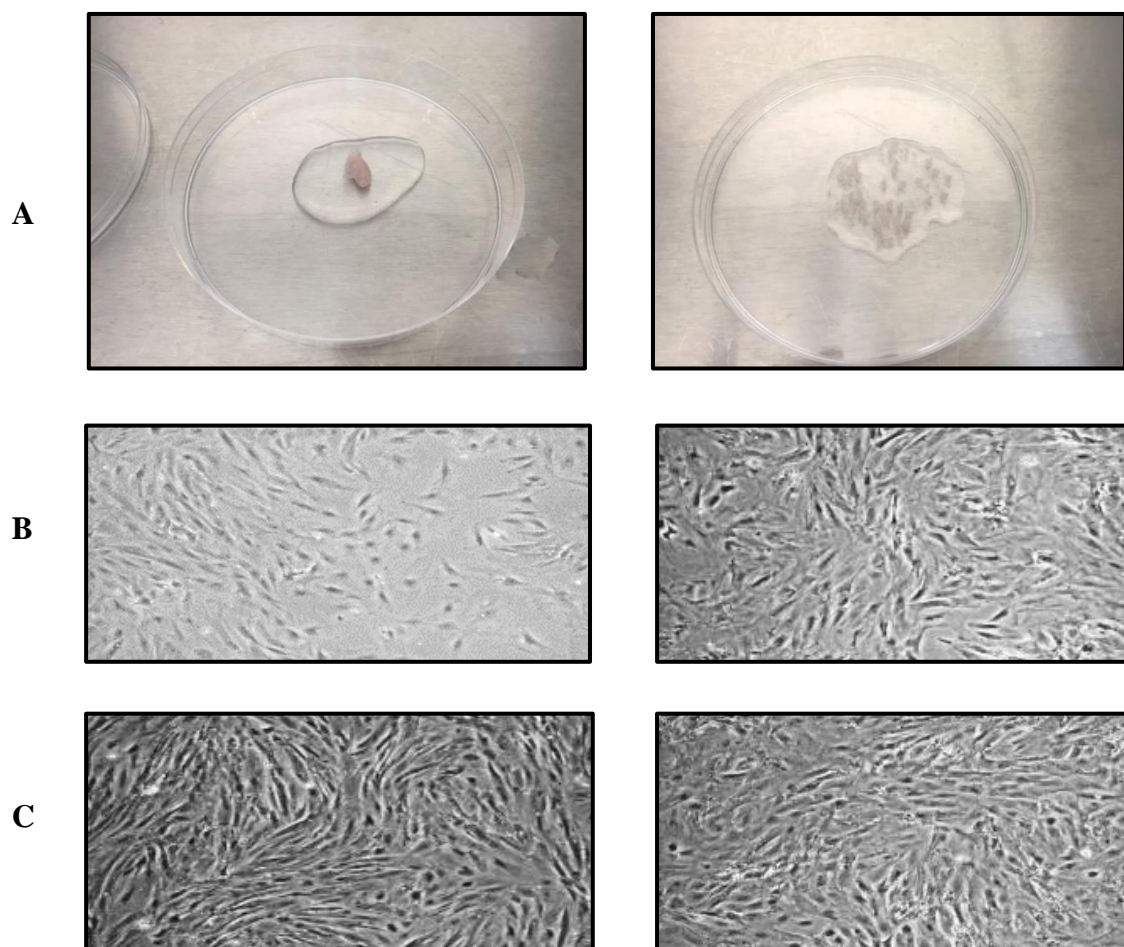
Figure 2-1: Wister rat whole urinary bladder and DSMC photographed at different stages

Wister rat whole urinary bladder (A, left) and dissected into small pieces (A, right). DSMC cultured and photographed at different stages of growth; first day after isolation (B, left) and second day after isolation (B, right). DSM cells at different passages; passage 2 (C, left) and passage 3 (C, right). Magnification was 40 x for all images. All the cells were cultured inside 199 medium containing 10% (v/v) foetal bovine serum

(FBS) and 100 IU/mL penicillin and 100 µg/mL streptomycin. Cells were used for experiment when they were approximately 80% confluent.

2.1.3. Cell maintenance and culture

Cells were maintained at 37ºC in 5% CO_2 by changing the media every 48 h until nearly confluent. Once approximately 80% confluent, then the procedure of cell passaging was started by aspiration of the media. DSMC were washed twice with phosphate buffer solution PBS (Gibco, Life Technologies, and UK) to remove any residual media. Cells were detached from the surface of the flask using 0.25% trypsin-EDTA (Gibco, Life Technologies) being incubated at 37ºC in 5% CO_2 for no more than 5 minutes. During the procedure mild agitation was used to help detachment of cells. Complete detachment was determined under the microscope and immediately followed by addition of 199 growth media supplemented with 10% FCS, in order to deactivate the effect of trypsin. Then the cells were centrifuged at 1000 rpm for 5 min, before being re-suspended in fresh media. Cells were then seeded in new plates at an appropriate density.

2.1.4. Characterization of smooth muscle

To characterize the smooth muscle after isolation and to confirm the isolated cells displayed a smooth muscle phenotype, we initially used light microscopy to assess the morphological characteristics of the cultured smooth muscle cells at different passages (Figure 2-1 B and C). To further assess the isolated cells phenotype western blot analyses were performed on DSMC cells by using primary antibodies (Table 1) to detect the smooth muscle cells markers stained with antibodies against α-smooth muscle actin (SMC) (129) and calponin at concentrations recommended by the suppliers.

2.1.5. Immunocytochemistry of smooth muscle

To confirm the morphology of isolated cells were smooth muscle. Cells were seeded into 13 mm glass coverslips in 24-well/plate multi-dish and incubated at 37°C in a humidified air (5% CO_2) atmosphere until become approximately 80% confluent. Medium was removed and cells were washed twice with PBS (Dulbecco's phosphate buffered saline). Cells were then fixed with 4% paraformaldehyde for 10 min at room temperature. Following that cells were washed with sterile phosphate buffer solution (PBS for 3 x 5 min). Anti-α-actin (1:1000) or anti-calponin (1:10,000) (Sigma, Pool, Dorset, UK) diluted in PBS-T (PBS–Triton X 100 0.2% v/v the concentrations recommended by the supplier; (Table 1) were used to detect these proteins in an overnight incubation at 4°C. Next day, cells were washed with PBS for 3 x 5 min and incubated with goat anti-mouse fluorescein isothiocyanate-tagged secondary antibody (1:400 dilution) to detect the primary antibodies, for 2 h at room temperature in the dark. Cells were washed an extra three times in PBS before adding of the nuclear stain propidium iodide diluted (1:3000) in PBS for 5 min, cells were Covered with foil to keep out of the light. Cover slips were washed with PBS and mounted in Vectashield mounting medium (from Vector Laboratories), a glycerol-based mounting medium for preserving fluorescence. Finally, the slides sealed with clear nail varnish and then stored at 4 °C until imaged. alpha-actin and calponin staining was pictured using an Olympus FV500 scanning laser confocal microscope (Olympus Corp., Lake Success, NY) by 488-nm laser excitation. Images were taken using a ×60 objective.

2.1.6. Cell counting

Cells were counted using a haemocytometer whish 20 µL of suspension cells were placed in the haemocytometer and cells were counted in each 2 x 1 mm^2 corner squares. The number of cells was calculated by taking the average of each square (total number of cells divided by 2) and multiplied by a factor of 10^{x4}, which gives the number of cells per mL of cell suspension (Figure 2-2).
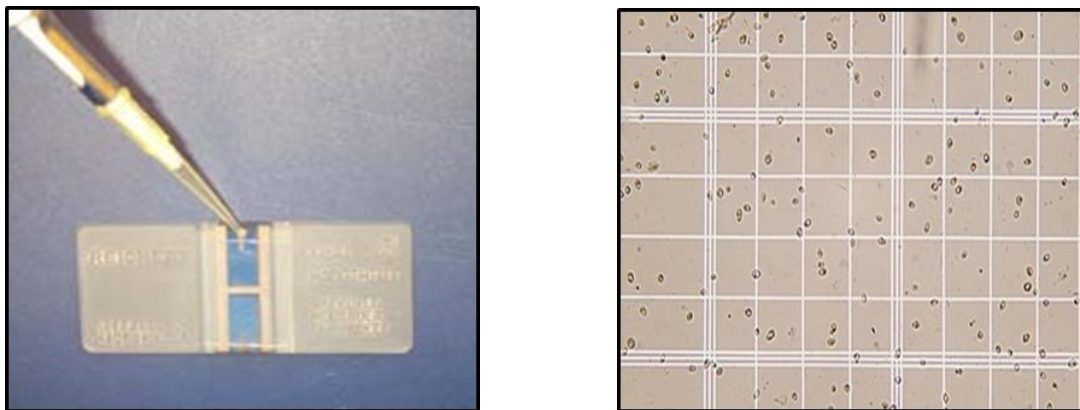
Figure 2-2: Cell counting using haemocytometer method

The above pictures explain how the haemocytometer count the cells, (left) shows how suspension cells were placed in the haemocytometer; (right) shows the number of cells in square. The picture is adapted from Wikipedia.

2.1.7. Homogenization of bladder

Whole bladder were isolated from Wistar rats, and the outer membranes removed as described above, minced and transfected to lysis buffer (50 mM Tris·HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.25% w/v sodium dodecyl sulphate), prior to homogenization using an Ultra Turrax homogenizer on full power for 30 s on ice. Insoluble material was removed via centrifugation (14,000 rpm, at 4°C), and the supernatants removed and the protein concentration determined by the method of Bradford (include the reference). Finally, the supernatants were aliquoted and stored at -80°C until required for western blotting. Protein Analysis and Western Blot

2.1.7.1. Bradford Assay

In order to equalise protein loading for all SDS-PAGE gels protein levels were determined in all cell and whole bladder lysates using the method of Bradford (1976). Briefly, a BSA standard curve (range 0.032, 0.064, 0.125, 0.25, 0.5, 1, 2 mg/mL) prepared in 0.1M NaOH, was used as a reference for determining the protein concentrations from cells or tissue lysates. Twenty micro litre of the bovine serum albumin standard curve were mixed and added to plastic cuvettes (size 1.5 mL), followed by 1 mL of Bradford reagent, and incubated at room temperature for 15 min. The absorbance was measured at 595 nm using a spectrophotometer (Gene spectrophotometer, Amersham Biosciences), and protein concentration were plotted against absorbance to generate standard curves using the GraphPad Prism v7.2 software (San Diego, CA).

2.1.7.2. Sodium dodecyl sulphate Polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

The percentage of acrylamide in gel was chosen depending on the size of the protein of interest. Lysates were mixed with SDS-containing loading buffer (5 × loading buffer) (40 µg) of each lysate protein was loaded per well (protein levels were determined via the method of Bradford as described above), on a 10% of gel alongside 2 protein markers was loaded in the first well of the gel: standard pre-stained protein ladder and biotinylated markers were used for orientation of the relative size of the separated

proteins in the cell lysate. Gels were then resolved in running buffer (0.25M Tris-HCl, 1.92M Glycine- 1% v/v SDS) and proteins were separated by electrophoresis at 150V. Proteins were transferred onto nitrocellulose membrane (Amersham) of poor size 0.45 µm equilibrated in semi-dry transfer buffer (48 mM Tris, 39 mM glycine, 0.01% w/v SDS, 20% v/v methanol) for 5 min prior to transfer using a semi-dry transfer cell (Bio-Rad). Transfer time was dependent of the molecular weight of the protein of interest at 21 V. Following transfer, non-specific binding sites were then blocked by incubation of the nitrocellulose membrane in 5% (w/v) non-fat milk powder in TBST buffer (50 mM Tris, 150 mM NaCl, 0.1% v/v Tween-20) or 5% (w/v) BSA in TBST for 1 h at room temperature. At the end of 1 h blocking the membranes were incubated with the appropriate primary antibody (**see Table** 1) either in TBST with 5% (w/v) milk for GRK proteins or with BSA 5% (w/v) in TBST for arrestin proteins overnight at 4°C. The following day membranes were washed (3 × 15 minutes) with TBST, before application of an appropriate secondary antibody in TBST with 5% milk (Table 1) for 1 h on a rocking platform. Membranes were washed (3 × 15 minutes in TBS-T) to remove any excess non-bound antibody, then the membranes were incubated with ECL detection reagent (enhanced chemiluminescent reagent from GE Healthcare) for 5 minutes. Finally blots were placed in cellophane, folded and sealed before being exposed to Hyperfilm (Amersham Hyperfilm, From GE Healthcare) in a light-tight cassette. Films were developed using a hyper processer (Amersham Biosciences).

GRK and arrestin proteins expression were determined, using standard immunoblotting protocols as described above. Arrestin2 and arrestin3 expression was determined using an anti-arrestin2 antibody (A1CT) (a kind gift from Prof. R.J. Lefkowitz, Duke University, Durham, NC, to Dr Willets). The A1CT antibody was raised against arrestin2 but also detects arrestin3 with lower affinity enabling detection both proteins (85,108,130).

Table 2-1: Antibodies, dilutions used for Western Blot and their suppliers

Target protein	Primary antibody dilution	Secondary antibody	suppliers	Secondary antibody dilution
GRK2	1 : 1000	Anti-rabbit, Anti-biotin	Santa Cruz	1 : 1000 1 : 2000
GRK3	1 : 1000	Anti-rabbit, Anti-biotin	Santa Cruz	1 : 1000 1 :2000
GRK5	1 : 500	Anti-rabbit, Anti-biotin	Santa Cruz	1 : 1000 1 : 2000
GRK6	1 : 1000	Anti-rabbit, Anti-biotin	Santa Cruz	1 : 3000 1 : 2000
Arrestin 2/3 (A1CT)	1 :1000	Anti-rabbit	Lefkowitz laboratory	1: 1000
α-actin	1 :1000	Goat anti-Mouse	Santa Cruz	1:400
calponin	1 :10000	Goat anti-Mouse	Santa Cruz	1:400

2.1.8. Measurement of intracellular $[Ca^{2+}]_i$ and IP_3 in DSMCs

2.1.8.1. Confocal microscopy theory

Confocal laser scanning microscopy (CLSM) allows high-resolution optical images with depth selectivity to be obtained by a process known as optical segmenting. A laser light beam is focused onto a fluorescent sample over the objective lens. The combination of reproduced and released light is taken by the same objective and is referred to the dichroic mirror. The imitated light is diverged by the mirror whereas the emitted fluorescent light goes through a confocal orifice (pinhole) to decrease the "out of focus" light (Figure 2-3). The pinhole blocks light approaching from objective points external the focal plane and excludes it from detection. The directed light permits over the emission sieve and continues to the photomultiplier. With the aim of producing a perfect image, the solo point is scanned in an X-Y mode as the laser beam is moved over the sample. To achieve information about the total sample, the laser beam needs to be focused through the sample or to change the sample near to the laser beam in technique known as scanning.
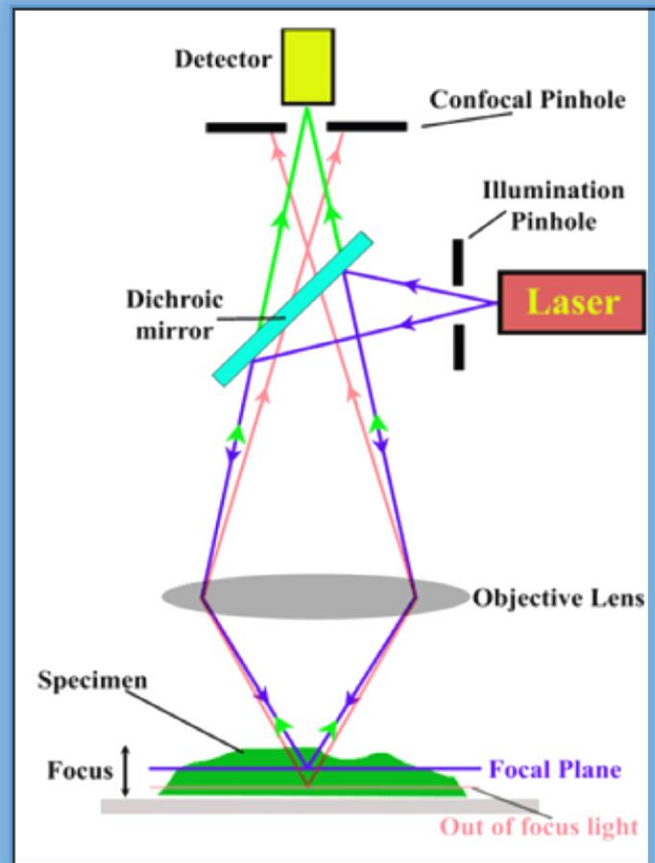
Figure 2-3: The picture shows confocal imaging

Laser beam illuminated from a light source goes over the objective lens, which passages up and down. Reflected light from the goal object passes over a half mirror and a pin hole, and reaches the light-receiving element. Fluorescent light (i.e. signal) passes back through the dichroic reflector and then passes through the second (exit) pinhole which is confocal. The exit pinhole can be made small enough that any light coming from areas away from the vicinity of the illuminated point will be congested by the aperture, accordingly provided that further attenuation of out of focus interference. A photomultiplier detector (PMT) delivers a signal of the light transient as the sample is scanned. A computer is used to control the successive scanning of the sample and to bring the image for display. Image adapted from Wikipedia.

2.1.8.2. Single cell confocal microscopy methodology

Forty eight hours after isolation DSMC were loaded with Ca^{2+}-sensitive dye Fluo 4-AM (4 µM) at room temperature for 60 minutes in the dark. To assess changes in intracellular Ca^{2+} cells were mounted onto the microscope and cells were maintained at 37°C using a Peltier unit, with continually perfusion of modified Krebs-Henseleit buffer composition in (mM); NaCl 134, KCl 6, $MgCl_2$ 1, glucose 10, HEPES 10 $CaCl_2$ 1.3, pH 7.4). Drugs were applied via the perfusion system. Images were taken every 3 sec using an Olympus FV500 Nikon C1Si laser scanning confocal inverted microscope (Olympus Corp., Lake Success, NY) with 488-nm laser excitation. Images were taken using a × 60 objective (Figure 2-4).
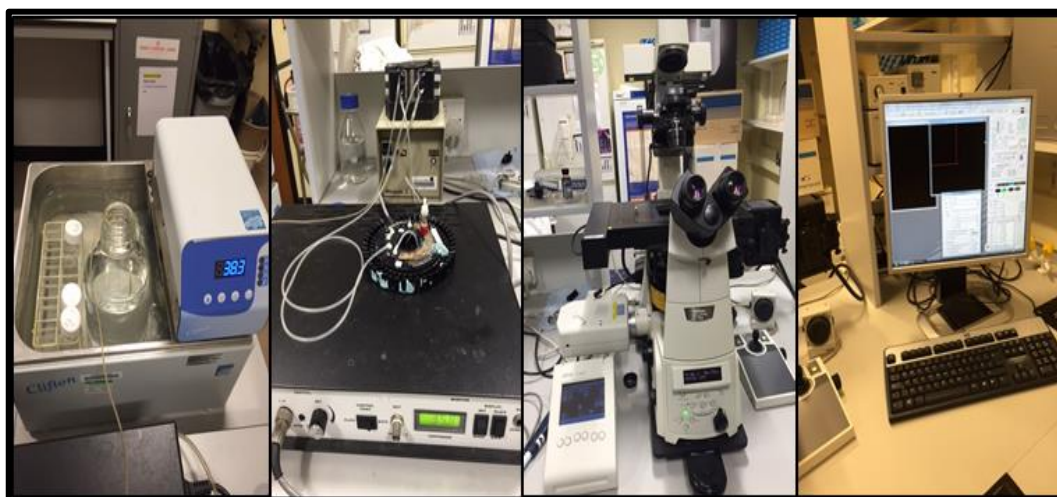
Figure 2-4: Laser scanning confocal inverted microscope using (X60 oil immersion objective).

This figure shows from right to left; water path, perfusion system, confocal microscope, and computer screen. This system used for the measurement of intracellular $[Ca^{2+}]_i$ and IP_3 in DSMCs and during assessed receptor desensitization.

2.1.8.3. Assessment of P2Y receptors stimulated Ca^{2+} signalling in DSMC (NOVO star imaging system)

DSMC were plated into 96-well plates and grown to (80% confluency). Cells were loaded with the Ca^{2+} sensitive dye (4 μM Fluo4-AM at room temperature for 1 h). Cells were washed with Kerbs buffer prior to challenge. Selective P2Y$_{2/4/6}$-receptor agonists (Tocris, UK), then the antagonist was added and the changes in intracellular Ca^{2+} levels. [Ca^{2+}]$_i$ were determined as the relative change in fluorescence using a NovoStar imaging system (BMG Labtech, Aylesbury, UK) excitation at 485 and emission of 520.

2.1.9. Cell Transfection

2.1.9.1. DSMCs transfected with red florescent protein (RFP)

DSMCs were transfected with red fluorescent protein (RFP) to help identify which cells were transfected with the DMN. To assess the potential roles that GRKs play in the regulation of P2Y-receptor signalling, DSMC were co-transfected with eGFP-PH (0.5 μg) and red florescent protein (RFP) 0.25 μg, In order to assess the transfected cells with DMN. The following dominant-negative mutant (DNM), catalytically inactive GRK mutants: ^{D110A,K220R}GRK , ^{D110A,K220R}GRK3, ^{K215R}GRK5, ^{K215R}GRK6 (0.5 μg), or control plasmid (pcDNA3) (Figure 2-5).
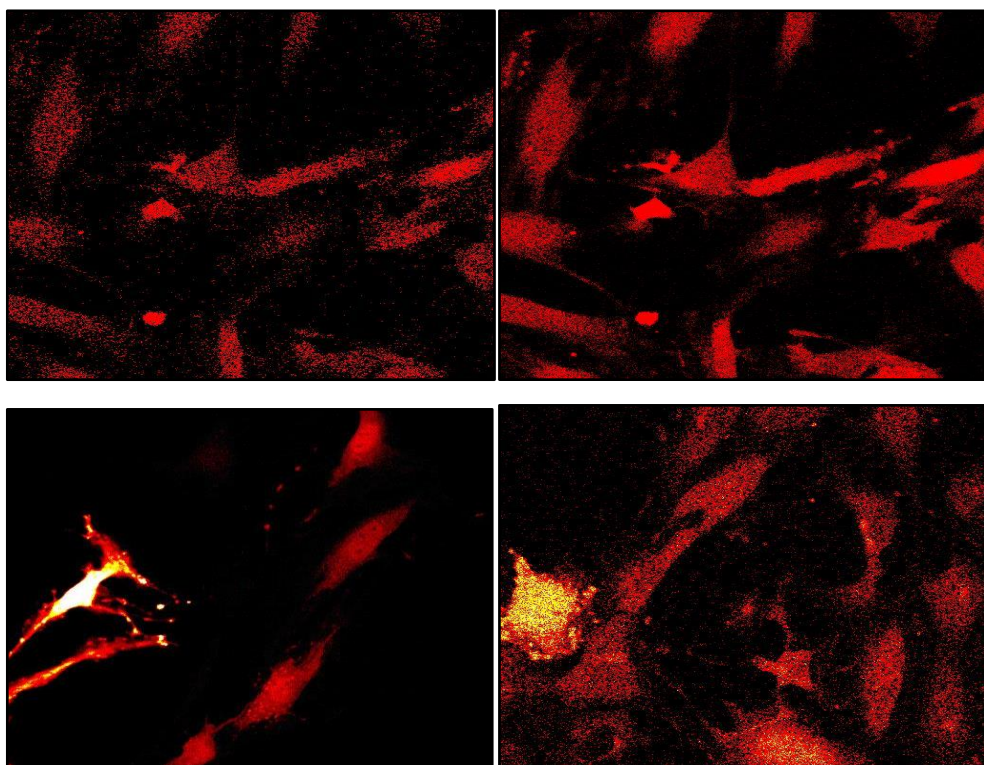
Figure 2-5: Detrusor smooth muscles collected from four different animals at different dates.

DSMC were co-transfected with eGFP-PH (0.5 mg) and red florescent protein (RFP) 0.25 µg transfected. This demonstrates that the red coloured are the transfected cells with the red florescent protein.

2.1.9.2. GRK2 and Arrestin knockdown by using siRNA

Detrusor smooth muscle cells were transfected using the Amaxa nucleofection technique (Lonza, Cologne, Germany), according to manufacturer's instructions. In brief, cells were harvested with 0.25% (w/v) trypsin, counted using a haemocytometer as described above so that each sample transfection contained $2x10^6$ cells. The cells were centrifuged at 1000 rpm for 5 min, and medium was carefully removed. The cell pellet was re-suspended in 100 µL of the transfection buffer (Lonza, Cologne, Germany), before addition of either negative control (non-targeting), anti-GRK2 (5′-GCAGGUACCUCCAGAUCUCtt-3′), anti-arrestin2 (5′-GCCACUGACUCGGCUACAAtt-3′) or anti-arrestin3 (5′-GCCUUCUGUGCCAAAUCUAtt-3′) siRNAs (Applied Biosystems, UK) at a final concentration of 10 nM, were added as required. Next cells were transferred to Amaxa-certified cuvettes using filtered pipette tips and transfection was performed using an

Amaxa/Lonza nucleofector device, set to program D-033 (which has been previously optimised in our laboratory as the protocol that gave maximal transfection of smooth muscle cells). Immediately after transfection, 0.5 mL of pre-warmed (37°C) supplemented 199 media was added to each cuvette, and the cells were transferred into Eppendorf tubes using plastic Pasteur pipettes provided. Cells were incubated at 37°C in a humidified air: 5% CO_2 atmosphere for approximately 10 min prior to seeding onto 6 well/plates. Arrestin or GRK2 depletion was assessed 48 h after nucleofection (LonzaAG, Cologne, Germany) by immunoblotting using either anti-arrestin1/2, or anti-GRK2 antibodies. Arrestin and GRK2 expression was quantified using the GeneGnome image analysis system (Syngene, Cambridge, UK).

2.1.10. Mechanical cell stretching

The cells were grown on a type I collagen-coated silicon membrane (Bio flex, from international corporation). Mechanical deformation of the cells was performed using a Flexer cell strain unit (FX- 4000) a device designed to apply precise and reproducible biaxial strains to the membrane on which the cells were grown. Design, calibration, and description of the equibiaxial strain system for cultured cells have been previously reported (20). The cells were seeded and incubated for 24 h before being serum starved for a further 24 h. Biaxial strain was applied by stretching the elastic membrane cyclically for 24 to provide 10% and 30% strain. So the details of the strain level estimation, the design of the Tensor stretch instrument, and computer program are optimised by our group in the lab. The strain level of 10% and 30% were used in this study because, this condition was found to be sufficient to produce molecular changes without inducing apparent cell injury. For comparison control non-stretched cells were cultured in the 6 wells plates collagen-coated under the same circumstances but without exposing them to mechanical strain. After completion of the stretch technique, control and stretched cells were scraped off and processed for protein assay and subsequent immunoblotting. To investigate the GRK and arrestin expression in detrusor smooth muscle cells from at least six individual wells were pooled to extract sufficient quantities analysis. Stretch and control experiments were processed at the same time and analysed identically.

2.1.11. Quantification and Data Analysis

Data were statistically calculated after assess the normality test. A one-way ANOVA test was used to compare among the groups followed by appropriate Post hoc test to test the significance between two groups. When datasets were not normally distributed, non-parametric statistical (Mann-Whitney) test was used as an alternative test. Student t-test was undertaken when only one group treated compared with control group. All data considered statistically significant when P values were ≤ 0.05. Immunoblot band intensities were quantified using the Syngene GeneGnome system with Gene Tools software. Concentration-response relationships were analysed by non-linear regression using GraphPad Prism 7 software (San Diego, CA).

2.1.12. Buffers

2.1.12.1.   Smooth muscles dissection buffer

NaCl	137 mM
KCl	5.4 mM
Na_2HPO_4	0.44 mM
NaH_2PO_4	0.42 mM
Glucose	10 mM
HEPES	10 mM
$MgCl_2$	1 mM

2.1.12.2.   Running gel composition 10% (per 2 gels)

Distilled H_2O	7.9 mL	
Acrylamide	6.7 mL	
1.5M Tris HCl	5 mL	
SDS	200 μL	
Do not add AMPS or TEMED until immediately before use		
10 % AMPS	200 μL	
TEMED	8 μL	In metal yellow cupboard

ml=millilitre, μl=microlitre

2.1.12.3.   Stacking gel composition (per 2 gels)

Distilled H_2O	5.5 mL	
Acrylamide	1.3 mL	On bench shelf
1M Tris	1 mL	Do not confuse with 1.5M
SDS	80 μL	On bench shelf
Do not add AMPS or TEMED until immediately before use		
10% AMPS	80 μL	Make when required (see point 3)
TEMED	8 μL	In metal yellow cupboard

M=molar, ml=millilitre, μl=microliter

2.1.12.4. Running buffer

10x TGS running buffer	100 mL
Distilled H$_2$0	900 mL

Running buffer was supplied as a 10X concentrate, which was diluted to 10X working stock in distilled water (dH$_2$O) TGS is 25mM Tris, 192mM glycine and 0.1%SDS

2.1.12.5. Semi-dry blotting buffer

Distilled H$_2$0	600 ml	
Tris-Base	48 mM	5.8 g
Glycine	39 mM	2.9 g
SDS	0.037 %	0.37 g
Methanol	20 %	200 mL
Distilled H$_2$O	Make up to 1 L	

L=litre, mL=millilitre, g=gram

2.1.12.6. TBS-T

5M NaCl	60mL
1M Tris	20mL
Distilled H20	1950mL
pH adjusted to 7.5 by slowly adding 1M NaOH	
Tween 20	1mL

M=molar, mL=millilitre

2.1.12.7. Krebs-Henseleit buffer

Nacl	134 mM
Kcl	6 mM
MgCl$_2$	1 mM
KH$_2$PO$_4$	1.2 mM
Glucose	10 mM
HEPES	10 mM
CaCl$_2$	1.3 mM
pH 7.4	

mM=milliomolar

2.1.12.8. Lysis Buffer

pH	7.4
HEPES	20 mM
NaCl	200 mM
EDTA	10 mM
Triton-X 100	1%
Benzamidine	0.2 mg/mL
Leupeptin	0.1 mg/mL
Phenylmethylsulphonylfluoride	0.5 mM

mM= millimolar, mg= milligram, mL= millilitre

Chapter 3

3. **Characterisation of purinergic receptor subtype expression and activity in rat detrusor smooth muscle**

3.1. Introduction

Adenosine triphosphate (ATP) and adenosine diphosphate (ADP) are co-released with ACh from parasympathetic nerves (43), and activate purinergic receptors to initiate bladder detrusor smooth muscle contraction. In 1985, Burnstock and Kennedy classified P2 receptors into ionotropic (P2X) and metabotropic (P2Y) receptor families, responsive to nucleotide ligands and distributed on every mammalian cell. There are two types of purinergic receptors that respond to ATP or UTP identified in the bladder, P2X (ion channels) and P2Y (GPCR) (54).

Purinergic receptors are involved in both motor and sensory functions (131). These receptors are located at or close to the luminal surface of the urinary bladder, suggesting that ATP and UTP may have an important role in chemical signalling of bladder contraction and relaxation (54). P2X receptors are ligand-gated cation channels and when activated generate a depolarising Na^+ and Ca^{2+} current that activates L-type Ca^{2+} channels to generate an action potential and therefore cause a further rise in $[Ca^{2+}]_i$ inducing smooth muscle contraction (132).

In an adult human bladder; $P2X_1$ was the predominant purinergic receptor established at the mRNA level (61). $P2X_1$ receptor activation generates an inward, depolarising current of Na^+ and Ca^{2+} sufficient to activate L-type Ca^{2+} channels to generate an action potential and promote Ca^{2+} influx (132). The influx of Ca^{2+} can either directly activate the contractile proteins, or more effectively raise the intracellular $[Ca^{2+}]_i$ further by Ca^{2+} induced Ca^{2+} release (CICR) from Ca^{2+} stores. On the other hand, P2Y receptors are coupled to G-proteins (26), which are believed to result in relaxation of the detrusor smooth muscle when activated via cAMP-dependent PKA activity (58). In relation to the urothelium, expression and activation of urothelial $P2Y_2/P2Y_4$ receptors could have a role in autocrine/paracrine signalling throughout the urothelium (32).

In addition, functional $P2Y_6$ receptor expression within the suburothelial myofibroblasts which may play an important role in integrating or amplifying the response to bladder distention has also been reported (32). By activation of a population of sub-urothelial bladder nerves, urothelial-derived ATP release during distension could trigger sensation of fullness and pain or induce changes in bladder activity (131).

3.2.Aims

Purinoceptors (P2) contribute to several areas of bladder function including sensation, neural signalling and voiding contraction. Therefore any anatomical or functional defects in purinergic receptor signalling may be connected with a range of urological diseases such as over active bladder and urinary incontinence. At present little is known regarding the mechanisms or regulation of bladder smooth muscle by GPCR signalling. The main objectives described in this chapter were to investigate the pattern P2 receptor expression ionotropic (P2X) and G protein-coupled (P2Y) receptors in DSMCs. and to examine P2Y receptors mediated changes in intracellular calcium concentration $[Ca^{2+}]_i$ in detrusor smooth muscle cells of rat bladder by using (NOVOstar) imaging system (see *the Methods section for more details*).

3.3. Method

Detrusor smooth muscle cells (DSMC) were isolated from male Wistar rats by enzymatic digestion using papain and collagenase. DSMC were cultured in 199 media supplemented with 10% foetal calf serum, penicillin and streptomycin (as described in material and method). For Immunocytochemistry of smooth muscle cells were fixed with 4% paraformaldehyde for 10 min at room temperature, followed washed by PBS for 3 x 5 min. As primary antibody we used α-actin (1:1000) or calponin (1:10000) diluted in PBS-T (PBS –Triton X 100 (0.2%)) incubated overnight at 4 $^{\circ}$C. Next day cells were washed with PBS for 3 x 5 min and incubated with goat anti-mouse secondary antibody (1:400) for 1 h and covered with foil to keep out of the light at the room temperature. Cover slips were washed with PBS and mounted on Vectashield and sealed with clear nail varnish and then imaged. Two days after cells isolation DSMC were loaded with the calcium sensitive dye Fluo4-AM (4 μM) at room temperature for 60 min, using confocal imaging in single cells in the presence of various P2 receptor agonists and antagonists. Increase in intracellular $[Ca^{2+}]_i$ were defined as F / F_0 where F was a florescence at any given time, and F_0 was the initial basal florescence Average height of Ca^{2+} spikes was calculated from the basal spikes, to spikes in the presence of agonist. All the agonists were added via the perfusion system. Secondly, DSMC were plated into 96-well plates and loaded with the Ca^{2+} sensitive dye (4 μM fluo4-AM at room temperature for 1h) using the 488-nm. Cells were challenged with selective P2-receptor agonists (Tocris, UK) and antagonist and the changes in intracellular Ca^{2+} levels $[Ca^{2+}]_i$ were determined as the relative change in fluorescence using a NovoStar imaging system (BMG Labtech, Aylesbury, UK).

3.4. Data analysis

All concentration–response curves were generated and EC_{50} values determined using non-linear regression analysis software Prism, version 7 (Graph Pad Software Inc., San Diego, CA, USA). Data were analyzed using one-way ANOVA, followed by appropriate test Significance was accepted when $*P < 0.05$.

3.5. Results

3.5.1. Characterization of DSMC cultures

In order to determine the purity of the DMSC isolated cultured cell populations a number of parameters were examined. Firstly, cell morphology was examined using light microscopy and confirmed expected smooth muscle cell characteristics, specifically; an ellipsoid shape with tapered ends and a single central nucleus (Figure 3-1 A). In addition, confluent cells were tightly packed and formed a "hill and valley" appearance in culture (Figure 3-1 A). Next western blotting analysis was used to determine expression of the smooth muscle cell markers such as α-actin (133) and calponin in cell lysates derived from isolated DSMC. Proteins were first separated on 10% SDS-PAGE gels (Figure 3-1 B), transferred to nitrocellulose membranes (**see** Materials and Methods), before being probed with antibodies against the actin severing protein calponin or smooth muscle specific α-actin. The anti-calponin antibody produced a specific band at 33 kDa and the anti-α-actin a band at 42 kDa (Figure 3-1 B), which was present in both DSMC lysates and extracts from whole bladder lysates. To determine the purity of DSMC cultures cellular expression of calponin and α-actin were examined using immunocytochemistry (Figure 3-2) (details in chapter 2 materials and methods section).

(A)

Day 1

Day 2

Day 4

(B)

Bladder lysate   **DSMC lysate**

Calponin 33KDa

α-actin 42KDa
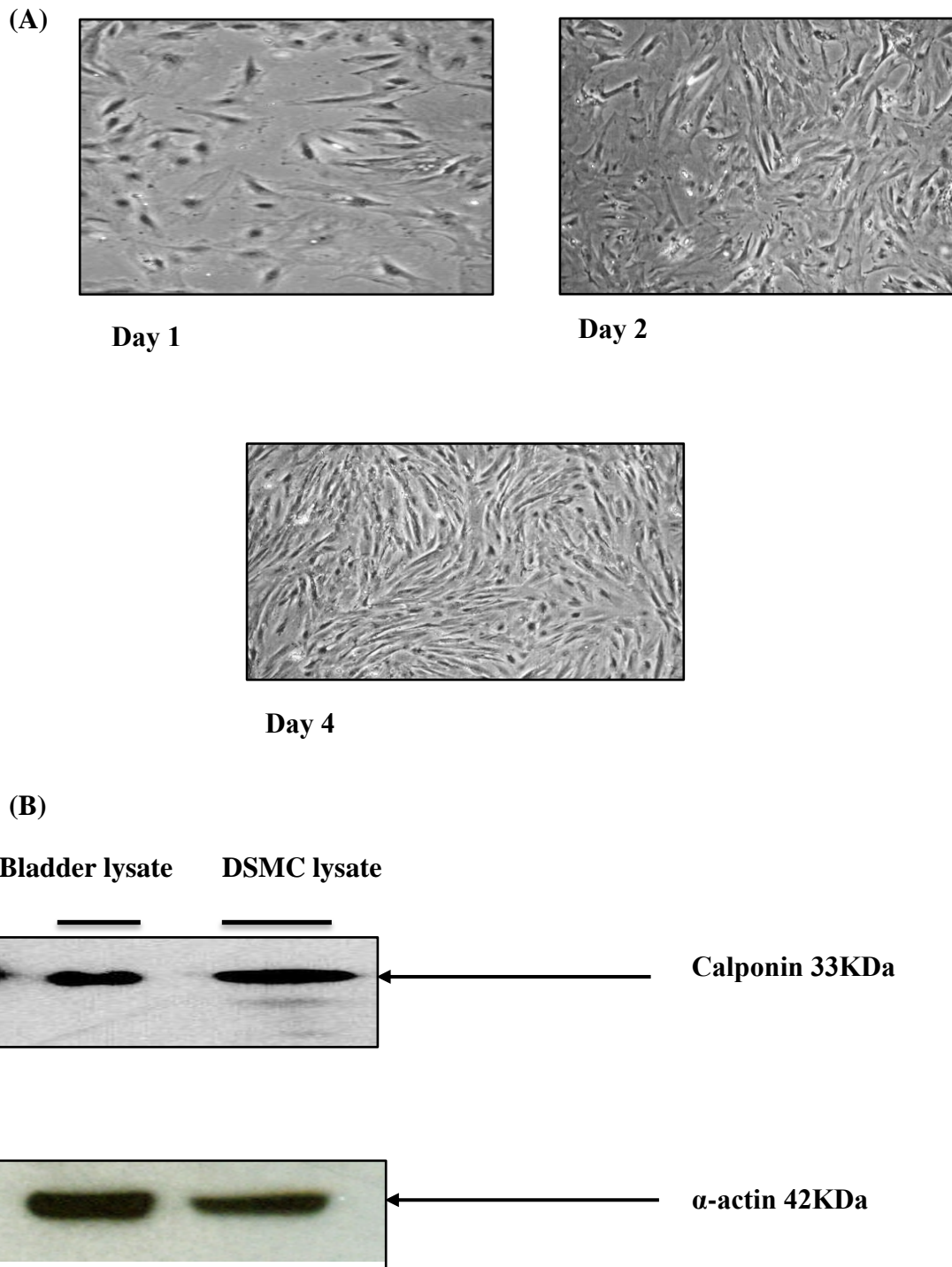
Figure 3-1: Characterization of bladder detrusor smooth muscle cells (DSMC).

To confirm smooth muscle cells phenotype, DSMC were examined morphologically and smooth muscle cell markers were examined by western blotting. **(A):** Representative photomicrographs show DSMC at different stages of growth. Magnification 40 X for all images of DSMC in culture. (**B):** Western blots showing

protein expression for α-actin and calponin in rat bladder and cultured DSMC lysates, as described in Materials and Methods.

3.5.2. Immunocytochemistry of smooth muscle
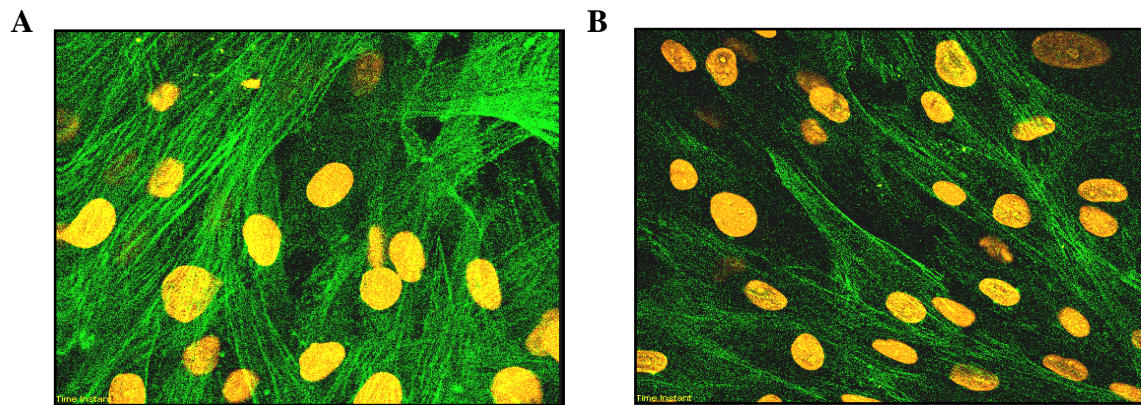
Figure 3-2: Immunocytochemical pictures of bladder smooth muscle cell

The above figure showing (green stain: FITC-conjugated anti-αSMA antibody) filaments of α-smooth muscle actin (A) using a fluorescence microscope (x40). (B) Shows calponin in DSMC the position of the nucleus is recognized by using a yellow stain (propidium iodide) after 7 days in culture.

3.5.3. Intracellular Calcium Imaging

3.5.3.1. Ca^{2+} imaging stimulate the cells with purinergic agonists and antagonists to identify the P2 receptors on isolated detrusor smooth muscle cells

ATP and UTP are potent agonists for both (P2Y and P2X) receptors (58). Previous studies have suggested that a variety of P2X and P2Y receptors are expressed in DSMC (57), these receptors are able to mediate changes in intracellular Ca^{2+} and therefore induce contraction. Consequently we examined whether DSMC respond to the purinergic agonists ATP and UTP. DSMC loaded with the Ca^{2+}-sensitive dye Fluo4 (see Methods and materials, chapter 2) were challenged with high concentrations of ATP or UTP (100 μM) for 30 sec and the changes in intracellular Ca^{2+} levels were detected as a change in cellular fluorescence using confocal microscopy (Figure 3-3). Our result suggest that when DSMCs of rat bladder challenged with ATP and UTP produced transient elevations in intracellular calcium $[Ca^{2+}]_i$ suggesting the presence of P2Y and/or P2X receptors (Figure 3-3).

A

B

C

D
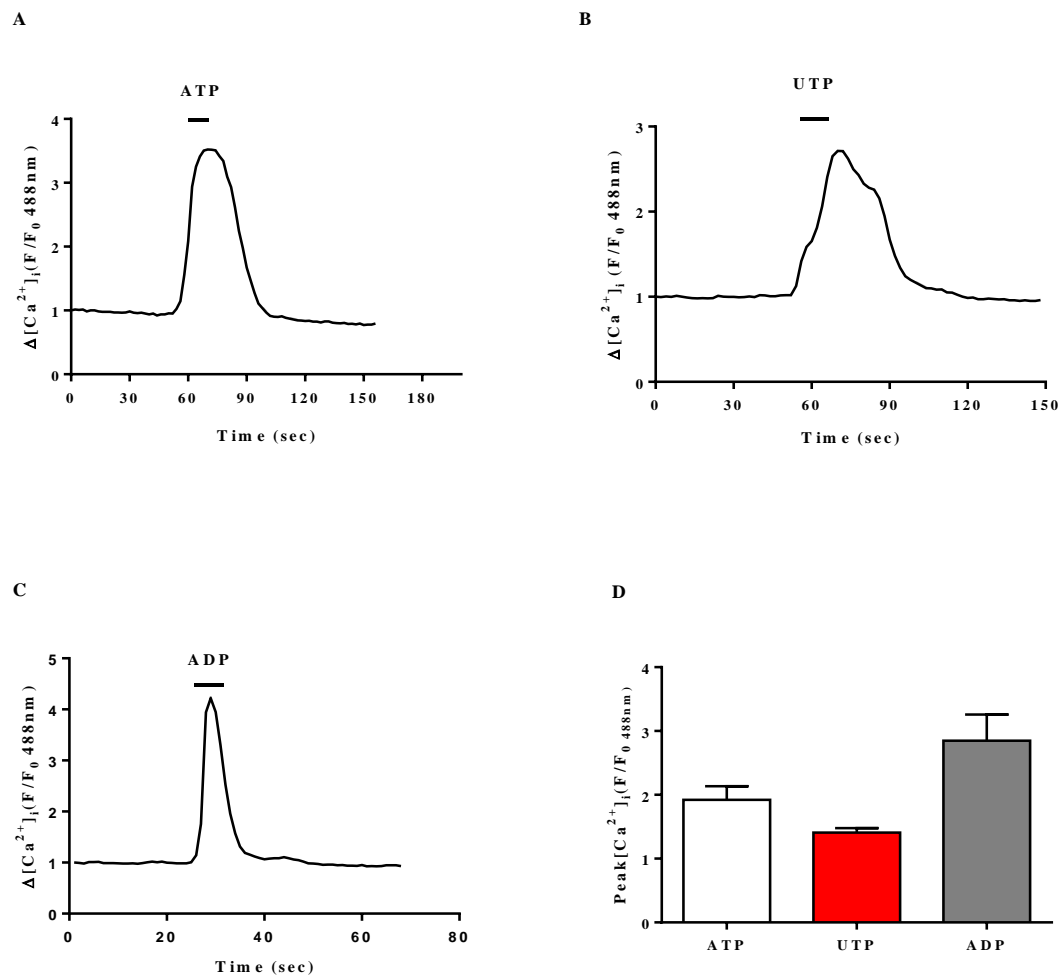
Figure 3-3: Representative traces showing changes in intracellular Ca^{2+} levels

When DSMC loaded with the Ca^{2+} sensitive dye Fluo4-AM (4 μM), were challenged for 30 sec with ATP (100 μM, A), UTP (100 μM, B), or ADP (10 μM, C). Cumulative data (D) shows the means ± SEM [Ca^{2+}]$_i$ changes from 20-38 cells, from at least 4 separate bladder preparations.

3.5.4. Characterization of ATP and UTP-mediated Ca^{2+} signalling in isolated detrusor smooth muscle DSMC

DSMC loaded with the Ca^{2+}-sensitive dye Fluo-4 (details in chapter 2 methods section) were challenged with different concentration of ATP and UTP (1, 10, 100 μM and 1mM for 30 sec) with 5 min washout between agonist treatments (Figure 3-4). Unfortunately, this protocol is not good to measure concentration response curve, because the change of intracellular calcium was high when the cells were challenged with 10 μM by ATP or ADP, and low when used 1000 μM. An alternative protocol was therefore introduced to examine concentration responsiveness.

A

ATP

B

UTP

C

D
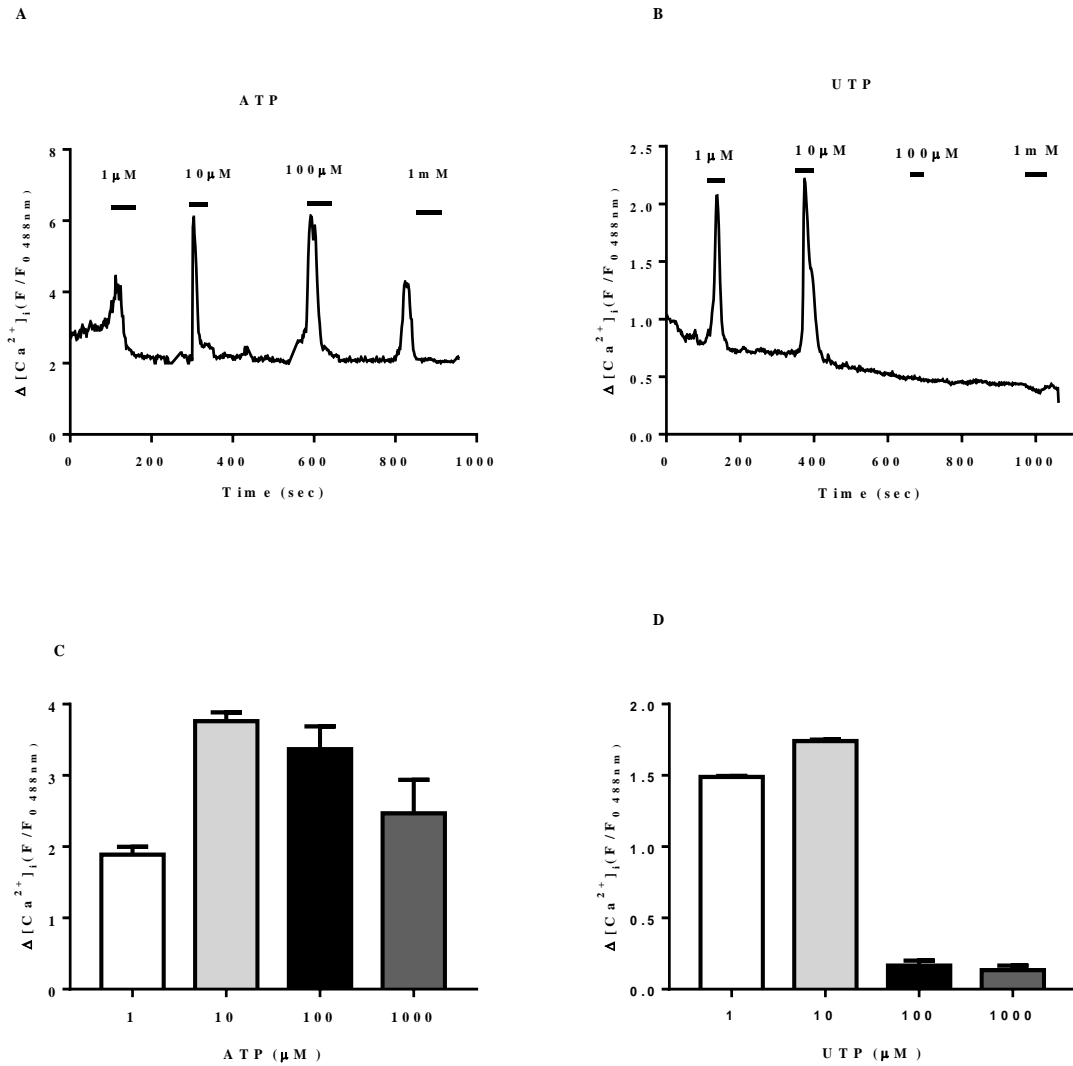
Figure 3-4: ATP- and UTP-stimulated -concentration-response curves

Representative traces showing changes in intracellular Ca^{2+} levels when DSMC loaded with the Ca^{2+} sensitive dye Fluo4-AM (4 μM) were challenged with increasing concentrations (1, 10, 100 μM and 1 mM; for 30 sec) of either ATP (A) or UTP (B). Cumulative data (C and D) ATP and UTP respectively shows the means ± SEM $[Ca^{2+}]_i$ changes from at least 3 separate bladder preparations.

3.5.5. Characterization of UTP-mediated Ca^{2+} signalling in isolated detrusor smooth muscle DSMC

This alternative protocol to measure concentration-dependent contractions of detrusor smooth muscle (Figure 3-5) was induced by UTP addition, with maximal contraction seen at UTP concentrations at 1000 µM. DSMC loaded with the Ca^{2+} sensitive dye Fluo4-AM (4 µM) were challenged with increasing concentrations of UTP (3, 10, 100 µM and 1 mM) for 30 seconds separately (EC_{50} values of 12.5 µM for UTP).
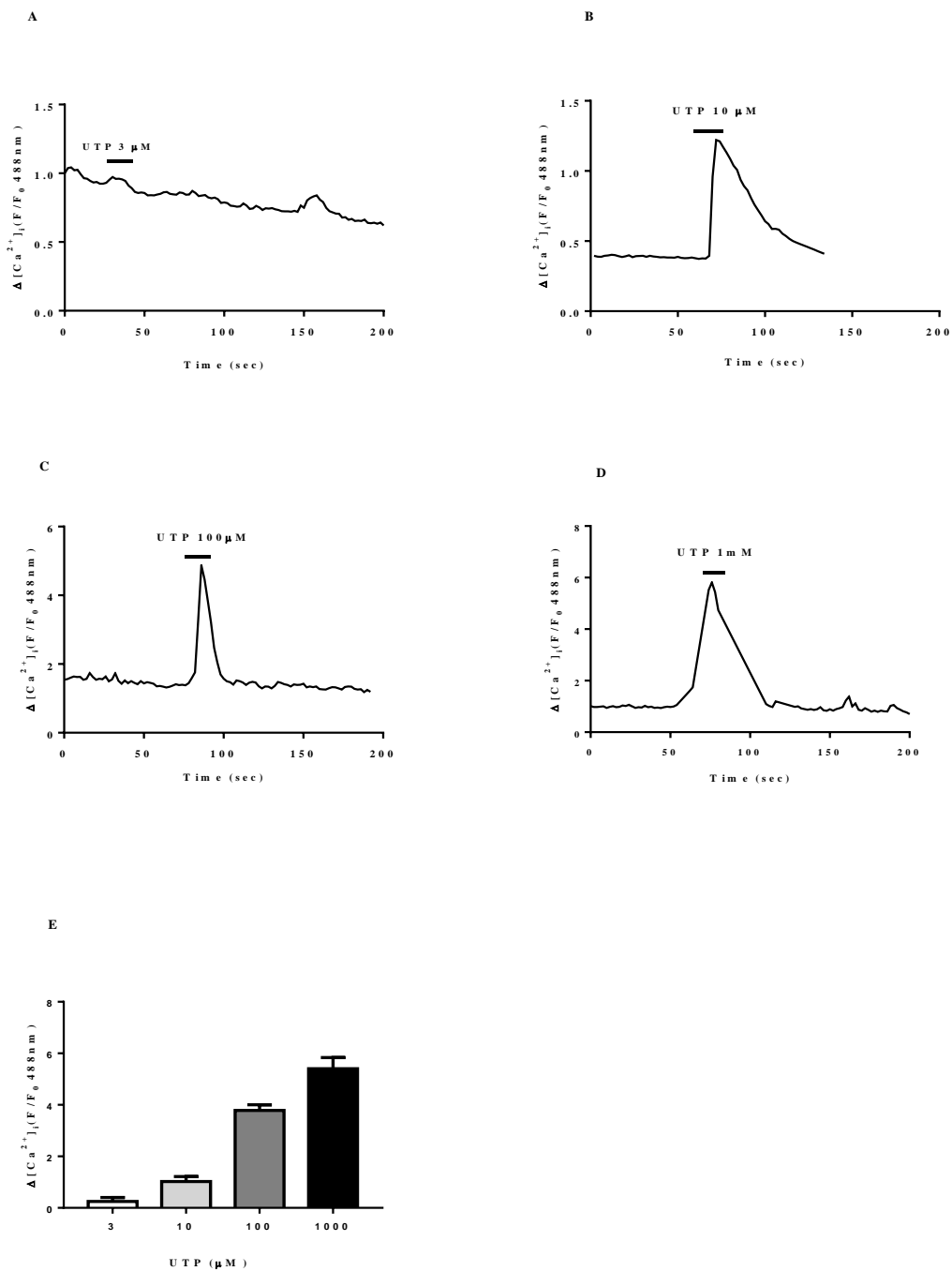
Figure 3-5: Representative traces showing changes in intracellular Ca²⁺ levels

When DSMC loaded with the Ca^{2+} sensitive dye Fluo4-AM (4 μM) were challenged with increasing concentrations of UTP (3, 10, 100 μM and 1 mM) for 30 sec (A, B, C and D). Cumulative data (E) shows the means ± SEM $[Ca^{2+}]_i$ changes from at least 3 separate bladder preparations(EC_{50} =12.5 μM for UTP).

3.5.6. Ca^{2+} signalling in isolated detrusor smooth muscle (DSMC)

To characterize P2Y receptor mediated intracellular calcium concentration [Ca^{2+}]$_i$ changes in detrusor smooth muscle cells of rat bladder. DSMC were loaded with Fluo4-AM (4 µM) and then challenged with agonist and antagonist-induced [Ca^{2+}]$_i$ changes monitored using a NOVOstar imaging system (Figure 3-6).
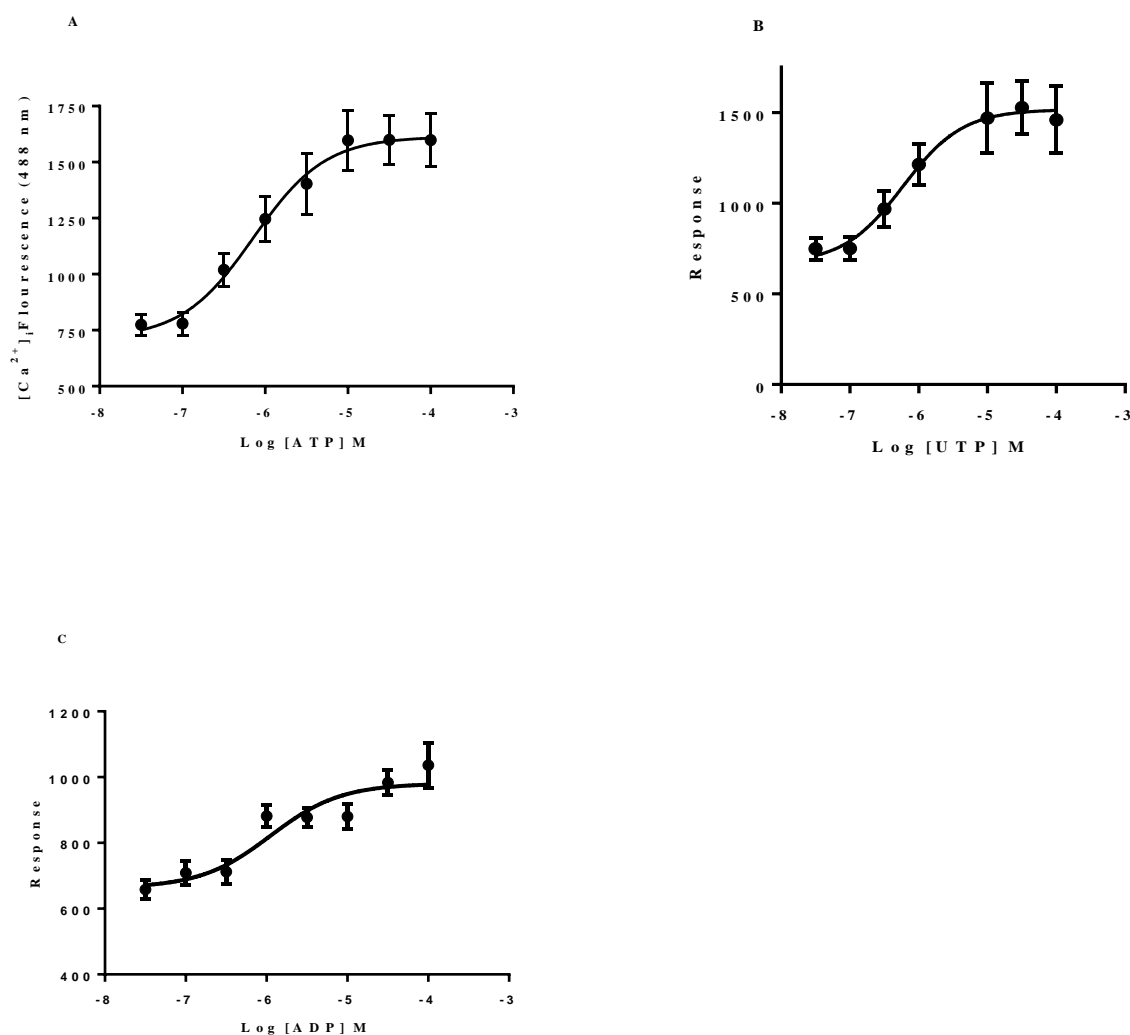
Figure 3-6: Concentration-dependent curves showing [Ca^{2+}]$_i$ elevation in response to ATP, UTP and ADP in DSMC.

DSMC in 96 well plates were loaded with Fluo-4-AM (4 μM, for 1 h at the room temperature in the dark). DSMC were treated with increasing concentration of ATP, UTP and ADP (0.1 nM to 100 μM), and agonist induced [Ca^{2+}]$_i$ changes monitored using a microplate reader (NOVOstar) imaging system. Florescence changes were measured as the maximum [Ca^{2+}]$_i$ response to agonist minus the basal [Ca^{2+}]$_i$ florescence, and used to generate concentration-response curves. Data are shown as means ± SEM from three different animals EC$_{50}$ values 12.5 μM for UTP, EC$_{50}$ values 50nM for ADP.

3.5.7.   Selective P2Y$_1$ receptor agonist MRS2365- mediated elevation of [Ca^{2+}]$_i$

As ADP could stimulated changes in intracellular Ca, which suggest the presence of P2Y1 receptors we looked to confirm these data by repeating experiments with the P2Y1 selective agonist agonist MRS2365 (*N*)-methanocarba-2-MeSADP. Fluo4 loaded DSMC were challenged with various concentrations (0.1 nM to 100 µM) of MRS2365 (Figure 3-7). Concentration-response analysis revealed an EC$_{50}$ value of 9.4 µM for MRS2365 (Figure 3-7).
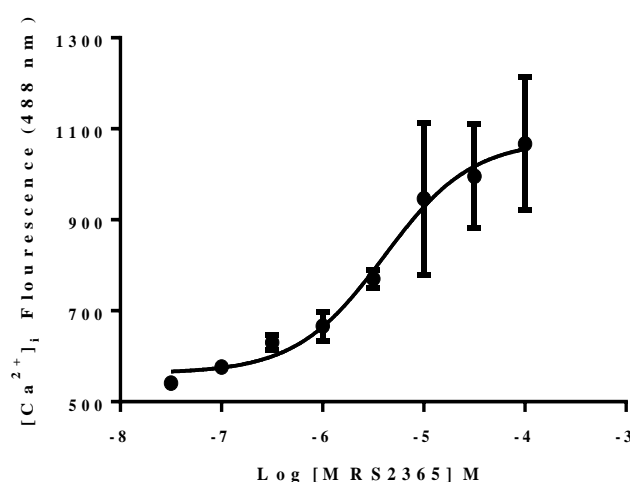
Figure 3-7: Calcium response curves of P2Y$_1$ receptor challenged with MRS2365

DSMC seeded into 96 well plates were loaded with Fluo-4-AM (4 µM, for 1 h) before challenge with various concentration of the P2Y$_1$ selective agonist MRS2365 (0.1 nM to 100 µM). [Ca^{2+}]$_i$ changes were monitored using a microplate reader (NOVOstar) imaging system. Florescence changes were measured as the maximum [Ca^{2+}]$_i$ response to agonist minus the basal [Ca^{2+}]$_i$ florescence, and used to generate concentration-response curves. Data are shown as means ± SEM from three different preparations.

3.5.8. UTP-mediated Ca^{2+} signalling and AR-C118925XX as P2Y$_2$ receptors antagonists

Since UTP was able to elicit increased [Ca^{2+}]$_i$, and both P2Y$_2$ and P2Y$_4$ receptors are known to interact with UTP, we examined whether the P2Y$_2$ selective antagonist AR-C118925XX could affect UTP-stimulated changes in [Ca^{2+}]$_i$. Here, Fluo4 loaded cells were challenged with different concentration of UTP (0.1 nM to 100 µM) to assess changes in receptor responsiveness. Treatment of the DSMC with the P2Y$_2$ antagonist AR-C118925XX (10 µM) for 1 h at the room temperature inhibited UTP-induced receptor signalling and caused the shift to the right of the dose response curve (approximate EC$_{50}$ values of UTP is 12.5 µM) (Figure 3-8). The change is the basal in the presence of AR-C118925XX may indicate a degree of auto-fluorescence for this compound.
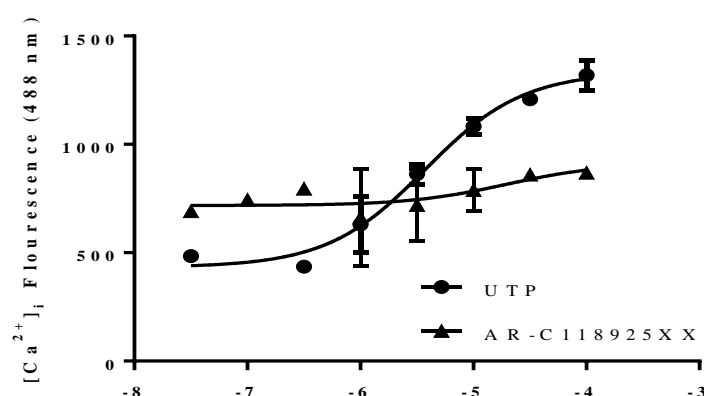
Figure 3-8: Calcium response curves of P2Y$_2$ receptor challenged with antagonists (AR-C118925XX) in the presences of UTP.

Cells in 96 well plates were loaded with Fluo-4-AM (4 µM, for 1 h at room temperature in the dark). DSMC treated with various concentration of UTP (0.1 nM to 100 µM). For the inhibitor experiment the cells were loaded with Fluo-4-AM and with antagonists AR-C118925XX (10 µM) for 1 h at room temperature in the darkness. Cells were exposed to UTP-induced [Ca^{2+}]$_i$ changes monitored using a microplate reader (NOVOstar) imaging system. Florescence change were measured as an index of change in [Ca^{2+}]$_i$ the maximum florescence change was used to graph the concentration-response curves. Data are shown as means ± SEM from three different animals.

3.5.9. Identification of P2X receptors expression mediated Ca^{2+} signalling in isolated DSMC by using P2X antagonist (2', 3'-O-trinitrophenyl-ATP (TNP-ATP)

DSMC stimulated with purinergic receptor antagonists 2', 3'-O-trinitrophenyl-ATP (TNP-ATP). ATP can activate a large number of P2Y receptors and also P2X ion channels, therefore it is likely that many of these receptor subtypes may contribute to the observed signal. Indeed, a previous study highlighted the presence of $P2X_1$ and $P2X_2$ receptors in detrusor smooth muscle (134) In this study, I investigated whether P2X receptors contributed to the ATP generated Ca^{2+} signalling in DSMC or not. We used the non-specific P2X receptor antagonist, 2', 3'-O-trinitrophenyl-ATP (TNP-ATP) to block P2X activity. DSMC were challenged with ATP (100 μM) for 30 sec and then either incubated with buffer or TNP (10 nM) for 5 min, followed by ATP (100 μM) for 30 sec. The profiles of ATP-stimulated $[Ca^{2+}]_i$ changes in the presence or absence of TNP were similar (Figure 3-9 A, B, C). These data indicate that DMSC may not express P2X receptors after two days in culture (Figure 3-9).
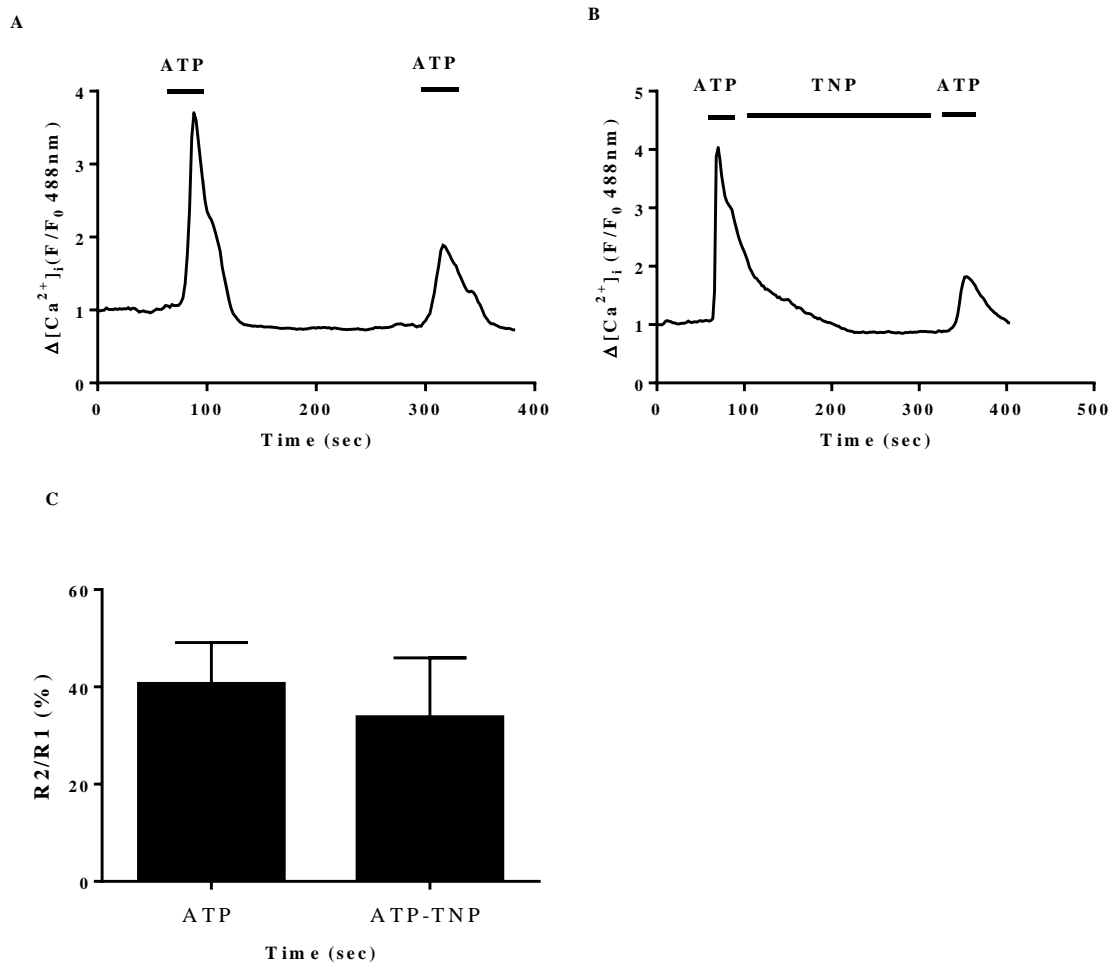
Figure 3-9: Effects of the non-selective P2X antagonist TNP on ATP-stimulated calcium signals.

A) Representative trace shows the changes in $[Ca^{2+}]_i$ in Fluo4-AM loaded DSMC challenged with ATP (100 µM, 30 sec, R1) followed by a 5 min washout period, prior to a second ATP (100 µM, 30 sec, R2) challenge. B) Representative trace showing the effects of adding TNP (10 nM) for 5 min during the washout period. Cumulative data show the reduction in the second ATP response (R2) compare to first (R1). Data represent means ± SEM $[Ca^{2+}]_i$ changes from 10 cells from three or more different bladder preparations. No statistical difference was observed in the R2/R1 ratio in the presence or absence TNP (Mann-Whitney test).

3.5.10. Determination of whether P2X receptors mediate ATP-induced $[Ca^{2+}]_i$ changes in DSMC

To further examine whether cultured rat DSMC expressed functional P2X receptors, cells were challenged with the non-selective P2X receptor agonist α,β-methylene adenosine 5'-triphosphate. DSMC were loaded with calcium dye Flou-4 and initially challenged with α,β-methylene adenosine 5'-triphosphate (20 μM) for 30 sec, before being exposed to ADP (10 μM) for 30 sec as control to confirm calcium signalling in the smooth muscle. The addition of α, β-methylene adenosine 5'-triphosphate failed to promote changes of $[Ca^{2+}]_i$ signalling, however, robust changes were observed following addition of ADP. These data indicate that DMSC do not express functional P2X receptors after two days in culture (Figure 3-10).
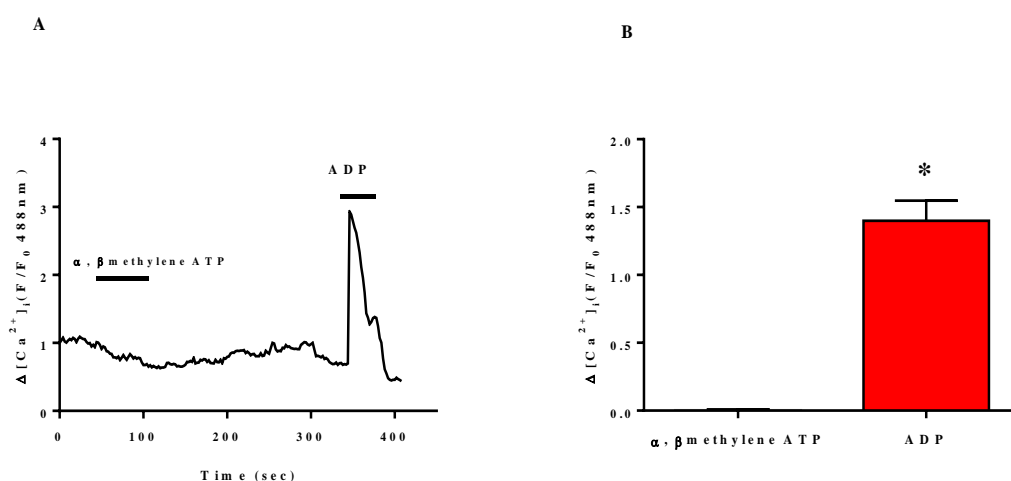
Figure 3-10: The effects of the non-selective P2X receptor agonist on $[Ca^{2+}]_i$ in Fluo4-AM loaded DSMC

(A) Representative trace showing the effects of the non-selective P2X receptor agonist α, β-methylene adenosine 5'-triphosphate (20 μM, 30 sec) on $[Ca^{2+}]_i$ in Fluo4-AM loaded DSMC. After 5 min DMSC were then challenged with ADP (10 μM, 30 sec) to activate $P2Y_1$ receptors as a positive control. (B) Cumulative data show significant increases in $[Ca^{2+}]_i$ signals after stimulation with ADP (*$p<0.05$, Wilcoxon test; when compared to α, β-methylene adenosine 5'-triphosphate treatment). Data are expressed as means ± SEM $[Ca^{2+}]_i$ changes from 15-20 cells from three or more different animals.

3.5.11. Pharmacological profiling of P2Y receptor activity in the generation of Ca^{2+} signalling in isolated rat DSMC

Previous reports suggest that rat DSMC express a number of different P2Y receptors, including $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$, which may contribute to the detected Ca^{2+} signals (32). To investigate which P2Y receptor subtypes predominantly mediate PLC/Ca^{2+} signalling in DSMC, cells were loaded with the Ca^{2+} sensitive dye Fluo4-AM (4 μM), and stimulated with a variety of different P2Y subtype selective agonists. DSMC were challenged with the MRS2365 is a selective full agonist of $P2Y_1$ receptor (100 nM) for 30 sec (Figure 3-11). DSMC also were challenged with the $P2Y_4$ and $P2Y_6$ receptors selective agonists (MRS4062 100 nM) and (MRS2693 10 nM), respectively. Our data suggested that MRS2365 ($P2Y_1$ receptor agonist) evokes Ca^{2+} signalling, yet addition of MRS4062 and MRS2693 did not produce any $[Ca^{2+}]_i$ signals in cultured rat DSMC. Indicating that the presence of $P2Y_1$ but not $P2Y_4$ or $P2Y_6$ receptors. It is worth noting that stimulation of DSMC with very high concentrations of the selective $P2Y_4$ (MRS4062; 10 μM) or $P2Y_6$ (MRS2693; 10 μM) receptor agonists produced increased $[Ca^{2+}]_i$ signals (Figure 3-11). However, at these concentrations it is likely that the agonists are interacting with other receptors. These data suggest that the existence of $P2Y_1$ receptors and absence of $P2Y_4$ and $P2Y_6$ in our DSMC cultures.
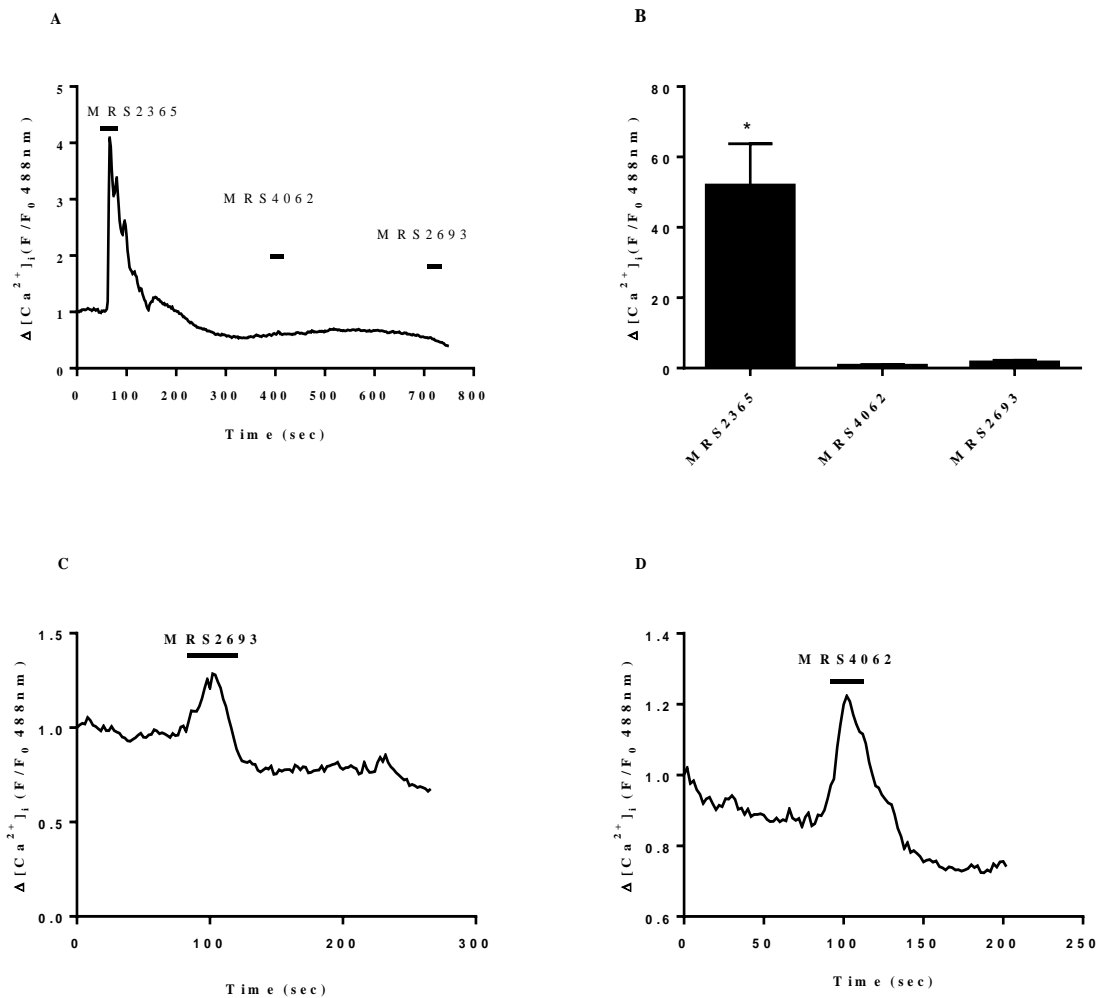
Figure 3-11: Pharmacological profile of P2Y receptor activity in DSMC.

(A) Representative trace showing the $[Ca^{2+}]_i$ changes in Fluo4-AM loaded cells, following challenge with either the $P2Y_1$ (MRS2365, 100 nM), $P2Y_4$ (MRS4062, 100 nM) and or $P2Y_6$ (MRS2693, 10 nM), selective agonists. (B) Cumulative data shown as means ± SEM $[Ca^{2+}]_i$ changes from 20-30 cells from three separate preparation animals. Significant changes in $[Ca^{2+}]_i$, *$p<0.05$ were found in the presence of MRS2365 (Student's *t*-test when compared to basal and MRS4062 or MRS2693 treated DSMC). Representative traces show the changes in $[Ca^{2+}]_i$ when DSMC were exposed to high concentrations of (C) MRS4062 (10 μM) or (D) MRS2693 (10 μM).

3.5.12. Effects of the P2Y$_1$ selective antagonist MRS2279 on ADP–stimulated calcium signalling in isolated DSMC

MRS2279 (2-chloro-N^6-methyl-(N)-methanocarba-2`-deoxyadenosione-3`,5` bisphosphate) is a selective high affinity competitive antagonist of the P2Y$_1$ receptor. In order to confirm the presence of P2Y$_1$ receptor in DSMCs. cells were stimulation with ADP (10 μM), for 30 sec with 5 min washout period between agonist additions (Figure 3-12 A) as control, then in (Figure 3-12 B) an identical protocol with the inclusion of the P2Y$_1$ selective antagonist MRS2279 (20 μM) during the washout period, our result suggested that DSMCs do indeed express endogenous P2Y$_1$ receptors.
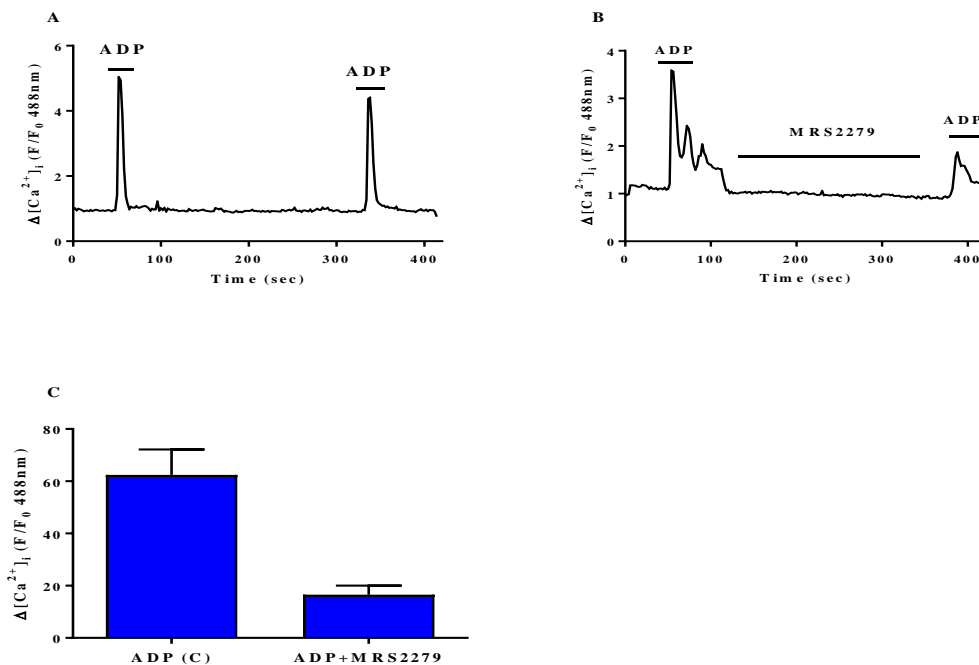
Figure 3-12: Effects of the P2Y$_1$ selective antagonist MRS2279 on ADP stimulated calcium signalling in isolated DSMC.

Representative traces showing the [Ca^{2+}]$_i$ changes in Fluo4-AM loaded cells, following challenge with either (A) stimulation with ADP (10 μM), for 30 sec with 5 min washout period between agonist additions; or (B) an identical protocol with the inclusion of the P2Y$_1$ selective antagonist DSMC MRS2279 (20 μM) during the washout period. C) Cumulative data showing the % decrease in the peak calcium response when comparing first and second additions of ADP. Data represent means ± SEM [Ca^{2+}]$_i$ changes from 15-25 cells from four different animals.

3.6.   Discussion

The urinary bladder is innervated by the sympathetic and the parasympathetic nervous systems which regulate the contraction of DSMC and thus continence. Previous evidence suggests that ATP released during parasympathetic activity stimulates $P2X_1$ receptors to mediate excitation of efferent and muscle contraction of the human bladder. In addition, the $P2X_3$ subtype is reported to mediate excitation of bladder sensory afferents (57), suggesting that P2X receptors may regulate bladder contraction. To assess this possibility this chapter characterized the mediator and receptors systems responsible for purinergic-stimulated contraction of rat detrusor smooth muscle.

Here different concentrations (1, 10, 100 μM and 1 mM for 30 sec) of ATP and UTP were used with 5 min washout between agonist treatments (Figure 3-4). Unfortunately, this protocol was not appropriate to accurately determine concentration response data, as obvious receptor desensitization was seen when cells were were challenged with higher UTP concentrations. These findings are reflected by Boland et al (1993) who found that mouse bladder smooth muscle cells produced a weak contractions when continuously challenged with ATP up to 1 mM (135). They concluded that it might be either due to the extracellular lysis of ATP by the ectonucleotidases (135), or due to the tachyphylaxis of the purinoceptors (135). Thus an alternative protocol was introduced to examine concentration responsiveness, whereby DSMC were stimulated with individual concentrations of UTP (3, 10, 100 μM and 1 mM) for 30 second; the highest response was seen when cells were challenged with UTP (1000 μM).

In this study, ATP, UTP and ADP produced transient raises in $[Ca^{2+}]_i$ in DSMC, which suggests the presence of P2Y and/or P2X receptors. Lewis et al (1998) reported that TNP-ATP was a potent P2X receptor antagonist which inhibited αβ-methylene ATP provoked contractions of arterial smooth muscle cells derived from rat mesenteric artery with an IC_{50} of approximately (30 μM) (136). Here, I used TNP-ATP as a P2X receptor antagonist to block any P2X receptors present in DSMC. These cells were incubated with TNP (IC_{50} = 10 nM) for 5 min, followed by ATP (100 μM) for 30 sec, this resulted in a similar response of the ATP-stimulated $[Ca^{2+}]_i$ in the presence or absence of TNP-ATP, suggesting that rat DSMC did not express P2X receptors after two days of cell culture. Nonetheless, previous studies utilising different approaches were able to detect P2X purinergic receptors in rat tissues. For example, Northern blotting analysis (137-

139) showed the presence of P2X receptor transcripts, whilst the cellular localization of P2X receptors has been described by immunohistochemical methods (134,140,141), or through immunoreactivity techniques (141). Moreover, P2X receptors were also identified using antibodies against the P2X receptors (140). In this study, I challenged rat DSMC with the non-selective P2X receptor agonist (α,β-methylene adenosine 5'-triphosphate) and used ADP (10 μM) as a control. The addition of α,β-methylene ATP failed to promote any changes of $[Ca^{2+}]_i$ signalling, whereas robust $[Ca^{2+}]_i$ changes were observed following the addition of ADP (10 μM) (Figure 3-10). Again, these data indicate that rat DSMC did not express functional P2X receptors after two days of cell culture. Lewis et al (1998) reported that α,β-methylene ATP provoked contractions of arterial smooth muscle cells (136), this might indicates that P2X receptors present in the arterial smooth muscle cells but not DSMC. Vial et al (2001) also found that α,β-methylene ATP (100 μM) as a P2X receptor agonist failed to cause contractions of the epididymis, and seminal vesicle smooth muscle cells (142), P2X receptors were below the level of detection in the smooth muscle of the small bowel and in woman reproductive tract (142), whilst others reported a different degree of P2X receptor distribution in urinary bladder smooth muscles (30,56,137). Furthermore, Birder et al (2004) found altered expression of $P2X_1$ receptors in urinary bladder smooth muscles with evidence of cystitis (30). In addition, O'Rielly et al (2001) found more expression of $P2X_1$ receptor (61) in an obstructed than in a normal bladder smooth muscle and suggests there was an increase in purinergic function in the unstable bladder (61). In contrast, there was no evidence of functional P2X receptor activity in rat DSMC after two days of cell culture; hence the time from cell isolation to experimentation might be the contributing factor for the loss of P2X receptor expression reported in this chapter.

In 1994, it had become obvious that the P2X receptor may not be the only purinergic receptor isoforms that are present in the urinary bladder smooth muscle cells and other contractile purinergic receptor, such as P1 and P2 receptors had been identified in rat (143) and P2 receptors human bladder smooth muscle cells (144). P1 purinergic receptors in smooth muscle usually mediate relaxation (145), although in a number of smooth muscle preparations adenosine can cause contraction (146,147). It has also been shown that P2 purinergic receptors in smooth muscle are subdivided into P2X which mediates (contraction) and P2Y which mediates (relaxation) (148). However, the physiological role of P2Y receptors has always been controversial. Depending on the

different tissue types, previous studies showed that P2Y receptors mediated contraction of rat colon (149) and small bowel (ileum) smooth muscle (150). On the other hand, P2Y receptors have been reported to cause relaxation in smooth muscle of rabbit mesenteric artery (151). There are different subtypes of P2Y purinergic receptors including; $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{13}$ and their expression is variable in numerous tissues including arterial and bladder smooth muscles, as well as platelets (30,108,152). The agonist profile obtained in my study provides functional evidence for the presence of $P2Y_1$ and $P2Y_2$ receptors, but not expression of P2X, $P2Y_4$ or $P2Y_6$ in cultured rat DSMC. Nevertheless, previous studies reported the expression of $P2Y_1$, $P2Y_2$, and $P2Y_4$ in the urothelium of cat (30), and $P2Y_6$ receptor expression has also been reported in the urothelium of the guinea pig (153).

The following P2Y purinergic receptors stimulated with the following neurotransmitters had been reported in vascular smooth muscle (ATP/UTP $P2Y_2$, UDP $P2Y_6$ and ADP $P2Y_{12}$ receptors), endothelium (ADP $P2Y_1$, UTP/ATP $P2Y_2$, UDP $P2Y_6$), myocardium (ATP/UTP $P2Y_2$, UDP $P2Y_6$, ATP $P2Y_{11}$), and platelets (ADP $P2Y_1$, ADP $P2Y_{12}$) (166). Von Kugelgen (2006) and Abbracchio et al (2006) confirmed the expression of $P2Y_{2,4,6}$ receptors in rat myocardial fibroblasts using RT-PCR and immunocytochemistry (167,168). Similarly, Talasila et al (2009) provided evidence of the co-expression of the same P2Y receptor subtypes described above and others such as $P2Y_{1,11}$ in the same tissues (169). Immunocytochemical studies, provided evidence for the expression of $P2Y_2$ and possibly $P2Y_4$ receptor staining in the normal rat urinary bladder. However, $P2Y_2$ immunoreactivity was present throughout the rat urinary bladder, including detrusor smooth muscle, underlying nerve fibres, and urothelium (154). Other studies used western blotting to investigate the expression of P2Y receptors in urothelial, detrusor smooth muscle, and whole bladder protein lysates was also indicated the presence of $P2Y_2$ (32) and to a lesser extent $P2Y_4$ (32).

In rat cultured aortic smooth muscle cells, ATP was reported to stimulate inositol phosphate accumulation and calcium mobilization, although the receptor subtype which mediated the response to ATP was not completely identified (155). Consequently, it appeared possible that α,β-meADP encouraged the contraction of rat urinary bladder smooth muscle was via P2Y-purinoceptor (143) and McMurray et al (1998), reported that marmoset DSMC had two receptors for ATP; which were P2X receptors that

mediate contraction and P2Y receptors that mediate relaxation (58). Therefore, it may be possible that ATP may induce either contraction or relaxation, depending on the type of receptor that interacts with ATP. Govindan et al (2010) reported that P2Y receptors induce intracellular calcium signals in vascular smooth muscle cells and regulate contraction (156). Similarly, in this study I found that the ADP activated the $P2Y_1$ receptors which evoked an increase of $[Ca^{2+}]_i$ signals in DSMC.

To confirm the presence of $P2Y_1$ receptor in rat DSMC, cells were challenged with selective $P2Y_1$ receptor agonist (MRS2365), a full agonist of $P2Y_1$ receptors (157). Our result showed $[Ca^{2+}]_i$ signals were evoked by ADP and $P2Y_1$ selective agonist (MRS2365; $EC_{50} = 0.40$ nM), suggesting that $P2Y_1$ purinergic receptors present in rat DSMC. Previous study showed that this $P2Y_1$ receptor agonist (MRS2365; $EC_{50} = 8.56 \pm 0.06$) has evoked concentration-dependent increase in $[Ca^{2+}]_i$ (156). On the other hand, $P2Y_1$ receptors antagonist (MRS2279) is a selective antagonist with high affinity to the $P2Y_1$ receptor; this antagonist confirmed the ability to block $P2Y_1$ receptor in human platelets and rat brain membrane (158,159). In this study, I applied 20 μM of MRS2279 to rat DSMCs, there was block to $[Ca^{2+}]_i$ signalling and this suggest the DSMC express the $P2Y_1$ purinergic receptor.

Rat DSMC were also challenged in this study with both $P2Y_4$ and $P2Y_6$ receptor selective agonists (MRS4062; 100 nM) and (MRS2693; 10 nM) respectively. Both of these agonists did not make any changes to $[Ca^{2+}]_i$ signals in cultured rat DSMC, these findings indicate that the DSMC lack the expression of $P2Y_4$ and $P2Y_6$ receptors. I also used $P2Y_2$ receptor antagonist (AR-C118925XX; 10 μM) to assess its effect on $[Ca^{2+}]_i$ signalling and confirm the presence of $P2Y_2$ receptors in cultured rat DSMCs, and this also blocked the $[Ca^{2+}]_i$ signalling. This suggested that $P2Y_2$ receptor present in rat DSMC that investigated in this study. In a previous study, the $P2Y_2$ receptor antagonist (AR-C118925XX) induced a concentration-dependent block of adenosine 5′-o-(3-thiotriphosphate) (ATP-γS) induced mucin secretion from human bronchial epithelial cells (160).

Kumari et al (2003) showed the responses to ATP and UTP in rat VSMC could potentially be mediated by either $P2Y_2$ or $P2Y_4$ receptors (161). The authors also extrapolated the EC_{50} of UTP in VSMCs as 10.1 μM (161). Henriquez et al (2018) reported in an abstract presentation, there was an increased venous smooth muscles

contraction in response to UTP (EC_{50} = 6,9 ± 2,45 µM) of rats with pulmonary arterial hypertension, as compared to rats with no evidence of pulmonary arterial hypertension (EC_{50} = 19 ± 5.01 µM). In the same way, ATP-dependent venous contraction was strongest in rats with pulmonary arterial hypertension (EC_{50} = 14,3 ± 1.9 µM) as compared to healthy rats (EC_{50} = 28.6,3 ± 1.1 µM) (162). This is comparable to the EC_{50} values of 12.5 µM of UTP reported in this study.

Wu et al (2004) reported that ATP, ADP, and UTP lead to transient influx of intracellular Ca^{2+} through P2Y purinergic receptor stimulation in guinea pig suburothelial myofibroblast (163). Similarly, Liang et al (2008) showed that stimulation of $P2Y_2$ purinergic receptor by ATP and UTP induced Ca^{2+} release from endoplasmic reticulum and Ca^{2+} influx in human heart valvular myofibroblast (164). The contractile effects of UTP on vascular smooth muscle cells indicated that P2Y receptors present in the vascular smooth muscle cells and mediate contraction (165).

The agonist profile in this study of purinergic receptors response provided functional proof for the presence of $P2Y_1$ and $P2Y_2$ receptors, and no expression of P2X, $P2Y_4$ and $P2Y_6$ in cultured rat DSMC. Indeed P2X receptor expression in bladder remains unclear and raises the possibility that urinary bladder smooth muscle cells may express a variety of different P2X receptor (134). However, the data in this thesis suggest that these cultured cells provide a good model to study P2Y-stimilated calcium imaging. However, the evidence for the existence of P2X receptor in human bladder is limited (170).

3.7. Conclusions

Initial immunocytochemical studies indicated that the cells isolated from the bladder in this study expressed the smooth muscle markers calponin and α-actin, indicating that they were indeed detrusor smooth muscle and not urothelial cells. When exposed to purinergic (ATP, UTP or ADP) agonists DSMC produced transient changes in $[Ca^{2+}]_i$ indicating the presence of purinergic receptors. Further application of selective P2Y and P2X agonist and/or antagonists highlighted that the changes in $[Ca^{2+}]_i$ were mediated through the action of $P2Y_1$ and $P2Y_2$, but not $P2Y_4$, $P2Y_6$ or P2X receptors. Moreover, repeated applications of ATP, UTP or ADP resulted in tachyphalaxis, which is indicative of receptor desensitization.

Chapter 4

4. **Desensitization and re-sensitization of purinergic receptors and Expressions of GRK protein in DSMCs of the rat bladder**

4.1. Introduction

In the previous chapter the data showed that when DSMC were exposed to repeated agonist stimulation the ability to produce calcium transients decreased, which is indicative of GPCR desensitization (171,172). GPCR desensitization is a fundamental process which occurs with the vast majority of receptors to prevent excessive or inappropriate signalling (94,171), and also plays a role in the regulation of signal duration, intensity and quality. Indeed, over activity of GPCR signalling is likely to contribute to the unwanted dysregulation of bladder contractions observed in overactive bladder. Therefore, since uncontrolled overactive GPCR signalling to initiate inappropriate contractions may underlie the pathophysiology of overactive bladder, it is important to investigate the regulation of purinergic receptor contractile signalling in DSMC.

Previous studies show that frequent agonist stimulation of purinergic receptors often leads to reduced responsiveness (desensitization) to additional agonist stimulation (109). Usually agonist occupied GPCRs undergo desensitization by recruitment of one or more members of a family of seven serine/threonine kinases, termed GRKs. GRKs mediate the phosphorylation of key serine and/or threonine residues within the third intracellular loop and/or C-terminal tail of the GPCR. GPCR phosphorylation by GRKs increases receptor affinity for arrestin2 and or arrestin3, which can physically bind to GPCRs and suppress interaction between receptor and G proteins to inhibit signalling (94,108,109).

Despite this at present little is known regarding the mechanism or regulation of detrusor purinergic receptor signalling in the bladder. Therefore this chapter characterises the desensitization and re-sensitization kinetic of endogenously expressed $P2Y_1$ and $P2Y_2$ receptor mediated PLC signalling in DSMC. In addition, we have utilised a number of approaches to manipulate the activity of GRK isoenzymes in an attempt to determine whether they regulate the desensitization of P2Y receptor activity. Initial experiments utilised overexpression of catalytically inactive GRK mutants that act as dominant negatives to inhibit the action of endogenous GRK proteins (173,174). In all cases the lysine which binds ATP in the catalytic domain of the GRK (i.e. K220 in GRK2 and GRK3; and K215 in GRK5 and GRK6) are mutated to arginine (R) to produce either a K220R or K215R mutation. However, since both GRK2 and GRK3 are reported to bind

via their RGS domains, to GTP-bound active $G\alpha_q$ and suppress PLC signalling (90,103,175), we used the double mutated version (i.e. ^{D110A,K220R}GRK2 or ^{D110A,K220R}GRK3) where the aspartic acid residue at 110 was replaced with alanine to block this interaction (176), and allow the assessment of whether the catalytic activity of GRKs 2 and 3 were important in the regulation of P2Y signalling. In addition, in some experiments we also utilised previously characterised small interfering RNA constructs to deplete endogenous GRK activity (109), and finally assessed the ability of potential GRK inhibitors to block P2Y receptor desensitization.

4.2. Aims

In the previous chapter the expression of purinergic P2Y and P2X receptors in the detrusor smooth muscle was determined by using a variety of selective purinergic agonist and antagonists. Here we have attempted to determine whether endogenous GRKs play a role in the regulation of purinergic receptors signalling. To address this aim the following were examined.

1- The desensitization and re-sensitization kinetic of purinergic receptors in response to ATP, ADP and UTP challenge were determined.

2- The potential requirement for GRKs in the regulation of purinergic receptor desensitization was determined using molecular techniques to manipulate endogenous GRK activity and/ or expression.

3- Determine whether selective serotonin reuptake inhibitor (SSRI) paroxetine and fluoxetine (GRK2 inhibitor) prevent purinergic receptor desensitization-related signalling mechanisms at a molecular level in DSMCs of urinary bladder

4.3. Methods

4.3.1. Single cell confocal imaging techniques to study P2Y receptor desensitization

I have used confocal imaging techniques and particular inhibition of endogenous GRK isoenzyme activities to assess the interaction between GRKs and purinergic receptors in isolated cultured detrusor smooth muscle cells (DSMC) of urinary bladder. DSMC were loaded with the Ca^{2+}-sensitive dye Fluo4-AM (4μM, 60 min). Cells were maintained at 37°C using a Peltier unit and continually perfused with Krebs-Henseleit buffer (134 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1.2 mM KH_2PO_4, 10 mM glucose, 10 mM HEPES, 1.3 mM $CaCl_2$; pH 7.4). Real-time images were taken using a Nikon Eclipse TE200 microscope (Nikon, Tokyo, Japan) (X 40 objectives). Cells were excited at 388 nm, and Fluo-4 emissions (Em) were collected at >660 nm. The fluorescence ratio was measured from regions of interest within the cytosol. $[Ca^{2+}]_i$ changes are displayed as the fluorescence emission relative to basal (F/F_0). Agonists were applied via the perfusion line as indicated. Briefly, to determine P2Y receptor desensitization, DSMC were exposed to an approximate EC_{50} concentration of purinergic receptor agonist termed R1 (10 μM UTP or ATP), after 5-min washout receptor desensitization was induced by applying a maximal ATP or UTP concentration (100 μM) for 60 sec, termed R max, a further application of an approximate EC_{50} concentration of UTP or ATP was added either, 5, 10, or 15 min after the R max application. Agonist-induced changes in intracellular Ca^{2+} measured using an Olympus FV500 scanning laser confocal microscope as described in Methods. Reduced R2/R1 ratios are interpreted as an indication of receptor desensitization (37). In this chapter two types of receptor desensitization are that adding maximal agonist concentration $P2Y_1$ and $P2Y_2$.

4.3.2. Western Blot Analysis

Determination of GRKs expression in isolated DSMC and whole bladder. (GRK2, 3, 5 and 6) Protein expressions were determined using standard immunoblotting protocols as described in the general Methods section. Cell lysates (40 μg/lane) were separated by SDS-PAGE before Western transfer, using specific anti-GRK antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

4.3.3. Inhibiting Endogenous GRK Activities on DSMC by dominant-negative GRK mutants

DSMC transfected with pcDNA3 (vector control), $^{\text{D110A, K220R}}$GRK2 (176), $^{\text{K215R}}$GRK5, or $^{\text{K215R}}$GRK6 (177,178) The D110A, K220R double-mutant form of GRK2 was chosen for these experiments to prevent the phosphorylation-independent inhibition of PLC signalling caused by this isoenzyme through $G\alpha_{q/11}$-binding (176).

4.4. Data analysis

Data presented are from cultured DSMC obtained from three separate animals and are expressed as means ± SEM. Data were analysed using one-way ANOVA as indicated, with appropriate post hoc testing (Graph Pad Prism 7, San Diego, CA, USA). Followed by appropriate test Significance was accepted when $*P < 0.05$.

4.5. Results

4.5.1. Single-Cell Ca^{2+} Imaging and Receptor Desensitization Studies
4.5.1.1. Desensitization and re-sensitization kinetics of P2YR-stimulated PLC signalling in DSMC

Firstly, the desensitization and re-sensitization kinetics of P2Y receptor-stimulated PLC signalling in response to ATP were assessed. Initially, ATP was utilised as this ligand activates both P2Y$_1$ and P2Y$_2$ receptor populations, and should provide and idea of the global desensitization of the P2Y receptor-stimulated PLC signalling in the DSMC. Here in an attempt to induce maximal receptor desensitization, DSMC were challenged with a maximal concentration of ATP (100 µM, 30 sec; termed R1) followed by either a 5, 10 or 15 min wash period. After the wash out period DSMC were again challenged with a second maximal ATP concentration (100 µM, 30 sec; termed R2). Comparison of R1 responses to (R2) revealed that (R2) induced [Ca^{2+}]$_i$ signals were markedly reduced with a 5 min washout period, yet recovered gradually with an increasing washout period, suggesting an initial desensitization of receptor activity followed by re-sensitization of receptor responsiveness (Figure 4-1).
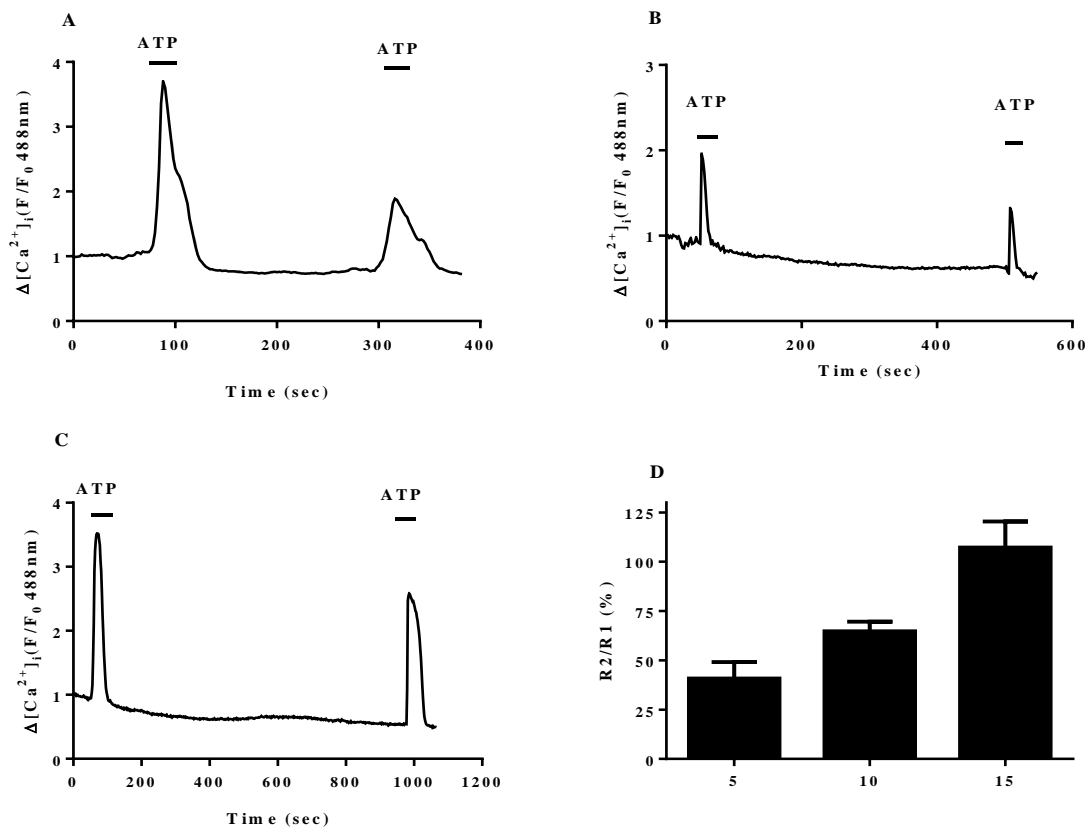
Figure 4-1: Assessment of P2Y receptor desensitization and re-sensitization in DSMC.

Representative traces show desensitization of purinergic P2Y receptor stimulated $[Ca^{2+}]_i$ signalling following ATP challenge. DSMC were loaded with Fluo4 and subjected to the following desensitization protocol: cells were challenged with ATP (100 μM) for 30 sec (termed R1), 5, 10 or 15 min before a second ATP challenge (100 μM) for 30 sec (termed R2). Representative traces show the relative loss of R2 response when compared to R1 with either a 5 (A), 10 (B) or 15 minutes (C) gap between agonist challenges. P2Y receptor desensitization was determined as the relative (%) change in R2 response when compared to R1. Cumulative data shows the means ± SEM $[Ca^{2+}]_i$ changes from n=18 cells, from at ≥3 separate bladder preparations.

4.5.1.2. Desensitization and re-sensitization of P2Y$_1$ PLC signalling mediated by ADP in isolated DSMC

The data from Chapter 3 indicate that rat DSMC express a mixed P2Y$_1$ and P2Y$_2$ receptor population, both of which contribute to the overall Ca^{2+} signal generated by ATP. Since ATP-stimulated Ca^{2+} signals obviously decrease following application of the R1/R2 protocol, it is likely that desensitization of one or both receptor subtypes underlies this change in receptor signalling. Therefore, to determine whether one or both receptors undergo desensitization, the relative loss of receptor activity was examined using selective P2Y$_1$ and P2Y$_2$ receptor agonists. Initial focus was on determining the kinetics of P2Y$_1$ receptor desensitization and re-sensitization, using ADP as the selective agonist. DSMC were loaded with Ca^{2+} sensitive dye Fluo-4 and stimulated with a maximal ADP (10 μM, 30 sec) concentration followed by 5, 10, or 15 min wash period. Comparison of R1 responses to R2 revealed that R2 induced [Ca^{2+}]$_i$ signals were markedly reduced with a 5 min washout period, yet they recovered gradually with increasing washout suggesting an initial desensitization of receptor activity followed by re-sensitization of receptor responsiveness (Figure 4-2).

To confirm the findings with ADP, desensitization and re-sensitization kinetics were determined in response to P2Y$_1$ receptor selective agonist MRS2365. Here, Fluo4 loaded DSMC were stimulated with MRS2365 (10 nM, 30 sec, R1) followed by a 5 and 10 min wash period, and then a second MRS2365 (10 nM, 30 sec, R2) was applied. Comparison of R1 to R2 responses revealed that R2 induced [Ca^{2+}]$_i$ signals were markedly reduced after 5 min washout period, yet they recovered gradually with increasing washout suggesting an initial desensitization of receptor activity followed by re-sensitization of receptor responsiveness (Figure 4-3).
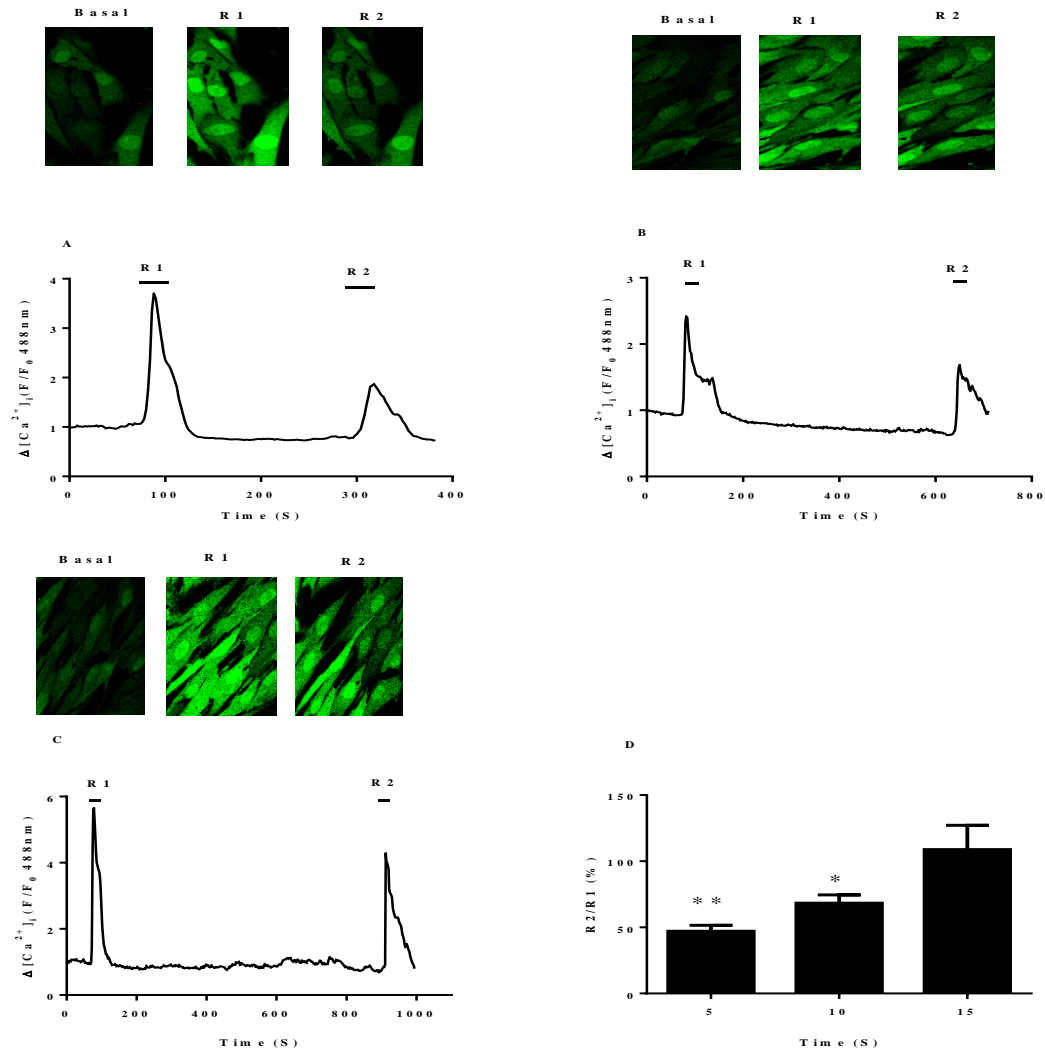
Figure 4-2: Desensitization and re-sensitization kinetics of P2Y$_1$ receptor stimulated [Ca^{2+}]$_i$ signalling following ADP challenge.

DSMC were subjected to the desensitization protocol outlined above for ATP (i.e. R1, R2 = 10 μM ADP, for 30 sec). Representative images and traces showing the extent of P2Y$_1$ receptor desensitization measured 5 min (A) or 10 min (B) challenge and 15 min (C), Cumulative data (D) showing changes in the R2/R1 ratio 5, 10, and 15 min challenge. Data are expressed as means ± SEM for the percent change in R2 relative to R1; n=20 cells for each time point, from at least four separate experiments; (**$P < 0.05$ Kruskal-Wallis test comparing R2/R1 ratios for 5 min and 10 min *vs*. 15-min).

A

B

C
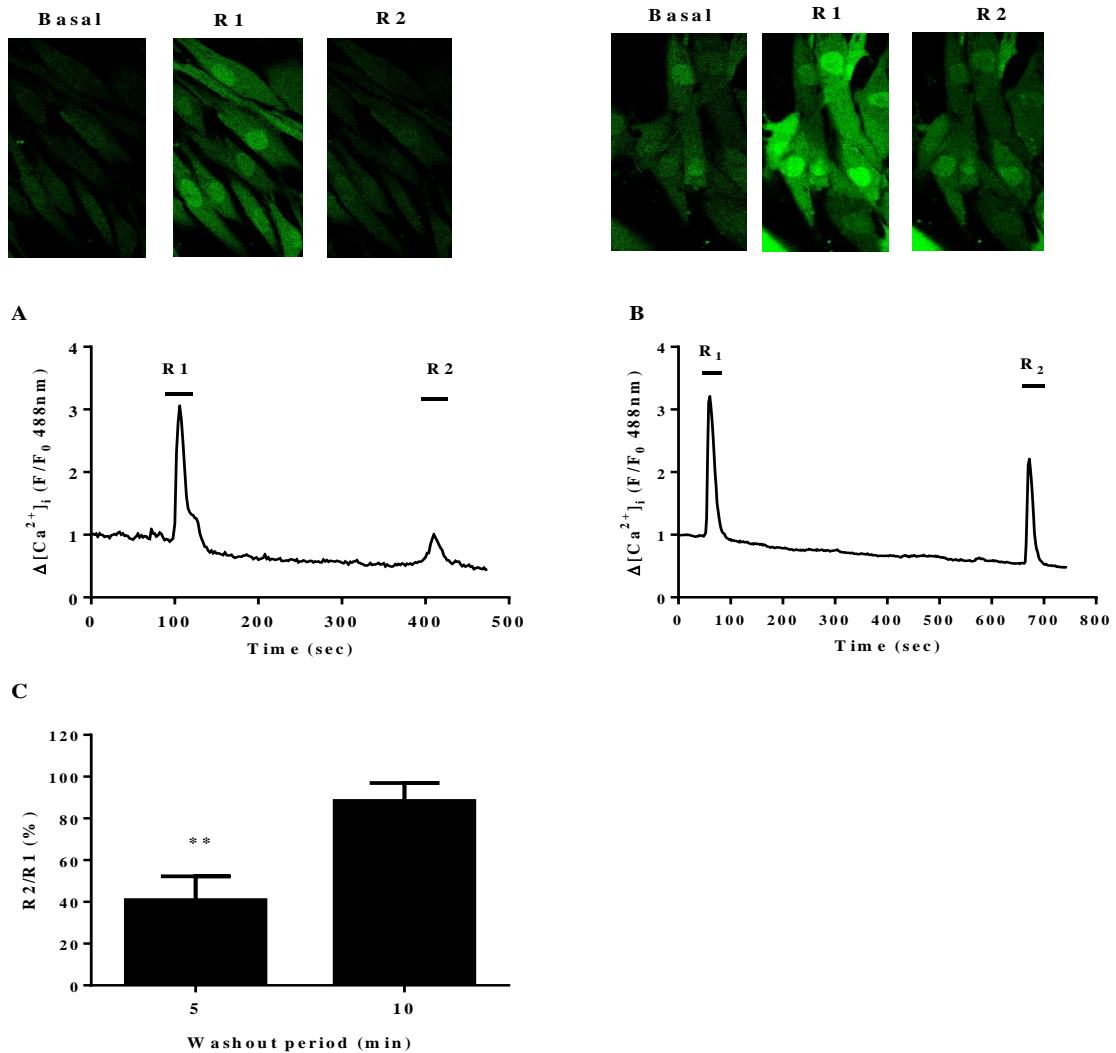
Figure 4-3: Assessment of P2Y$_1$ receptor desensitization and re-sensitization in DSMC.

Representative traces show desensitization of P2Y$_1$ receptor stimulated $[Ca^{2+}]_i$ signalling following MRS2365 challenge. DSMC were loaded with Fluo4 and subjected to the following desensitization protocol: cells were challenged with MRS2365 (10 nM) for 30 sec (termed R1) 5 or 10 min before a second MRS2365 challenge (10 nM) for 30 sec (termed R2). Representative traces show the relative loss of R2 response when compared to R1 with either a 5 (A) or 10 (B) min gap between agonist challenges. P2Y$_1$ receptor desensitization was determined as the relative (%) change in R2 response when compared to R1. C) Cumulative data show the means ± SEM $[Ca^{2+}]_i$ changes from n= 26 cells, from at least three separate bladder preparations statistical comparison was by one way ANOVA with post-hoc t-test and (**P<0.05) which is 5 min vs 10 min.

4.5.1.3. Characterisation of the desensitization and re-sensitization kinetics of P2Y$_2$ receptor-stimulated PLC signalling mediated by UTP signalling in isolated DSMC

Since P2Y$_1$ receptors are not activated by UTP (156) and our evidence suggests that neither P2Y$_4$ nor P2Y$_6$ receptor are present in our cultures we assumed that UTP is stimulating only P2Y$_2$ receptors in DSMCs. Previous work from our group has highlighted that in vascular smooth muscle cells purinergic calcium signals generated by P2Y$_2$ receptor-stimulated PLC signalling do not appear to desensitize if a maximal agonist concentration is used for the R2 and R1 applications (108), which is reflective of receptor reserve. However, using a slightly altered protocol where an approximate EC$_{50}$ concentration of UTP (1 μM, for 30 sec) is added prior to termed (R1), and after termed (R2), a desensitizing pulse of maximal agonist (100 μM, Rmax, for 60 sec), a reduction in the R2 compared to R1 response is seen (Figure 4-4). This reduction in R2:R1 ratio is indicative of receptor desensitization. Therefore, to determine the time course of P2Y$_2$ receptor desensitization and re-sensitization in DSMC were subjected to this protocol with the R2 dose of UTP added either 5 or 10 min after Rmax. Reduced R2/R1 ratios are interpreted as an indication of receptor desensitization. Comparing R2 to R1 responses showed that R2 values were significantly reduced with a 5 min washout period between Rmax and R2 (Figure 4-4 A, C). While if the washout period was extended to 10 min, R2 responses were similar to R1 indicating (Figure 4-4 B, C) a re-sensitization of P2Y$_2$ receptor activity.
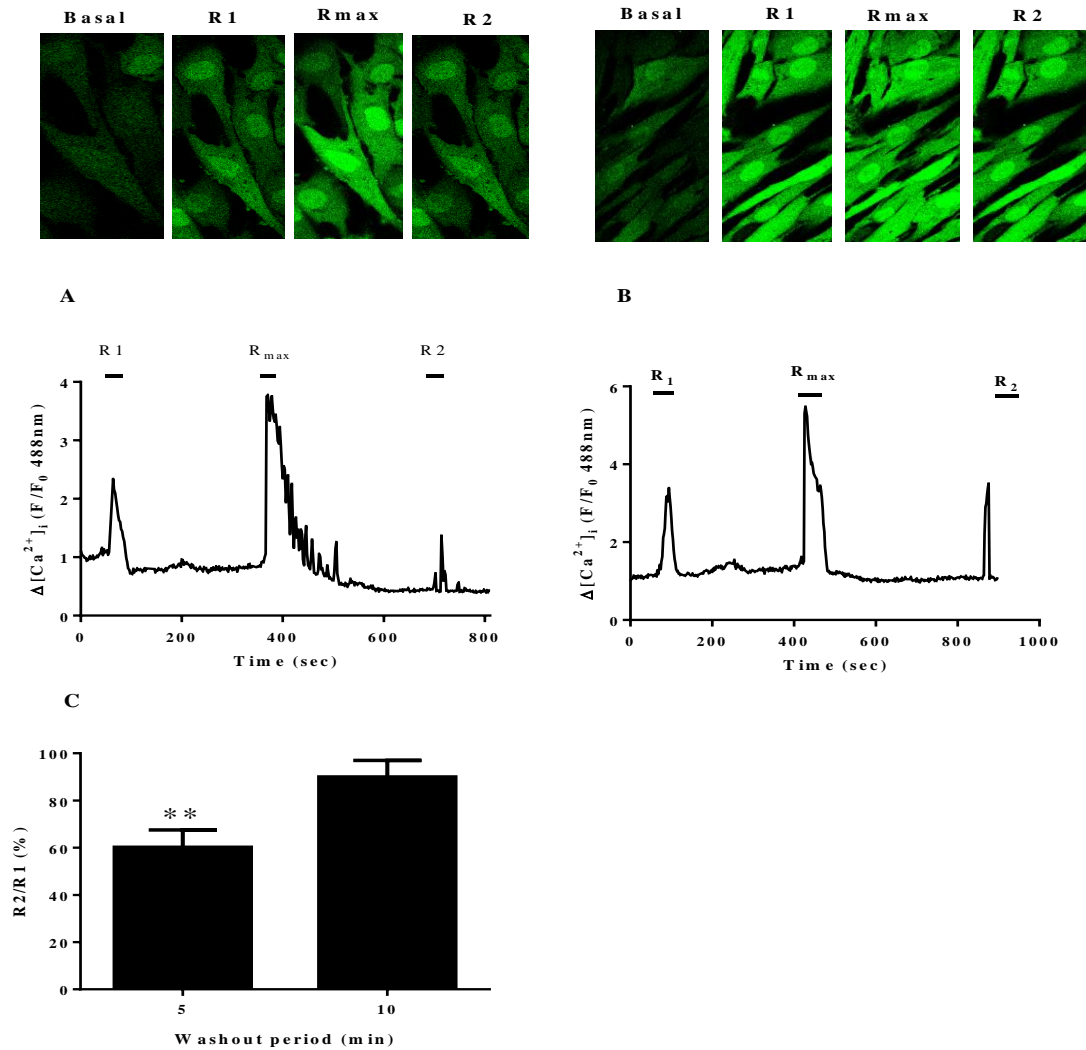
Figure 4-4: Desensitization and re-sensitization of P2Y$_2$ receptor-stimulated Ca^{2+} signalling in DSMC.

Cells loaded with the Ca^{2+}-sensitive dye Fluo4-AM (4 μM) were subjected to the desensitization protocol (R1, R2 = 10 μM UTP, for 30 sec; Rmax = 100 μM UTP, for 60 sec). A) Representative images and trace showing the decrease in R2 response compared with R1 when assessed 5 min after addition of the maximal UTP pulse (Rmax). B) Representative images and traces showing recovery of the R2 response to a similar level to R1 when the delay between Rmax and R2 was increased to 10 min. UTP was applied for the times indicated by the *bars*. C) Cumulative data show the means ± SEM [Ca^{2+}]$_i$ changes for the percentage change in R2 relative to R1 from 28 cells, from three separate bladder preparations. Statistical comparison was by t-test (**P<0.01) when comparing 5 min vs 10 min washout periods.

4.5.2. Identification of GRK protein expression in DSMC and whole bladder

G protein coupled receptor kinase expression were determined, using standard immunoblotting protocols as described in section 2.1.8; 40 µg of each lysate protein was loaded per well (protein levels were determined through Bradford assay as described above. After 1 hour blocking the membranes were incubated with the appropriate primary antibody either in TSBT with 5% milk for GRKs protein overnight at 4°C. After washing with TBS-T, membranes were incubated with a secondary antibody (details in chapter 2 methods section). Our data clearly show that purinergic agonist-stimulated changes in $[Ca^{2+}]_i$ desensitize and re-sensitize over time and since P2Y GPCR activation is responsible for these calcium signals, we wished to determine the molecular mechanisms responsible for P2Y receptor desensitization. As GPCR desensitization is usually mediated by the action of GRK proteins, we examined which GRKs proteins were expressed in DSMC and whole bladder. As we can see that the Western blots showing protein expression for GRK2, 3, 5 and 6 in DSMCs and whole rat bladder (Figure 4-5 and 4-6).
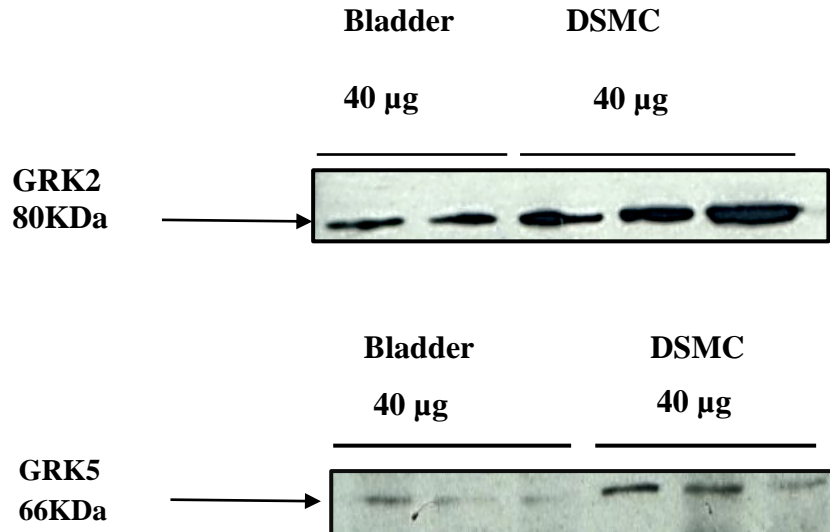
Figure 4-5: Western blots showing protein expression for GRK2 and GRK5 in cultured DSMC and whole bladder.

The immunoblot band was detected at 80 kDa and 66kDa for GRK2 and GRK5, respectively.

1.

GRK3
80KDa

C   **C**   **C**

GRK3
80KDa

B   **B**   **B**

2.

GRK5
66KDa

B   **B**   **B**

3.

GRK6
68KDa

B   **B**   **B**
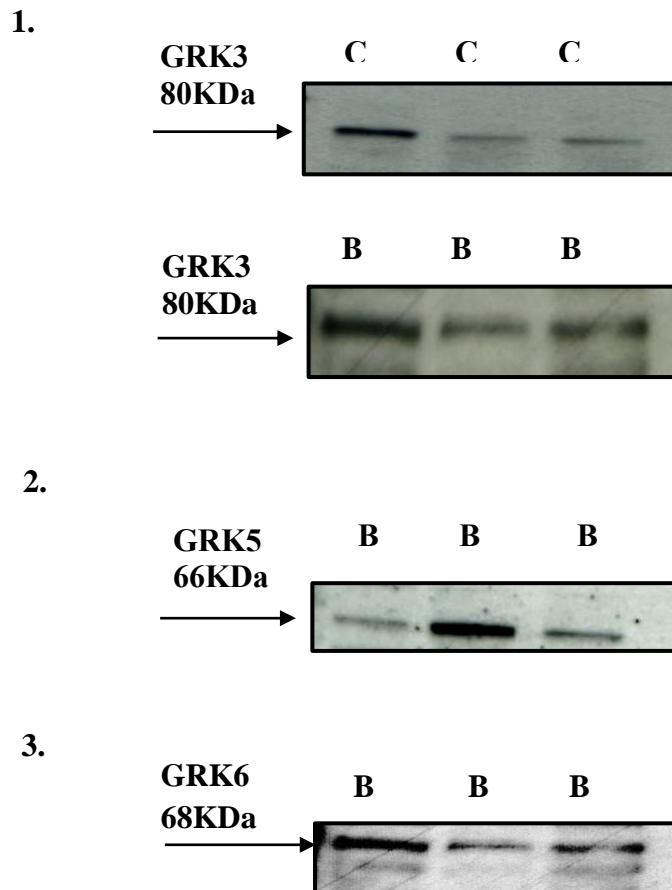
Figure 4-6: Western blots showing protein expression for GRK 3, 5 and 6 in cultured DSMC and whole bladder

The immunoblot a band for GRK3 expression was detected at 80 kDa for DSMC and whole bladder (1). For GRK5 protein expression and 6 in whole rat bladder, bands were detected at 66 KDa and 68KDa, respectively (2 and 3).

C= DSMC, **B**= Bladder

4.5.3. Effects of dominant-negative GRKs expression on P2Y$_1$- receptor PLC signalling in DSMC

GPCR activity is known to be regulated by G protein-coupled receptor kinases (GRK) (38). Therefore to assess P2Y receptor/GRK interactions in DSMC were transfected with dominant-negative/catalytically inactive versions of GRK proteins which are known to prevent the desensitizing actions of endogenous GRKs on GPCR activity (174). To examine whether P2Y receptor signalling is regulated by endogenous GRKs, specific GRK isoenzymes were inhibited by overexpressing catalytically inactive dominant-negative GRK mutants. This approach has been successfully used to cause selective inhibition of endogenous GRK activities in a variety of different cells (38,174).

Analysis of P2Y$_1$ receptor desensitization in DSMC co-transfected with RFP and pcDNA3 (control), ^{D110A,K220R}GRK2, ^{K215R}GRK5, or ^{K215R}GRK6 showed that the changes in R2 responses compared with R1 were comparable to those observed in non-transfected cells (Figure 4-7). However, the presence of ^{D110A,K220R}GRK3 significantly attenuated reductions in the R2 response when compared with R1 (Figure 4-7 C), suggesting that endogenous GRK3 activity plays a significant role in regulating ADP-stimulated P2Y$_1$-receptor-mediated PLC/Ca^{2+} signalling. These findings strongly suggest that GRK3 is a key mediator of ADP-induced P2Y$_1$ receptor desensitization.
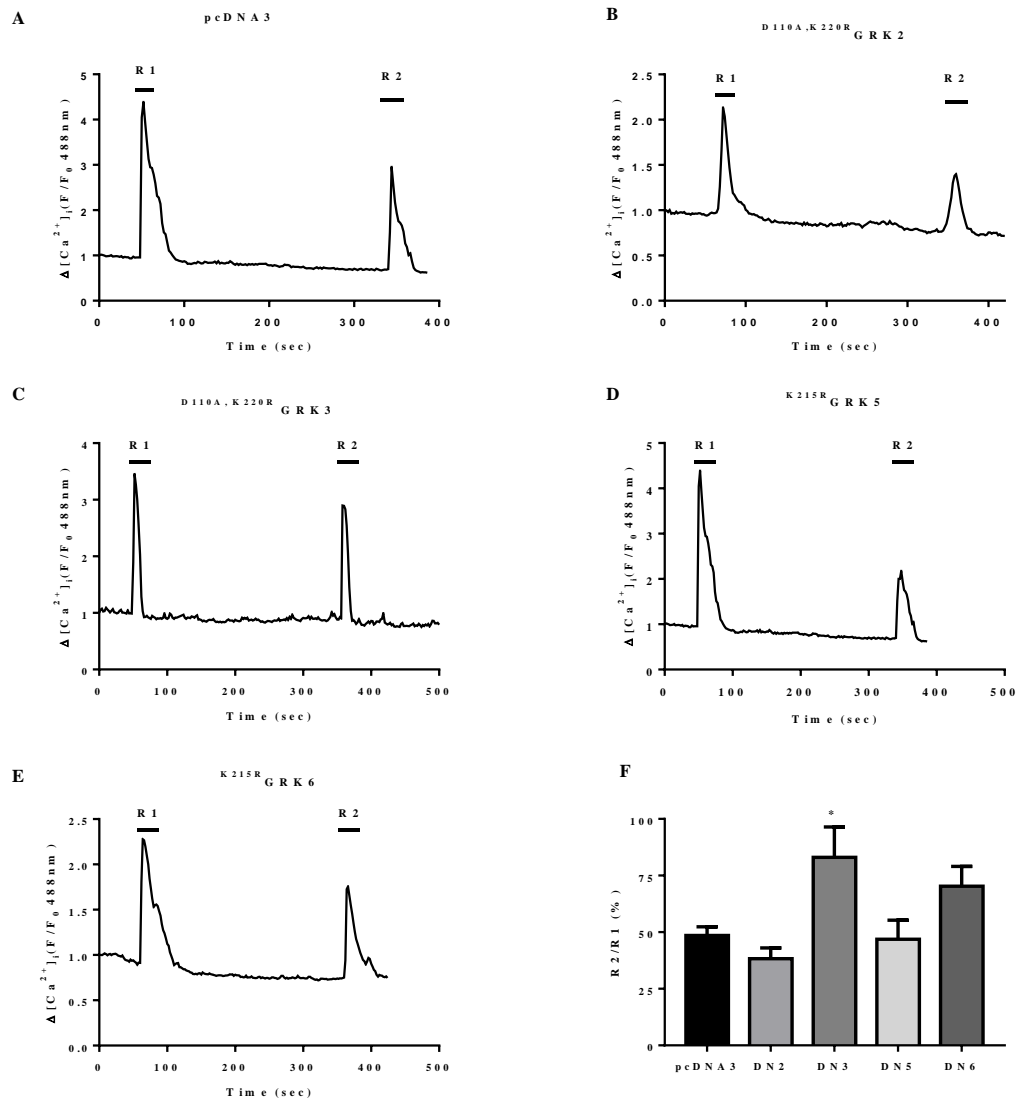
Figure 4-7: GRK3 inhibition decreases P2Y$_1$-receptor desensitization induced by ADP.

DSMC were transfected with pcDNA3 (control), ^{D110A,K220R} GRK2, ^{D110A,K220R}GRK3, ^{K215R}GRK5, or ^{K215R}GRK6 (0.5 μg). Cells were subjected to the standard R1/Rmax/R2 protocol. Representative traces show $[Ca^{2+}]_i$ changes in cells transfected with pcDNA3 (A), ^{D110A,K220R}GRK2 (B), ^{D110A,K220R}GRK3 (C), ^{K215R}GRK5 (D), or ^{K215R}GRK6 (E). P2Y$_1$ receptor desensitization was determined as the relative (%) change in R2 response compared with R1 (F). The cumulative data are presented as means ± SEM from n=25 cells from DSMC preparations from >3 different animals. Statistical significance is indicated as *P< 0.05 (one-way ANOVA, Dunnett's post hoc test).

4.5.4. G protein-coupled receptor kinase 2 regulates P2Y$_2$-purinoceptor mediated PLC signalling in DSMC

4.5.4.1. Effects of dominant-negative GRKs expression on P2Y$_2$ receptor PLC signalling in DSMC

Analysis of P2Y$_2$ receptor desensitization in DSMC co-transfected with RFP and pcDNA3 (control), ^{D110A,K220R}GRK3, ^{K215R}GRK5, or ^{K215R}GRK6 showed that the changes in R2 responses compared with R1 were comparable to those observed in non-transfected cells (Figure 4-8). However, the presence of ^{D110A,K220R}GRK2 significantly attenuated reductions in the R2 response when compared with R1 (Figure 4-8), suggesting that endogenous GRK2 activity plays a significant role in regulating UTP-stimulated P2Y$_2$-receptor-mediated PLC/Ca^{2+} signalling. These findings strongly suggest that GRK2 is a key mediator of UTP-induced P2Y$_2$ receptor desensitization.

4.5.4.2. Effects of inhibiting endogenous GRK2 activities in DSMC by depletion siRNA

To confirm and extend the data obtained in previous experiment using the catalytically inactive dominant-negative mutant (DNM) GRKs, in this experiment will study the effects of GRK knockdown using small interfering RNAs (siRNAs) on DSMC of rat bladder. DSMC were transfected with siRNAs designed to target GRK2. Optimal depletion of endogenous GRK2 was achieved 48 h after siRNA transfection at Concentrations of siRNA of 10 nM (Figure 4-9). As a result of the transfection with anti-GRK2 siRNA caused a marked reduction in GRK2 compare to the (NC) negative-control siRNA in cultured DSMC.
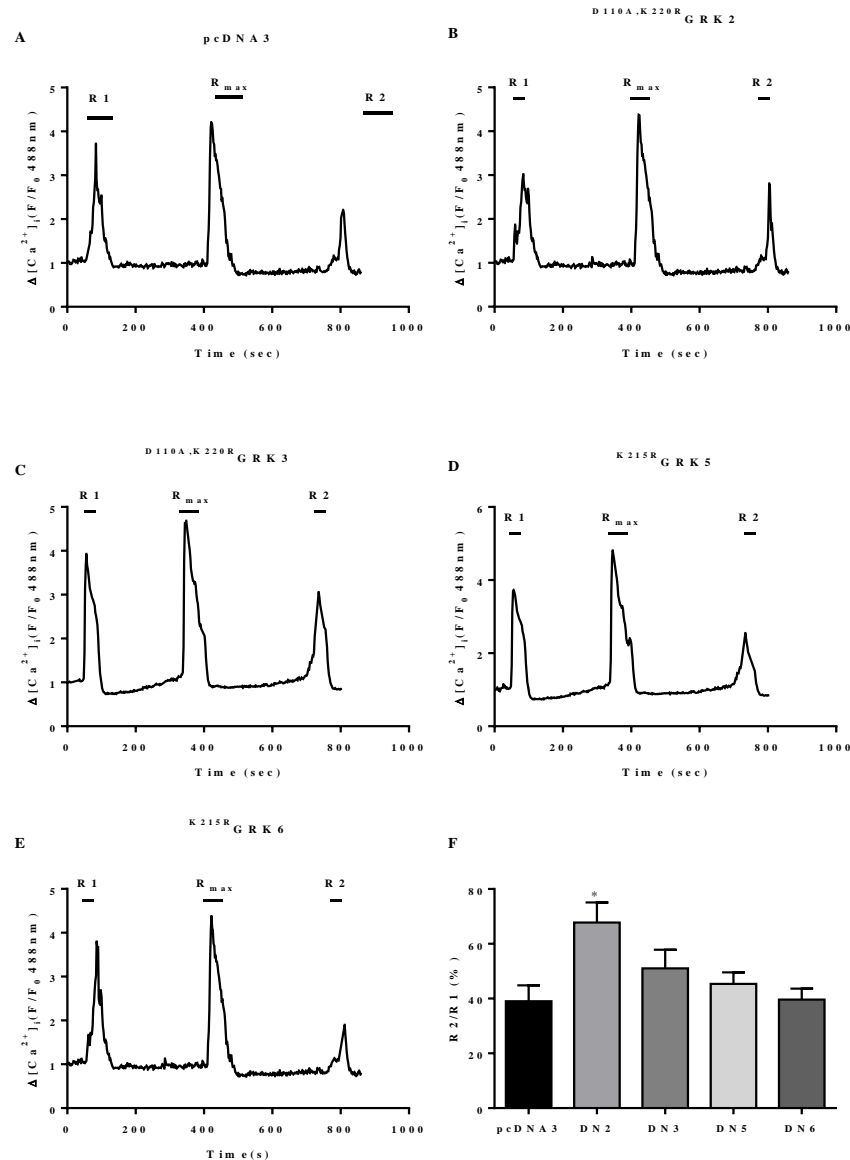
Figure 4-8: GRK2 inhibition decreases P2Y$_2$-receptor desensitization induced by UTP.

DSMC -transfected with pcDNA3 (control), [D110A,K220R]GRK2, [D110A,K220R]GRK3, [K215R]GRK5, or [K215R]GRK6 (0.5 µg). Cells were subjected to the standard R1/Rmax/R2 protocol. Representative traces show calcium changes in cells transfected with pcDNA3 (A), [D110A,K220R]GRK2 (B), [D110A,K220R]GRK3 (C), [K215R]GRK5 (D), or [K215R]GRK6 (E). P2Y$_2$ receptor desensitization was determined as the relative (%) change in R2 response compared with R1. F) Cumulative data are presented as means + SEM from 19-25 cells from DSMC preparations from ≥3 different animals. Statistical significance was indicated when *P<0.05 DSMC (one-way ANOVA, Dunnett's post hoc test).
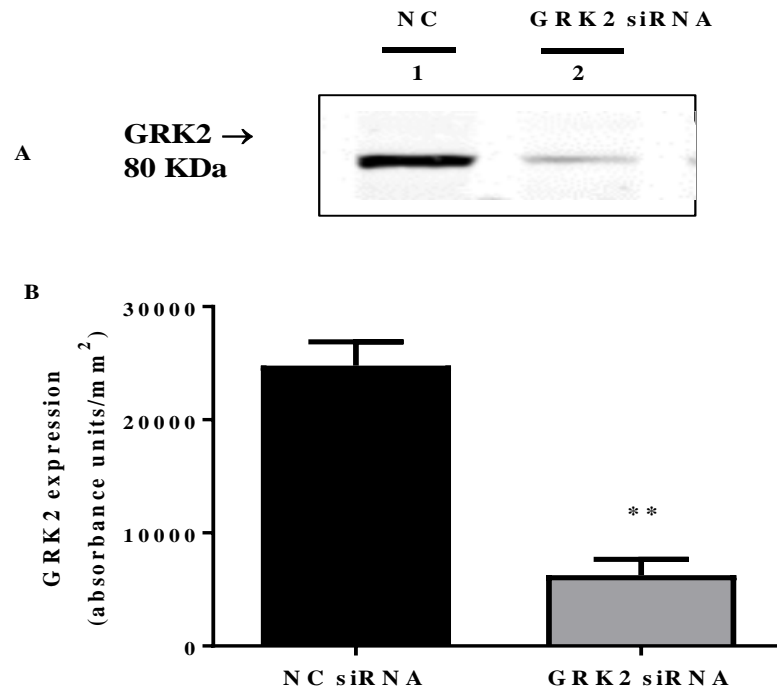
Figure 4-9: Anti-GRK2 siRNA causes endogenous GRK2 protein depletion.

DSMC were transfected with control or anti-GRK2 siRNAs (10 nM) using the Lonza Nucleofection technique. After 48 h, cells were lysed and 40 µg of protein loaded for SDS–PAGE separation and immunoblotting. (A) Representative western blots of GRK2 and negative-control siRNA are shown **lane 1**, (**NC**= negative control); **lane 2,** (GRK2-overexpressing). (B) Cumulative data showing that GRK2 siRNA significantly decreased GRK2 compare to the control in DSMC. Data are means ± SEM for 3 separate nucleofections from three separate cell preparation. Statistical significance by t-test is indicated as (**P<0.05 GRK2 siRNA vs. control cells).

4.5.5. Characterising the selectivity of the putative GRK2 inhibitor paroxetine to inhibit GRK2 mediated receptor desensitization.

Until recently there were no specific pharmacological inhibitors of GRKs. Previously a variety of molecular biological approaches were utilised to determine which GRK isoenzymes were responsible for GPCR desensitization in a variety of cell backgrounds such as myometrial smooth muscle cells (179). These techniques aimed to manipulate endogenous GRK activity by either enhancing activity through overexpression of individual GRK proteins, or inhibiting GRK activity via overexpression dominant-negative GRK mutants (174,176). More recently the advent of RNA interference (RNAi) technologies has allowed the selective depletion of target proteins which enables the specific knockdown of individual GRK isoenzymes (37,109). However, the inefficiency of transfection techniques to deliver plasmids, or RNAi into primary cells, often results in low protein expression or reduced suppression of endogenous protein expression, and thus false negative data. Therefore, the discovery of cell permeable small molecule GRK inhibitors would enable examination of GRK/GPCR interactions in all cell types. Indeed, recent work, especially by the (Tesmer group) has identified a number of different potential GRK inhibitors (180), including the selective serotonin reuptake inhibitor (SSRI) paroxetine. Paroxetine, has been on the market since 1992 (181) and is the most potent and one of the most specific SSRI (182). However, the discovery that the selective serotonin re-uptake inhibitor (SSRI) paroxetine can inhibit GRK2 function (183), prompted us to examine whether this drug could enable us to delineate a role for GRK2 in P2Y receptor desensitization in isolated DSMC. Indeed, not only has paroxetine been identified as a GRK2 inhibitor in *in vitro* studies, but has recently been shown to enhance βAR mediated contraction of cardiomyocytes (181), a process regulated by GRK2 (184) and improve cardiac function post myocardial infarction (180). Here the potential ability of paroxetine to inhibit the desensitization of P2Y mediated PLC signalling was determined. To rule out non-selective effects the structurally distinct SSRI fluoxetine (which does not inhibit GRKs was used as a control) (180).

To assess the specificity of paroxetine to inhibit GRK2 activity, initial experiments focused on an immortalised human smooth muscle cells line (ULTR) that expresses endogenous PLC-coupled receptors that are either exclusively regulated by GRK2

(histamine H_1) (174) or exclusively regulated by GRK6 (oxytocin) (130). To study histamine H_1 receptor desensitization an identical protocol was applied as that utilised for the $P2Y_2$ receptor, however, in this case cells were previously transfected with the extensively characterised IP_3 biosensor eGFP-tagged PH domain of PLC_δ (0.5 µg, for 48 h) (37,109,130,174). Receptor/PLC activity was assessed by measuring IP_3 production rather than intracellular calcium changes; however, we have previously shown that both outputs are valid and interchangeable readouts of GPCR/PLC activity and receptor desensitization (37,108,109,130,174). Following a 30 min pre-incubation with fluoxetine, the reduction in the R2:R1 ratio was similar to that achieved in vehicle pre-treated cells (means ± SEM; 43.8 ± 6.3, n=7 control vs 47.4 ± 7.9, n=5 fluoxetine), suggesting that fluoxetine was unable to alter histamine (H_1) receptor desensitization (Figure 4-10). However, inclusion of paroxetine reversed the reduction in the R2:R1 ratio, suggesting that paroxetine was able to prevent desensitization (Figure 4-10). To induce oxytocin receptor desensitization we amended our protocol, comparing the responses of two maximal concentrations of oxytocin (R1 and R2) either side of a 5 min washout period. Here, neither SSRI was able to prevent the desensitization of oxytocin receptor PLC signalling (Figure 4-11), suggesting neither compound could inhibit GRK6 mediated receptor desensitization.
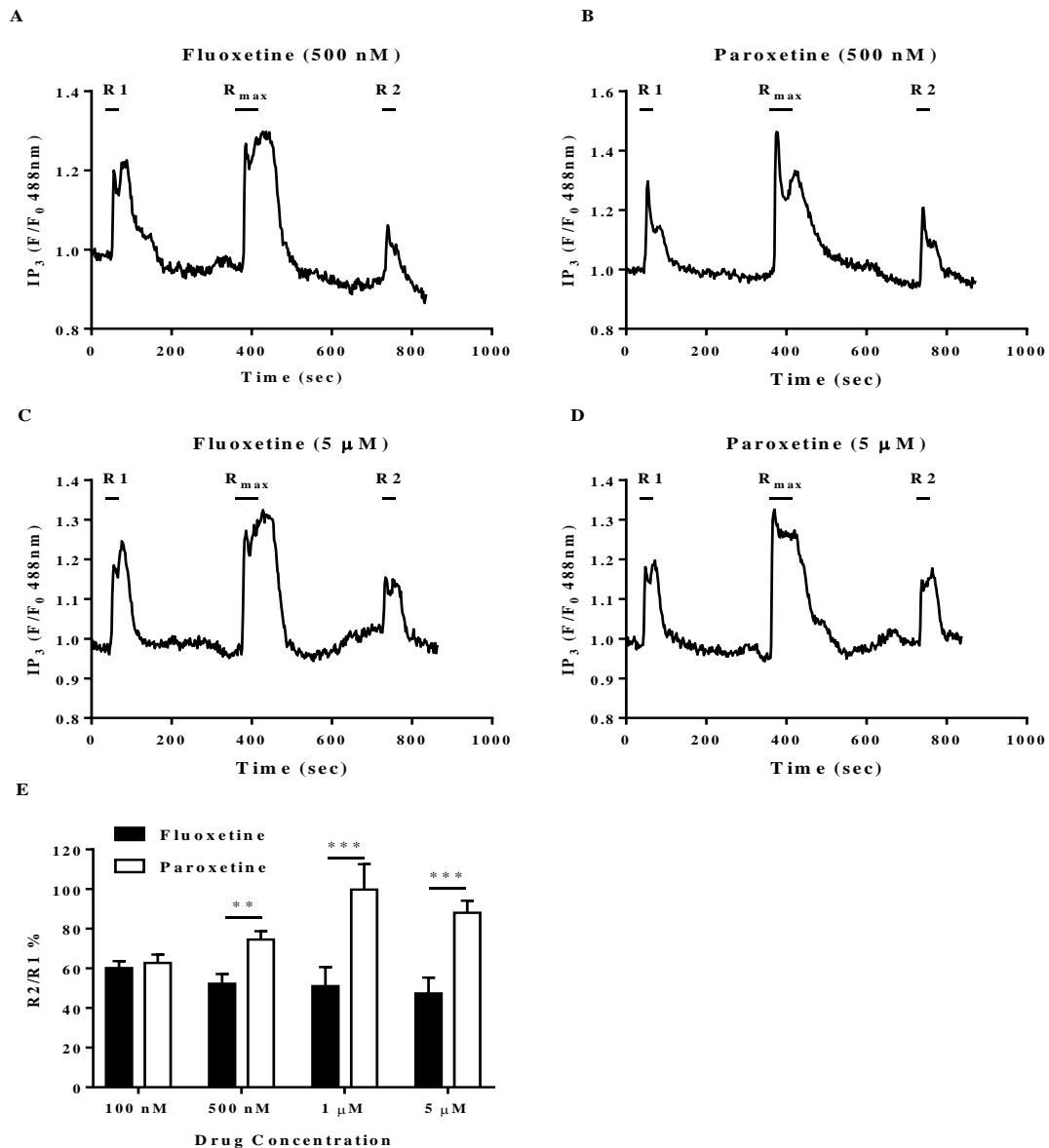
Figure 4-10: Paroxetine but not fluoxetine prevents histamine H_1 receptor desensitization in ULTR cells.

Cells were transfected with eGFP-PH (0.5 μg) and 48 h later incubated with various concentrations of fluoxetine or paroxetine for 30 min, prior to being subjected to the desensitization (R1, R_{max}, R2) protocol (see Methods). Representative traces show the changes in IP_3 for DMSC pre-treated with fluoxetine (A, 500 nM; C, 5 μM) or paroxetine (B, 500 nM; D, 5 μM). E) Cumulative data (means ± SEM) show the percentage changes in IP_3 (R2 relative to R1) from 19 cells for each treatment condition, from at ≥4 separate transfections. Statistical comparison was one-way ANOVA by t-test, and (***P<0.05) was considered significant.
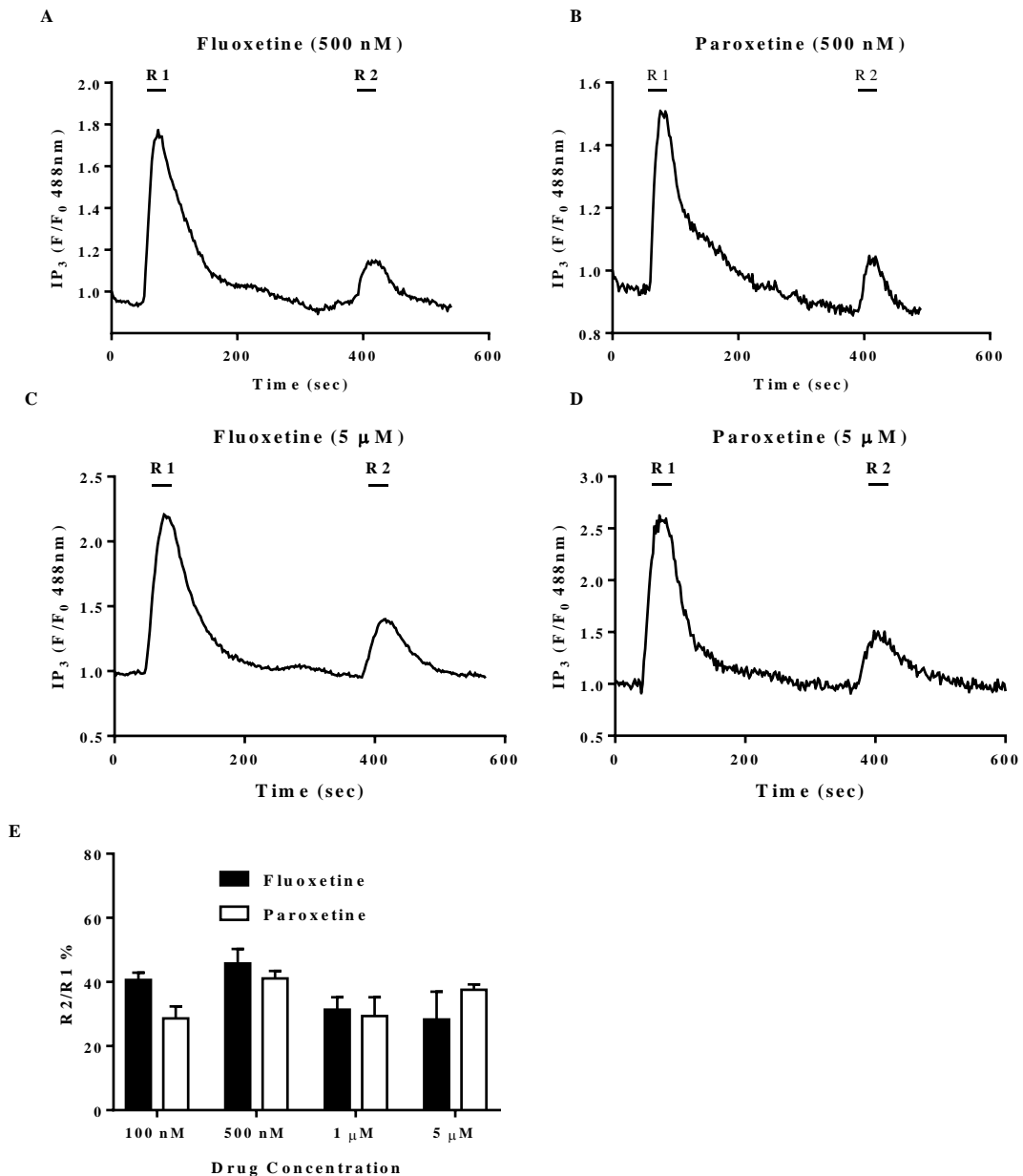
Figure 4-11: Paroxetine and fluoxetine do not affect the desensitization oxytocin receptor PLC activity in ULTR cells.

Cells were transfected with eGFP-PH (0.5 µg) and 48 h later incubated with various concentrations of fluoxetine or paroxetine for 30 min, prior to being subjected to the standard oxytocin desensitization protocol (see Methods). Representative traces show the changes in IP_3 for DMSC pre-treated with fluoxetine (A, 500 nM; C, 5 µM) or paroxetine (B, 500 nM; D, 5 µM). E) Cumulative data (means ± SEM) show the percentage changes in IP_3 (R2 relative to R1) from 15 cells for each treatment condition, from at ≥4 separate transfections.

4.5.6. Does the putative GRK2 inhibitor paroxetine prevent the desensitization of P2Y receptor PLC activity in DSMC?

As the ULTR data suggests that paroxetine inhibits the desensitization PLC signalling of GPCRs that are regulated by GRK2, but not GRK6, experiments were conducted that assessed whether paroxetine could also affect P2Y receptor/PLC signalling in DSMC. Since the DMN data highlighted a role for GRK2 in the desensitization of $P2Y_2$ but not $P2Y_1$ receptor signalling, one would expect paroxetine to block $P2Y_2$ but not $P2Y_1$ receptor desensitization. To test this theory DSMC were loaded with the Ca^{2+}-sensitive dye Fluo4-AM (4 µM) in the presence of vehicle control (DMSO), paroxetine (5 µM) or fluoxetine (5 µM) for 1 h, before being subjected to the standard $P2Y_2$ receptor desensitization protocol (R1, R2 = 10 µM UTP, for 30 sec; R_{max} = 100 µM UTP, for 60 sec). The reduction in the R2:R1 ratio was similar in vehicle control and fluoxetine (i.e. ~50%) treated cells as that seen in the absence of either compound (Figure 4-12) suggesting that fluoxetine was unable to alter $P2Y_2$ receptor desensitization (Figure 4-12). In contrast, inclusion of paroxetine reversed the reduction in the R2:R1 ratio, suggesting that paroxetine was able to prevent the desensitization of $P2Y_2$ receptor PLC activity in DSMC (Figure 4-12).

To examine the specificity of paroxetine's actions on $P2Y_1$ receptor activity we examined whether paroxetine could alter the desensitization of $P2Y_1$ receptor PLC signalling when the cells challenged by ADP. Using the standard R1:R1 desensitization protocol, the expected reduction in R2:R1 ratio was slightly less than in non-treated control cells (Figures 41 B). However, inclusion of fluoxetine or paroxetine failed to change the R2:R1 ratio when compared to vehicle treated control responses (Figure 4-13), which implies that neither SSRI prevented $P2Y_1$ receptor desensitization.
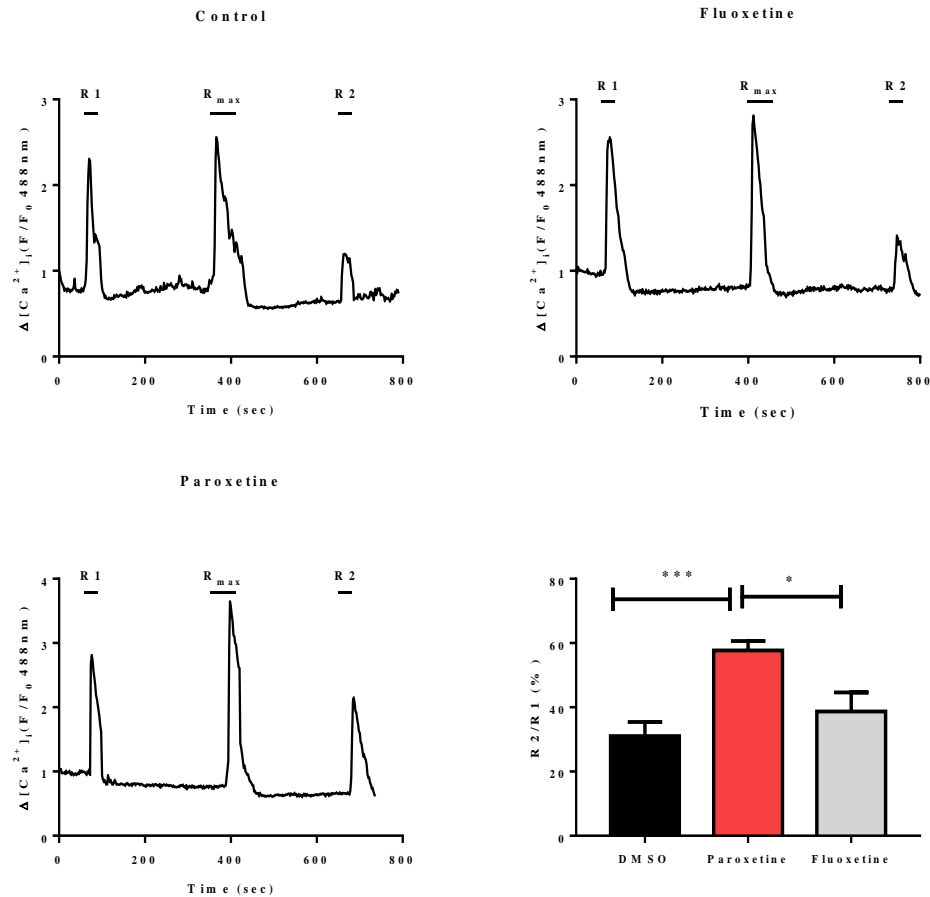
Figure 4-12: Paroxetine but not fluoxetine inhibits the desensitization of P2Y$_2$ receptor PLC activity in DSMC.

Cells were loaded with the Ca^{2+}-sensitive dye Fluo4-AM (4 μM) and paroxetine or fluoxetine for 1 h (5 μM), before being subjected to the standard desensitization protocol (R1, R2 = 10 μM UTP, for 30 sec; Rmax = 100 μM UTP, for 60 sec, with 5 min washout periods). Representative traces show the effects of vehicle control (A), fluoxetine (B) or paroxetine (C) pre-treatment on UTP-stimulated $[Ca^{2+}]_i$. D) Cumulative data show the means ± SEM $[Ca^{2+}]_i$ changes for the percentage change in R2 relative to R1 from 18 cells, from at least 3 separate bladder preparations. Statistical comparison showed that paroxetine significantly attenuated receptor desensitization (*P<0.05; ***P<0.01, one-way ANOVA, Dunnett's *post hoc*) when compared to control and fluoxetine treated cells.
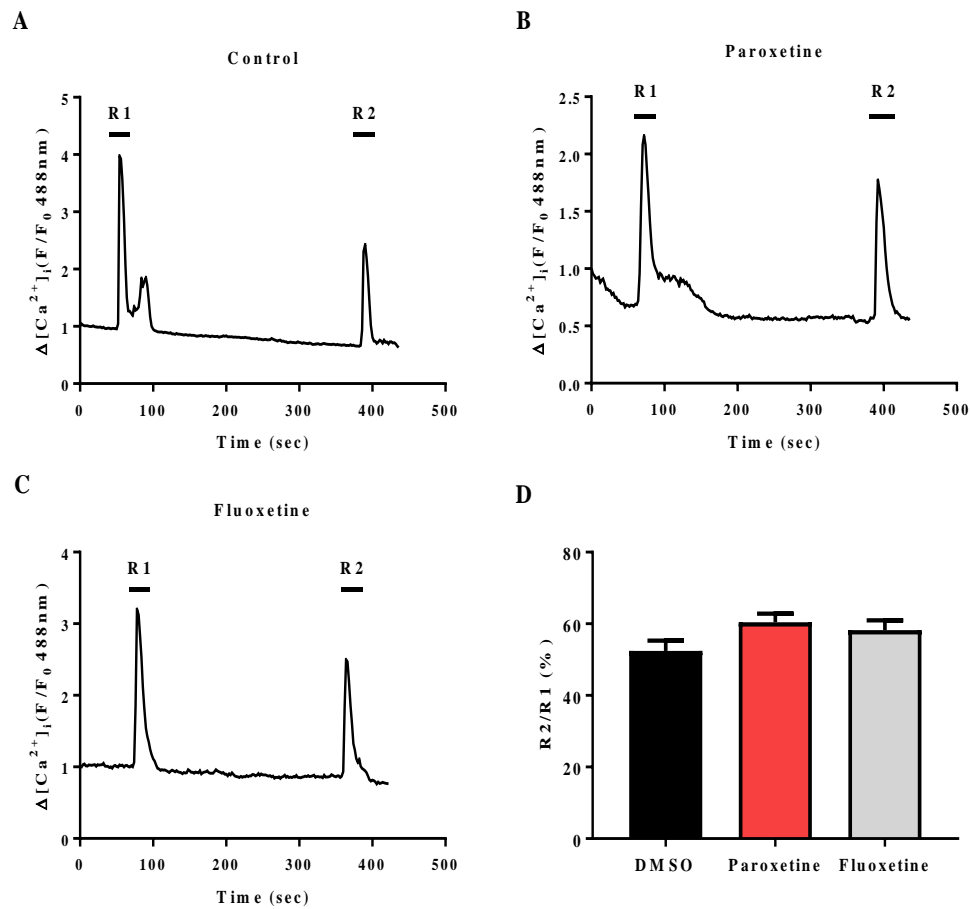
Figure 4-13: Assessment of the desensitization of P2Y$_1$ receptor PLC activity in DSMC by paroxetine and fluoxetine.

Cells were loaded with the Ca^{2+}-sensitive dye Fluo4-AM (4 μM) and incubated with paroxetine or fluoxetine (5 μM) for 1 h, before being subjected to the standard desensitization protocol (R1, R2 = 10 μM ADP, for 30 sec). Representative traces show the effects of vehicle control (A), fluoxetine (B) or paroxetine (C) pre-treatment on ADP-stimulated [Ca^{2+}]$_i$. D) Cumulative data show the means ± SEM [Ca^{2+}]$_i$ changes for the percentage change in R2 relative to R1 from 16 cells, from at least 3 separate bladder preparations.

4.6. Discussion

The data from previous chapters confirmed that nucleotide signalling such as ADP and UTP had mediated the P2Y purinergic receptors signalling in isolated rat DSMC. These findings confirmed that $P2Y_1$, $P2Y_2$ purinergic receptors promote increased $[Ca^{2+}]_i$ in rat DSMC, thus are likely to induce contraction of the urinary bladder. Consequently, it is likely that $P2Y_1$ and $P2Y_2$ receptors contribute to the control of detrusor smooth muscle contraction and thus bladder emptying. Ideally, it would have been interesting to examine the roles that $P2Y_1$ and $P2Y_2$ receptors play in the contraction of whole bladder however, this was not technically possible.

To our knowledge, no previous studies have addressed the regulation of GPCR protein regulated by G protein coupled receptor kinases (GRKs) in DSMC. Therefore to address this lack of information I used confocal microscopy imaging protocols to investigate receptor desensitization this provide evidence that when the receptors were exposed to ATP and UTP for 30 seconds followed by a 5, 10 or 15 min washout period, the receptor responsiveness recovered gradually with increasing washout time, suggesting an initial desensitization of the receptor activity followed by re-sensitization of receptor responsiveness. Previous work showed that UTP-induced $P2Y_2$-receptor PLC signalling was subject to desensitization in isolated mesenteric smooth muscle cells MSMC (109). Using similar protocols, I have shown that Ca^{2+} measured using (Fluo4) can be used to assess purinergic receptor desensitization in the DSMC. $P2Y_2$ receptor desensitization was determined as the relative (%) change in R2 response when compared to R1 responses showed that R2 values were significantly reduced with a 5 minutes washout period. While, if the washout period was extended to 10 minutes, R2 responses increased to R1 indicating a desensitization of $P2Y_2$ receptor activity. Desensitization experiments show that after a 5 minutes washout period $[Ca^{2+}]_i$ signals were markedly suppressed. $P2Y_2$ receptor responses had recovered gradually over time, and recovering totally after 15 minutes. However, $P2Y_1$ responses remained suppressed after 10 minutes. Interestingly, these findings suggest an initial desensitization of receptor activity followed by re-sensitization of $P2Y_2$, but not $P2Y_1$ receptor responsiveness.

Since GPCR desensitization is usually mediated through the action of GRK proteins we next investigated the expression of GRK iosozymes in DMSC. In the present study, for the first time I have shown the presence of GRKs 2, 3, 5 and 6 in DSMC. Since the co-

ordinated activity of receptor function is vital in regulating the contractile state of the bladder, I assume that GRK-mediated regulation of receptor function may play an important role in the maintenance of continence. As GPCR activity is known to be regulated by GRKs (38), therefore to assess P2Y receptor/GRK interactions in DMSC, cells were transfected with dominant-negative/catalytically inactive versions of GRK proteins which are known to prevent the desensitizing actions of endogenous GRKs on GPCR activity (37,38). The inhibition of GRK2 attenuated $P2Y_2$ receptor desensitization, whilst inhibition of GRK3 prevented $P2Y_1$ receptor desensitization. In previous study, the authors highlighted the roles of GRK2 as important regulator of UTP-stimulated $P2Y_2$ receptor responsiveness in arterial smooth muscle cells (109). However, Mundell *et al* found that desensitization of $P2Y_1$ receptor is more dependent on protein kinase C (PKC) activity in human platelets (187). Which suggests that the mechanisms of P2Y receptor regulation are dependent upon which cells they are expressed in.

More recently the advent of RNA interference (RNAi) technologies has allowed the selective depletion of target proteins which enables the specific knockdown of individual GRK isoenzymes (37,109). However, the inefficiency of transfection techniques to deliver plasmids, or RNAi into primary cells, often results in low protein expression or reduced suppression of endogenous protein expression, and thus false negative data. Therefore, the discovery of cell permeable small molecule GRK inhibitors would enable examination of GRK/GPCR interactions in all cell types. Indeed, recent work, especially by the (Tesmer group) has identified a number of different potential GRK inhibitors (180), including the selective serotonin reuptake inhibitor (SSRI) paroxetine. Discovery that the selective serotonin re-uptake inhibitor (SSRI) paroxetine can inhibit GRK2 function (183), which these data prompted us to examine whether this drug could enable us to delineate a role for GRK2 in P2Y receptor desensitization in isolated DSMC. Indeed, not only has paroxetine been identified as a GRK2 inhibitor in *in vitro* studies, but has recently been shown to enhance βAR mediated contraction of cardiomyocytes (181), a process regulated by GRK2 (184) and improve cardiac function post myocardial infarction (180). Here the potential ability of paroxetine to inhibit the desensitization of P2Y mediated PLC signalling was determined. To rule out non-selective effects the structurally distinct SSRI fluoxetine (which does not inhibit GRKs was used as a control) (180). Until recently, there were

no specific pharmacological inhibitors of GRKs. Previously a variety of molecular biological approaches were utilised to determine which GRK isoenzymes were responsible for GPCR desensitization in a variety of cell backgrounds such as myometrial smooth muscle cells (179). These techniques aimed to manipulate endogenous GRK activity by either enhancing activity through overexpression of individual GRK proteins, or inhibiting GRK activity via overexpression dominant-negative GRK mutants.

To assess the specificity of paroxetine in inhibiting GRK2 activity, initial experiments focused on an immortalised human smooth muscle cells line (ULTR) that expresses endogenous PLC-coupled receptors that are either exclusively regulated by GRK2 (histamine H_1) (174) or exclusively regulated by GRK6 (oxytocin) (130). To study histamine (H_1) receptor desensitization, an identical protocol was applied similar to that utilised for the $P2Y_2$ receptor. However, in this case the cells were previously transfected with the extensively characterised IP_3 biosensor eGFP-tagged PH domain of PLC_δ (0.5 µg, for 48 h) (37,109,130,174). Receptor/PLC activity was assessed by measuring IP_3 production rather than intracellular calcium changes; however, we have previously shown that both outputs are valid and interchangeable readouts of GPCR/PLC activity and receptor desensitization (37,108,109,130,174). Following a 30 min pre-incubation with fluoxetine, the reduction in the R2:R1 ratio was similar to that achieved in vehicle pre-treated cells, suggesting that fluoxetine was unable to alter histamine (H_1) receptor desensitization (Figure 4-10). However, inclusion of paroxetine reversed the reduction in the R2:R1 ratio, suggesting that paroxetine was able to prevent desensitization (Figure 4-10). To induce oxytocin receptor desensitization, I amended this protocol, comparing the responses of two maximal concentrations of oxytocin (R1 and R2) either side of a 5 min washout period. Here, neither SSRI was able to prevent the desensitization of oxytocin receptor PLC signalling (Figure 4-11), suggesting neither compound could inhibit GRK6 mediated receptor desensitization.

When DSMC were challenged with UTP, the reduction in the R2:R1 ratio was similar in vehicle control and fluoxetine (i.e. ~50%) treated cells as that seen in the absence of either compound (paroxetine and fluoxetine) (Figure 4-12) suggesting that fluoxetine was unable to alter $P2Y_2$ receptor desensitization (Figure 4-12). In contrast, the presence of paroxetine reversed the reduction in the R2:R1 ratio, suggesting that

paroxetine was able to prevent the desensitization of $P2Y_2$ receptor PLC activity in DSMC. However, inclusion of fluoxetine or paroxetine failed to change ADP stimulated R2:R1 ratio when compared to vehicle treated control responses (Figure 4-13), which implies that neither SSRI prevented $P2Y_1$ receptor desensitization when the cells were challenged by ADP. Indeed, this is not wholly unsurprising since our data suggests that GRK3 and not GRK2 regulates $P2Y_1$ receptor activity.

Our data indicates an important role of paroxetine as GRK2 inhibitor in regulation of DSMC contractility. Hence, I suggest that the paroxetine as a selective GRK2 inhibitor may have a great unique role as a chemical probe for a new therapy of OAB in future. Thal *et al* assessed the heart function in patients with heart failure, and identified that paroxetine as a selective inhibitor of GRK2 improved the heart function both *in vitro* and *in vivo*, yet these improvements were not observed for the SSRI fluoxetine (181). Our data suggests that in rat DSMCs agonist-mediated phosphorylation and regulation of the P2Y receptor is undertaken by endogenous GRKs. As a result, this data are suggesting that GRK2 other than GRKs may be more important for purinergic receptor desensitization.

4.7. Conclusion

To date, little is known regarding the mechanism or regulation of detrusor purinergic receptor signalling in the bladder. This chapter characterised the desensitization and re-sensitization kinetic of endogenously expressed $P2Y_1$ and $P2Y_2$ receptor mediated PLC signalling in rat DSMC. Both $P2Y_1$ and $P2Y_2$ receptors underwent desensitization which was maximal at 5 min and fully reversed after 10 or 15 min, respectively. GPCR desensitization is usually mediated by the action of GRK proteins and our data established that rat DSMC express GRKs 2, 3 5 and 6, matching the expression pattern seen in whole bladder lysates. Overexpression of catalytically inactive dominant-negative mutant (DNM) GRK2 significantly attenuated $P2Y_2$ receptor desensitization. In addition, the putative GRK2 inhibitor paroxetine was able to prevent the desensitization of $P2Y_2$ receptor PLC activity in DSMC. Collectively these findings suggest that GRK2 is the key kinase in regulating $P2Y_2$ receptor driven PLC activity. In contrast, DNM GRK3 attenuated $P2Y_1$ receptor desensitization, suggesting a selective role for GRK3 in the negative regulation of $P2Y_1$ receptor PLC activity.

Chapter 5

5. Characterising the roles that arrestin proteins play in the regulation P2Y-purinoceptor PLC signalling in DSMCs

5.1. Introduction

The process of GPCR desensitization occurs following agonist binding and activation of the receptor, which alters the receptor conformation and recruits one or more GRK isoenzymes. As mentioned in the previous chapter GRK proteins phosphorylate serine or threonine residues within the third intracellular loop and, or C-terminal tail of GPCRs. GRK-mediated phosphorylation initiates the process of desensitization by priming the GPCR to recruit arrestin proteins. Once bound to the GPCR, arrestin proteins physically uncouple the GPCR from the G protein to effectively prevent further signalling. However, arrestins themselves can not only inhibit certain GPCR activated signalling pathways but can also act as an agonist-adaptor scaffold to both recruit and re-direct GPCR signalling down alternative signalling pathways (85,109,188) .

In the previous chapter, the data identified the expression of GRKs 2, 3, 5 and 6 in DSMC. Subsequent transfection of DSMC with dominant-negative/catalytically inactive versions of GRKs 2, 3, 5 or 6, which are known to inhibit the desensitizing actions of their endogenous target GRKs on GPCR activity, highlighted that $P2Y_1$ receptor desensitization was attenuated by expression of the GRK3 dominant negative construct. In contrast, $P2Y_2$ desensitization was attenuated following inclusion of the GRK2 dominant negative. Considering that these dominant negative constructs function to inhibit the recruitment of their corresponding endogenous GRK to thus prevent GPCR phosphorylation, it is logical to assume that endogenous GRK3 is phosphorylating $P2Y_1$ and GRK2 phosphorylating $P2Y_2$ receptors. Since GRK mediated GPCR phosphorylation is a prelude to subsequently promote arrestin recruitment it is likely that both $P2Y_1$ and $P2Y_2$ receptors are also regulated by arrestin proteins in DMSC. However, at present no reports have demonstrated the presence of arrestin proteins in DSMC.

Although to date, no studies have examined the potential roles for arrestin proteins in the regulation of endogenous purinergic receptor/PLC signalling in DSMC, previous data suggests that both receptors are substrates for arrestin proteins (88). Indeed, our laboratory has identified a selective role for arrestin2, but not arrestin3 in the desensitization of $P2Y_2$ activated PLC signalling in rat arterial smooth muscle cells (108). Further studies utilising exogenous P2Y receptors expressed in model cells systems indicate that both $P2Y_1$ and $P2Y_2$ receptors are capable of recruiting arrestin

subtypes (189) collectively, these data suggest that agonist-occupied $P2Y_1$ and $P2Y_2$ receptors are capable of recruiting arrestin proteins, although the degree of selectivity and specificity seems dependent on the cell background and experimental conditions. Therefore, to examine the potential requirements and roles that arrestins play in the regulation of endogenously expressed $P2Y_1$ and $P2Y_2$ receptors, their ability to activate PLC signalling was determined in the absence of individual arrestin expression. To achieve knockdown of individual arrestin isoforms proteins were targeted with selective small interfering RNAs, which have been extensively characterised and utilised previous in our laboratory (108,109). These techniques were combined with confocal imaging of either the eGFP-PH biosensor to measure changes in (IP_3) or calcium to assess the effects of arrestin knockdown on $P2Y_1$ and $P2Y_2$ receptor signalling and desensitization.

5.2. Aims

This chapter aimed to investigated whether different arrestin isoforms regulate nucleotide (P2Y) receptor/PLC signalling in rat detrusor smooth muscle cells (DSMCs).

1- Determine whether arrestin proteins are expressed in the detrusor smooth muscle cells and whole bladder

2- Using selective small-interfering (si)RNAs to selectively deplete endogenous arrestin 2 or arrestin 3 expression and examine subsequent effects on the desensitization of ADP/P2Y$_1$- and UTP/P2Y$_2$-stimulated IP$_3$ and Ca^{2+} signalling.

5.3. Methods

5.3.1. Cell maintenance and culture

For imaging experiments DSMC were isolated and grown as described in chapter 2 methods section. However, for some studies including demonstration of whether the siRNA constructs were able to suppress the expression of individual arrestin expression a larger number of DSMC were required (as $2x10^6$ cells were required per transfection for these experiments). This was very challenging task, as attempting to subculture DSMC proved difficult with cells quickly becoming senescent. After attempting multiple culturing techniques and different media, I found that the most suitable media was 199 medium supplemented with 10% foetal calf serum (FCS), 100 IU/mL penicillin, 100 μg/mL streptomycin, maintained at 37ºC and 5% CO_2 in humidified conditions to grow DSMC.

5.3.2. Knockdown of endogenous arrestin2 and arrestin3 in DSMC

DSMC were grown until ~90% confluent in 2, $175cm^2$ culture flasks as described above. Cells were harvested with 0.25% (w/v) trypsin, and counted by using a haemocytometer and aliquoted so that each transfection contained $2x10^6$ cells. Before transfection, cells were centrifuged at 1000 rpm for 5 min, and media was removed carefully. The pellet was re-suspended into 100 μL of transfection solution (Lonza, Cologne, Germany), and 10 nM of negative-control (non-targeting), anti-arrestin2 (5′-GCCACUGACUCGGCUACAAtt-3′) (10nM), or anti-arrestin3 (5′-GCCUUCUGUGCCAAAUCUAtt-3) (10 nM) (Applied Biosystems, UK) added. The resulting solutions were transfected to individual cuvette (one per transfection) and transfection was performed using an Amaxa/Lonza nucleofector device; set to program D-033 (which we know provided maximal transfection of smooth muscle cells) (108). After transfection 500 μL of pre-warmed (37°C) culture 199 media was added to each cuvette, and the cells were transferred into eppendorf tubes using plastic pipettes provided. Cells were incubated at 37°C in a humidified air 5% CO_2 atmosphere for about 10 min prior to seeding onto 6 well/plates. One transfection contained enough cells to provide one confluent well of a 6 well culture plate. After 48 h cells were lysed assessed for protein content by the method of Bradford (1976). Arrestin protein expression were determined using standard immunoblotting protocols as described previously (46). Cell lysates (40 μg/lane) were separated by SDS-PAGE before Western

transfer. Arrestin2 and arrestin3 expressions were determined using an anti-arrestin2 antibody (A1CT) (gift from Prof R. J. Lefkowitz, Duke University, Durham, NC). The A1CT antibody was raised against arrestin 2 (1) but also detects arrestin 3 with a lower affinity enabling detection of both proteins see (Methods and Materials).

5.3.3.   Single cell imaging and receptor desensitization studies

Once the optimal concentration and incubation period was determined for maximal depletion of arrestin proteins (i.e. 48 h and 10 nM siRNA), we focused on assessing whether arrestin depletion could alter P2Y receptor activated PLC signalling. Here, acutely isolated cells were grown for 48 h prior to being co-transfected either negative control, anti-arrestin2, anti-arrestin3 siRNA and the pleckstrin-homology domain of $PLC_{\delta 1}$ tagged with enhanced green fluorescent protein (eGFP-PH, 0.5 μg). eGFP-PH is a biosensor that is known to measure changes in IP_3 concentrations following activation of G_q coupled GPCRs (37,109). After a further 48 h the ability of $P2Y_1$ or $P2Y_2$ receptors to stimulate PLC activity was measured using confocal imaging exactly as previously described (see Method and Material). Changes in cytosolic eGFP-PH fluorescence are displayed as the fluorescence emission (F)/initial basal fluorescence (F_0) (F/F_0). Additional Ca^{2+} experiments in the absence of eGFP-PH were conducted using Fluo-4-AM (4 μM, 60 min) and imaged as for eGFP. In some experiments to assess the ability of arrestins to regulate P2Y receptor activated Ca^{2+} signalling, DSMC cells were co-transfected with RFP and 10 nM of negative control, anti-arrestin2, anti-arrestin3 siRNAs. After 48 h, cells were loaded with Fluo4-AM as described previously (Materials and Methods). Since our previous data suggested that when co-transfected with two fluorescent markers >90% expressed both constructs, we assumed that any RFP-expressing cells also contained siRNA. Therefore, RFP cells were selected and Ca^{2+} changes measured in these cells.

5.4. Results

5.4.1. Expression of arrestin2 and arrestin3 proteins in DSMCs and whole bladder

The majority of non-visual cells express both arrestin2 and arrestin3 proteins. The A1CT antibody is known to detect both arrestin isoforms with higher affinity for arrestin2 over arrestin3 (109). As expected data in the figure below clearly show that arrestin2 (band at 50 kDa) is expressed in both the whole bladder and DSMC. Further exposure of the same blots also shows a band at (49 kDa) that corresponds to arrestin3, which is often difficult to detect due to the high immunoreactivity of the arrestin2 band. Indeed, clearly expression of AR2 higher in the whole bladder rather than DSMCs (Figure 5-1).
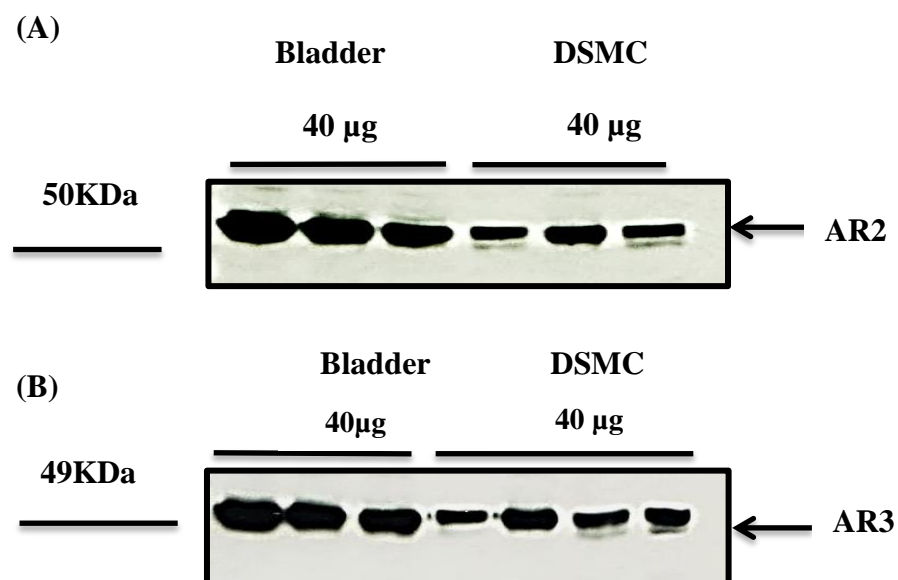
Figure 5-1: Arrestin 2 and arrestin 3 are expressed in rat bladder and DMSC.

Representative immunoblots show the expression of arrestin2 (**A**) and arrestin3 (**B**) in bladder and detrusor smooth muscle cells (DSMC). In both cases the upper band corresponds to arrestin2 (AR2; 50 kDa) and the lower band (49 kDa) corresponds to arrestin3 (AR3).

5.4.2. Effects of depletion of the siRNA on endogenous arrestin2 and 3 in DSMC

Although the anti-arrestin siRNA constructs used here have previously been optimised and shown to selectively deplete their target proteins in rat smooth muscle (108) their effectiveness in DSMC had not been determined. Therefore, DSMC were transfected with either anti-arrestin2 or anti-arrestin3 siRNA (10 nM) for 48 h and knockdown assessed using western blotting. Although not shown, the negative control siRNA had no effect on arrestin expression when compared to non-transfected cells or arrestin siRNA transfected cells. However, transfection with anti-arrestin2 siRNA produced a 75% reduction in arrestin2 expression with no effect on arrestin3 expression (Figure 5-2 A, C). In contrast, anti-arrestin3 siRNA had no effect upon arrestin2 expression but reduced arrestin3 expression by 80% (Figure 5-2 B, D).
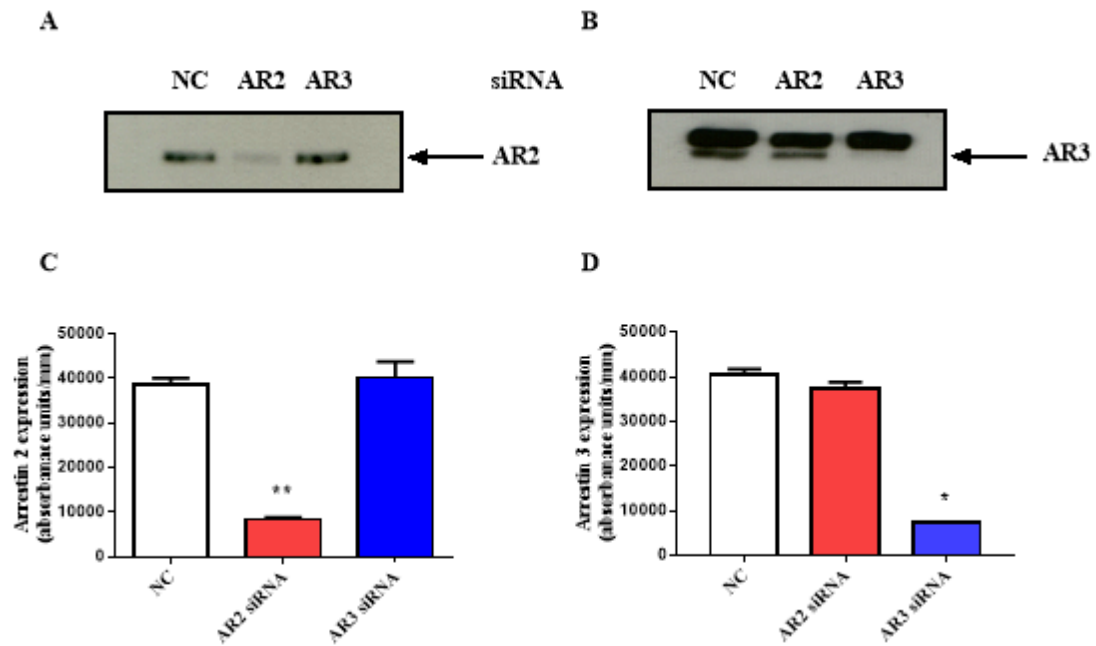
Figure 5-2: siRNA-mediated depletion of endogenous arrestin2 and arrestin3.

DSMC were transfected with negative control (NC), anti-arrestin2 (AR2) or anti-arrestin3 (AR3) siRNAs (10 nM). After 48 h cells were lysed and 40 μg of protein loaded for SDS–PAGE separation and immunoblotting. **A**) Representative Immunoblot showing arrestin2 depletion, (**B**) immunoblot showing depletion of AR3. Cumulative densitometric (means ± SEM; n=3 transfections from 3 separate animal preparations) data shows that arrestin2 (AR2) siRNA significantly decreased arrestin2 expression compared to the negative-control or anti-AR3 treated cells (**C**), and that AR3 siRNA significantly decreased arrestin3 expression compared to the control and anti-AR2 treated cells (**D**). Arrestin expression was quantified using the GeneGnome image analysis system (Syngene, Cambridge, UK). Statistical significance by t-test is indicated as (**p<0.01, AR2 siRNA vs NC; and *p<0.05 AR3 siRNA vs NC).

5.4.3. Effects of arrestin2 and arrestin3 depletion on ADP-Stimulated PLC Signalling and P2Y$_1$-receptor desensitization in DSMC

To assess the roles that arrestin proteins play in the regulation of P2Y receptor PLC activity, cells were co-transfected with eGFP-PH 0.5 µg; an extensively characterized IP$_3$ biosensor (108,109) and anti-arrestin2, anti-arrestin3, or negative-control siRNA (all at 10 nM; Applied Biosystems, Warrington, UK). Forty hours after transfection DSMC were loaded with Fura-Red (4 µM; 60 min) to enable simultaneous IP$_3$ and Ca^{2+} measurements. Receptor desensitization was determined using single cell confocal imaging as described previously. Changes in cytosolic eGFP-PH fluorescence are displayed as the fluorescence emission (F)/initial basal fluorescence (F$_0$) (F/F$_0$) Selective depletion of arrestin3 markedly reversed ADP-induced decreases in R2 relative to R1 which inhibit P2Y$_1$ receptor desensitization (Figure 5-3 C). Thus arrestin3 depletion had a big effect on R2/R1 ratios (Figure 5-3 E) and not arrestin2; this did not achieve statistical significance, suggesting that attenuation of the ADP signal is primarily effected by the arrestin3 isoform (Figure 5-3).
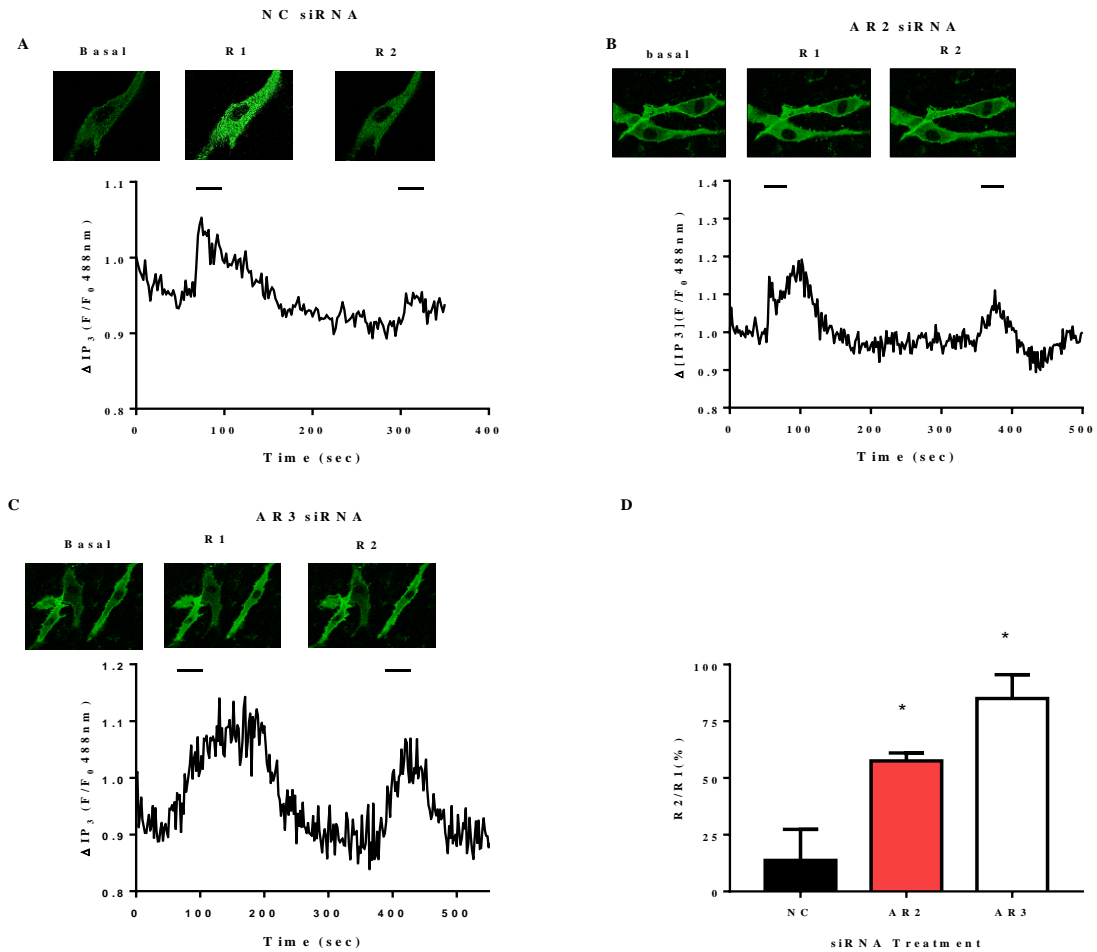
Figure 5-3: Arrestin knockdown prevents P2Y$_1$ receptor desensitization signalling.

DSMC were co-transfected with negative control (NC), anti-arrestin2 and anti-arrestin3 siRNAs and eGFP-PH (0.5 μg) using the nucleofection technique (see Material and Methods). After 48 h, DSMC were imaged using confocal microscopy, and agonist-stimulated changes in IP$_3$ were measured as the change in cytosolic fluorescence. Representative traces show ADP-stimulated (10 μM; R1 and R2; for 30 sec) IP$_3$ responses in NC (**A**), anti-arrestin2 (**B**), or anti-arrestin3 (**C**) DSMC. D) Cumulative data show the effects of arrestin depletion on the desensitization of R2 vs R1 responses n $_=$ 19 cells prepared from 4 different animals are shown after depletion of arrestin2 and arrestin3. Comparisons of NC siRNA-transfected and non-transfected cell responses are shown in. Arrestin2 depletion enhanced ADP-stimulated IP$_3$ signals when compared with negative control (*$P < 0.01$; one-way ANOVA; Dunnett's *post hoc* test).

5.4.4. Effects of isoform-specific arrestin knockdown on UTP-Stimulated P2Y$_2$ receptors signalling

Detrusor smooth muscle cells were transfected with arrestin2 or arrestin3 siRNA to examine the potential role that arrestin proteins play in regulating P2Y$_2$-receptor signalling in DSMC. Again DSMC were nucleofected with anti-arrestin2 and anti-arrestin3 or negative control (NC) siRNA (see Methods section) and subjected to the standard (R1/Rmax/R2) desensitization protocol. DSMC were challenged with approximate EC$_{50}$ concentration of UTP (10 μM) for 30 s before (R1) and after (R2) addition of a maximal UTP concentration (Rmax: 100 μM, for 30 s). The data shown inhibition of arrestin3 expression enhanced UTP-stimulated P2Y$_2$ receptor signalling (Figure 5-4). While suppression of arrestin2 was less effect on P2Y$_2$ receptor signalling (Figure 5-4) compare to the NC siRNA. As a result siRNA-mediated knockdown of arrestin3 attenuated UTP-stimulated P2Y-receptor desensitization, and arrestin2 depletion.
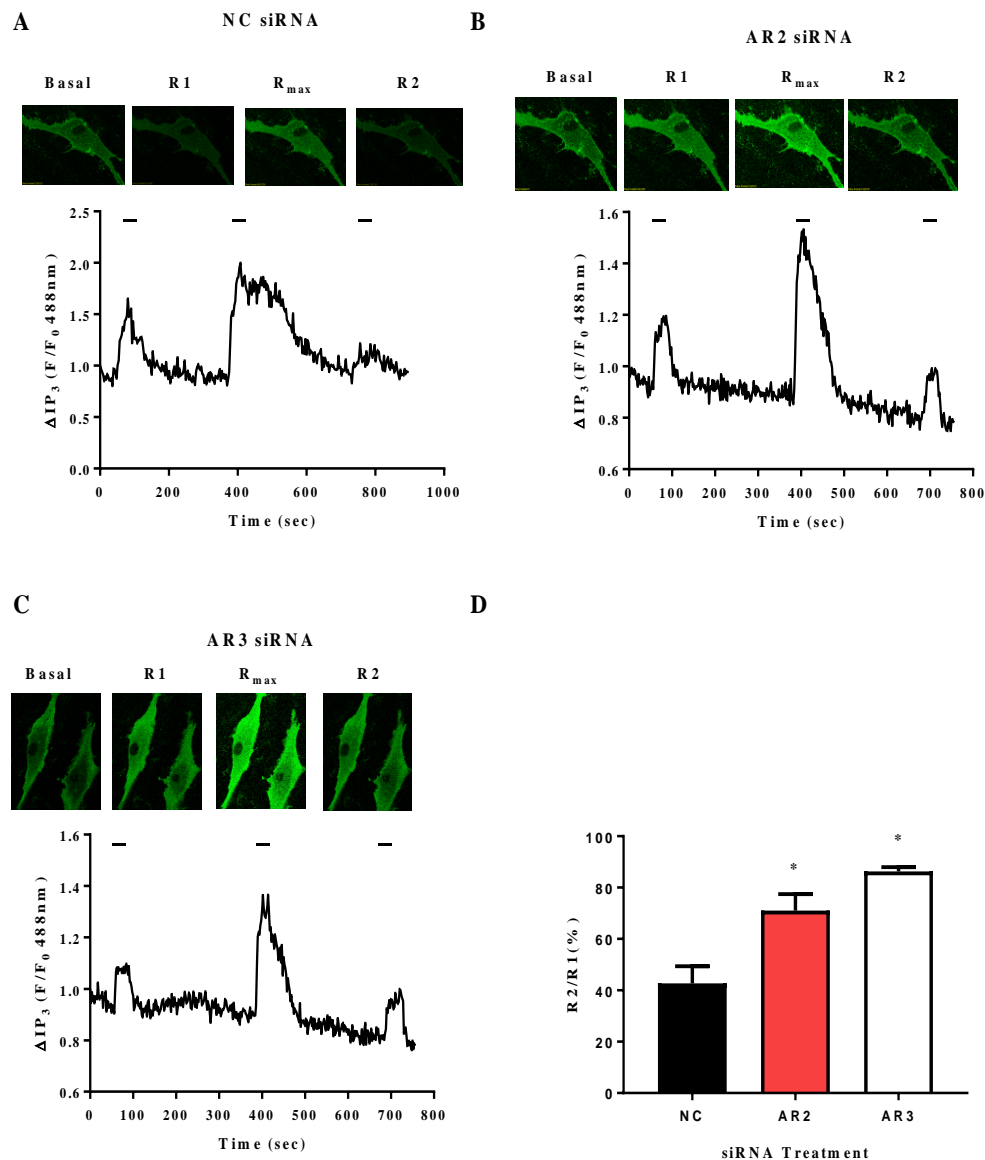
Figure 5-4: Arrestin knockdown prevents desensitization of P2Y$_2$ receptor PLC signalling.

DSMC were co-transfected with 0.5 μg of eGFP-PH and either negative control (NC), anti-arrestin 2 or anti-arrestin siRNAs (10 nM) using the Lonza nucleofection technique (see Methods). After 48 h cells were subjected to the standard R1/R$_{max}$/R2 desensitization protocol. AR3 depletion significantly enhanced UTP-stimulated IP$_3$ signals when compared with negative control, and arrestin 2- transfected cells significantly enhanced UTP-stimulated IP$_3$ signals when compared with negative control (NC) (*$p<0.01$; one-way ANOVA; Dunnett's *post hoc* test).

5.4.5. Differential effects of arrestin proteins depletion on P2Y receptors and stimulated PLC signalling by measuring intracellular calcium [Ca^{2+}]

In the preceding experiments the roles that arrestins play in the regulation of P2Y$_1$ and P2Y$_2$ receptor PLC function was determined by assessing changes in IP$_3$ production in DSMC. However, as IP$_3$ acts as a precursor of intracellular calcium changes, and increased intracellular calcium leads to detrusor muscle contraction and bladder emptying, the effects of arrestin depletion on purinergic receptor-stimulated calcium signals in isolated DSMC were examined. Here I utilised ADP and UTP to probe P2Y$_1$ and P2Y$_2$ receptor desensitization, in DSMC transfected with either negative-control, anti-arrestin2 or anti-arrestin3 siRNAs. As the Lipofectamine2000 transfection process does not transfect all cells (~30%), yet our data suggest that co-transfection occurs in >90% of cells which express two co-transfected fluorophores, we co-transfected DSMC with RFP and siRNA. DSMC expressing RFP were thus deemed to contain siRNA and were thus included in the dataset whilst cells only containing Fluo4 were omitted (Figure 5-5).
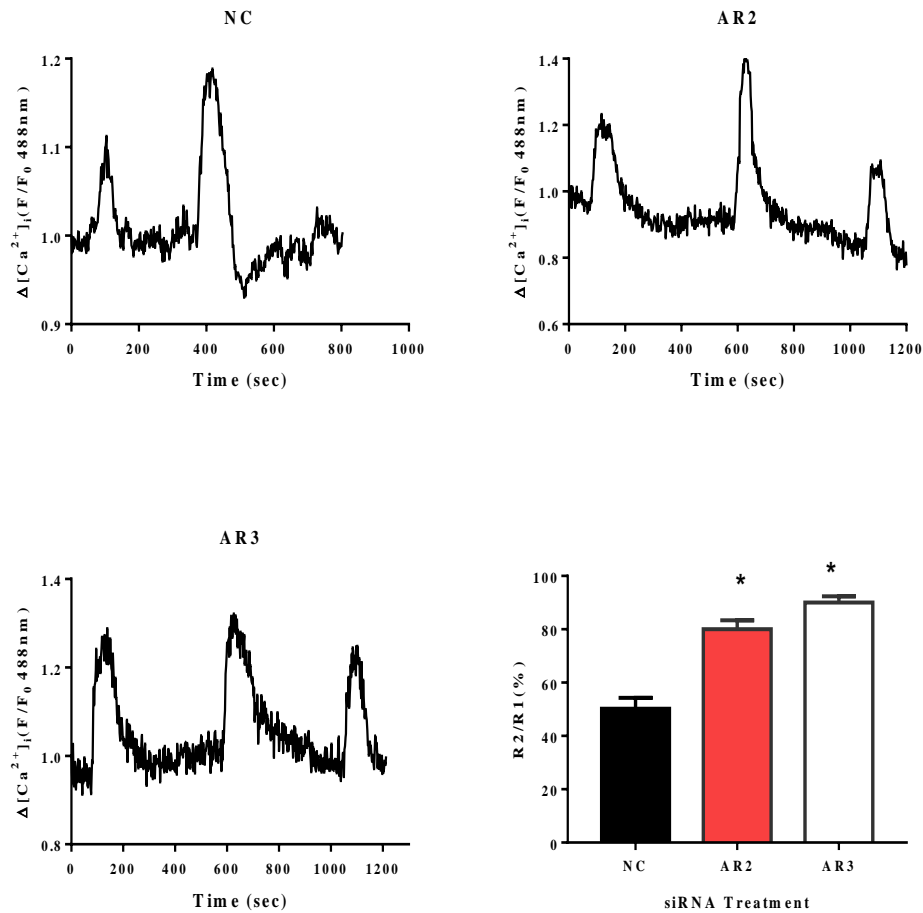
Figure 5-5: Arrestin depletion attenuates the desensitization of P2Y$_2$ receptor stimulated [Ca^{2+}]$_i$ signalling in DSMC.

DSMC were co-transfected with 0.5 µg of eGFP-PH and either negative control (NC), anti-arrestin2 (AR2) or anti-arrestin3 (AR3) siRNAs (10 nM) using the Lonza nucleofection technique. After 48 h cells were subjected to the standard R1/Rmax/R2 desensitization protocol. AR2 or AR3 depletion significantly (*$p < 0.01$; one-way ANOVA; Dunnett's *post hoc* test) attenuated the drop in R2:R1 ratio when compared with negative control transfected cells.

5.5. Discussion

The data presented in this Chapter confirms the presence of both AR2 and AR3 proteins in the detrusor muscle and whole rat urinary bladder. Furthermore, depletion of arrestin proteins after application of selective siRNA showed marked reduction in arrestin2 and arrestin3 expression compared to the (NC) negative-control siRNA in cultured DSMC. We have shown that siRNA-mediated depletions of endogenous arrestin3 in DSMC attenuated P2Y receptor desensitization, while arrestin2 knockdown was less effective on receptor desensitization. Fascinatingly, our data in previous chapter confirmed the inhibition of endogenous GRK2 attenuated $P2Y_2$ receptor desensitization, whilst depletion of GRK3 prevented $P2Y_1$ receptor desensitization. Interestingly, our study demonstrated the first report that $P2Y_1$ and $P2Y_2$ receptor signalling was suppressed after selective reduction of endogenous arrestin3 and arrestin2, an effect similar to that observed before for GPCRs in other cell backgrounds (79,102,179). These facts suggested that arrestins and GRKs-mediated regulation of receptor function that may play an important role in the maintenance of continence.

The interesting results from the previous studies where investigation of the expression of arrestin proteins in mesenteric smooth muscle cells and results from arrestins knockout mice have highlighted the roles for arrestins in the regulation of vascular smooth muscle contraction (108). In agreement with the data presented in this study arrestin2 also regulates $P2Y_2$ receptor PLC signalling in rat VSMC (37,109), suggesting a common role for this protein in the regulation of $P2Y_2$ receptor contractile signalling in smooth muscle cells. However, although arrestin regulation of $P2Y_2$/PLC activity in VSMC is exclusive (109), in DSMC arrestin3 appears equally adept at inducing $P2Y_2$ receptor desensitization. Prior evidence has shown that GRKs can inhibit the interactions between GPCRs and G proteins in a phosphorylation independent way, GRK2 binds to activated GTP bound form of $G\alpha_{q/11}$ but not to $G\alpha_S$, $G\alpha_i$ or $G\alpha_{12/13}$ to inhibit PLC-β activation (38). GPCR activation drives the intiation of multiple intracellular signalling routes, including second messenger pathways controlled by adenylyl cyclases, phospholipases and ion channels. Stimulation of GPCRs triggers their phosphorylation by GRKs (G protein-coupled receptor kinases). Arrestins bind to the phosphorylated receptors and block any interactions with G proteins (38), which leads to the loss of receptor responsiveness, termed desensitization (94) (Figure 5-6).

GRK/arrestin-mediated desensitization of GPCRs prevents prolonged or inappropriate signalling (50,191). Previous evidence suggests that the human bladder expresses GRK2, GRK3 and GRK4. GRK2 expression decreases with bladder obstruction, thus may suggesting that the failure of GPCR desensitization might be one of the mechanisms by which an overactive bladder is induced (89).
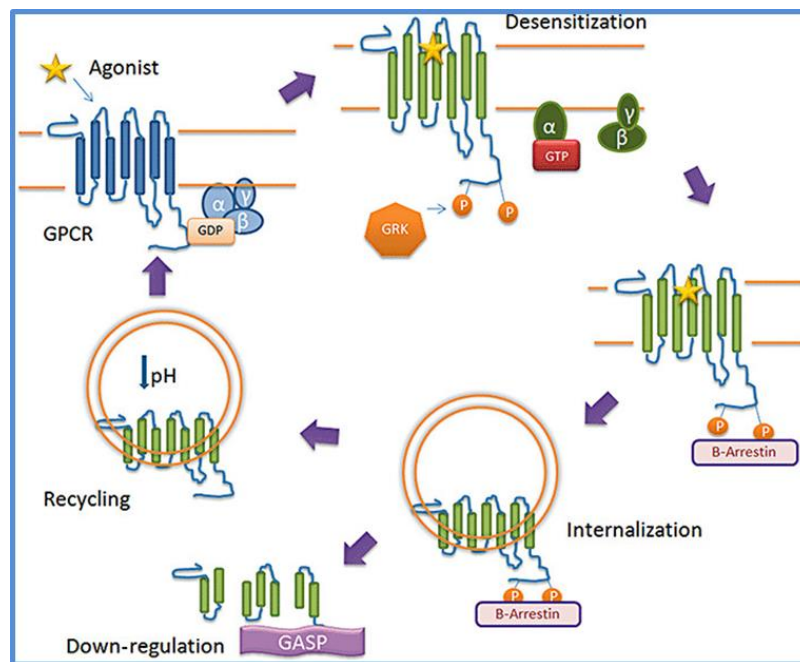
Figure 5-6: G-protein-coupled receptor (GPCR) desensitization, internalization and down-regulation mechanisms (Figure adapted from Smith et al 2010 (192).

After activation of the GPCR, GPCR kinase (GRK) phosphorylates the receptor, on C-terminal Ser/Thr residues. As soon as phosphorylated, β-arrestin coupled to the GPCR, desensitizing the receptor and causing the receptor to internalize, and then GPCRs may be reused back to the cell surface subsequent de-phosphorylation. Otherwise, GPCRs can be transferred to lysosomes and degraded (down-regulation), this process is facilitated by GPCR-associated sorting protein (GASP).

5.6. Conclusion

Our data confirmed the presence of both AR2 and AR3 proteins in rat detrusor muscle cells and whole bladders lysates. In addition, selective siRNA mediated depletion of individual arrestin expression showed that both arrestins were equally able to negatively regulate $P2Y_1$ and $P2Y_2$ PLC signalling in DSMC. These data suggest despite that fact that $P2Y_1$ receptors are substrates for GRK3 and $P2Y_2$ receptors being substrates for GRK2 the resultant phosphorylation patterns do not promote the selective recruitment of arrestin2 or arrestin3.

Chapter 6

6. **Characterising the effects of mechanical stretch on GRK and arrestin protein expression in isolated DSMC.**

6.1. Introduction

The data from previous chapters confirmed that nucleotide signalling (such as ADP and UTP) in isolated DSMC is mediated through metabotropic P2Y receptors. In brief, DSMC contractile responses were stimulated through $P2Y_1$ and $P2Y_2$ receptor which causes a rapid influx of Ca^{2+} to initiate smooth muscle contraction. Therefore, it is likely that $P2Y_1$ and $P2Y_2$ receptors contribute to the control of detrusor smooth muscle contraction and thus bladder emptying. Ideally, it would have been interesting to examine the roles that $P2Y_1$ and $P2Y_2$ receptors play in the contraction of whole bladder. Unfortunately, due to a lack of appropriate kit (i.e. organ baths) and technical knowledge, investigation of this process was not possible. However, during filling and emptying of the bladder, DSMC are exposed to mechanical stress, particularly during bladder filling. Indeed, previous studies (193) have applied mechanical stress to isolated DSMC in an attempt to replicate the strain placed on DSMC during the cyclic changes of bladder filling and emptying. In addition, application of mechanical strain can also replicate some of the changes associated with bladder pathophysiology such as over-active or obstructive bladder (129,193,194).

Obstructed bladder is characterised by extreme mechanical stress (mechanical overload), which is believed to be a pathogenic cause, inducing both structural and functional changes in detrusor smooth muscle (193,195). In addition, detrusor muscle over-activity and bladder hypertrophy are known responses to bladder outlet obstruction (195) and mechanical stretch stress is considered to initiate and induce these responses in the urodynamically overloaded bladder (196). Indeed, previous study using mechanical stress show that repetitive stretch of bladder smooth muscle cells results in the increased expression of a variety of growth factors and other specific proteins, which likely underlie the detrusor muscle hypertrophy and over-activity (196).

Mechanical cells stretch stress

GRKs

B-arrestin
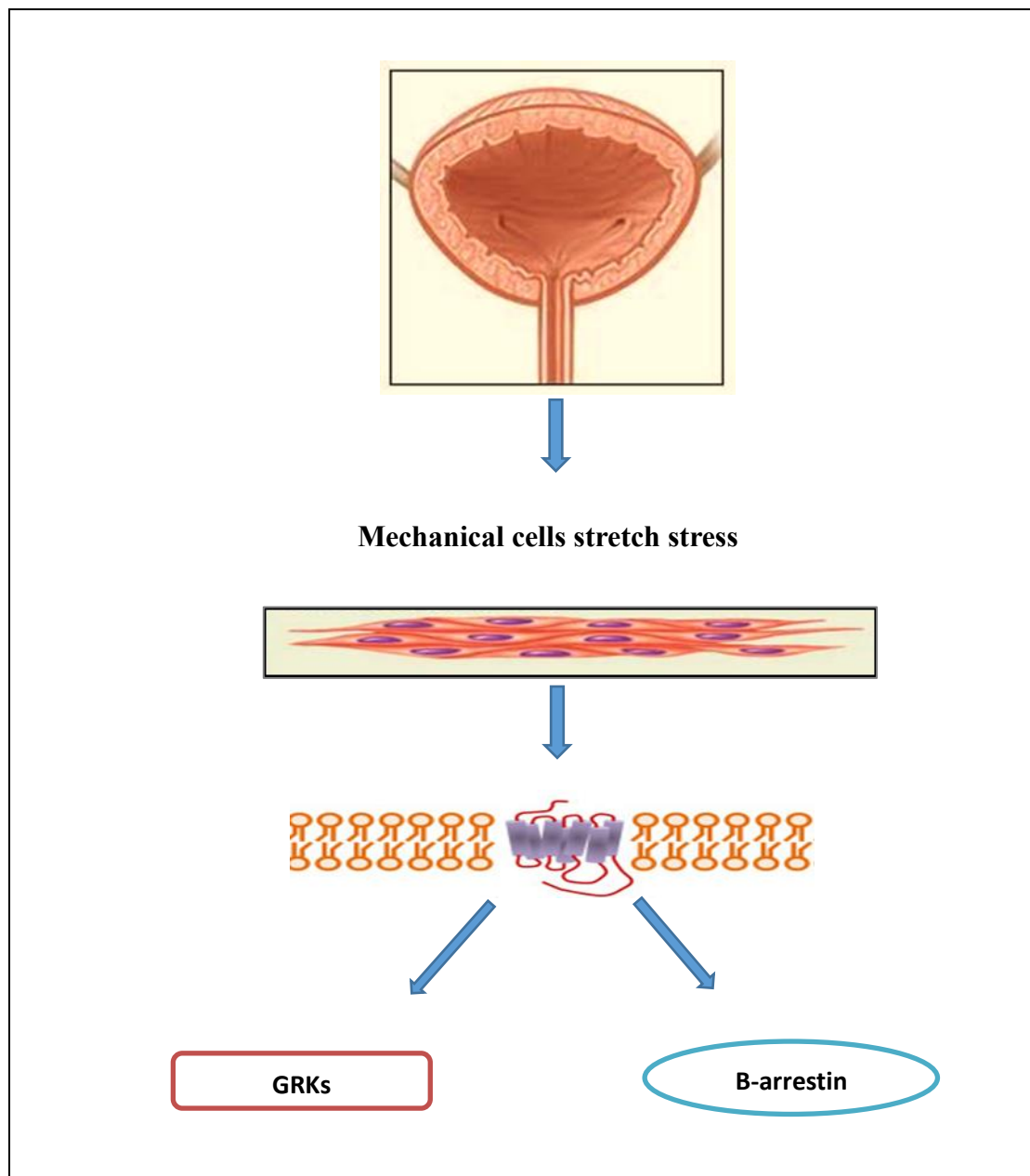
Figure 6-1: Flow diagram showing the protocol for isolation and mechanical stretching of DSMC.

Although current knowledge of the pathophysiology of the obstructive bladder is limited, it has been proposed that localized contractions result in the abnormal stretching of bladder smooth muscle (17,133). Previous studies reported that outlet obstruction dramatically induced obstruction bladder stimulates protein expression in bladder smooth muscle cells via increased mechanical stretch (133). We assume stretching cells induced different type of protein in DSMC such as G-protein coupled receptor kinase and arrestin protein.

Since in the obstructive bladder detrusor smooth muscle is continually stretched, this will persistently stimulate the nerve ending that sense the volume/size of the bladder. Thus neuronal feedback circuits prompt the brain to initiate the voiding process. Micturition is initiated through increasing activity of the parasympathetic nervous system, resulting in increased acetylcholine release, which in turn activates M_3 muscarinic receptors to increase intracellular calcium and induce bladder contraction. As ATP is usually packaged with acetylcholine in parasympathetic nerve terminals, both neurotransmitters are co-released, implying that purinergic receptors are also likely to activated during this time and thus likely play a role in bladder emptying. However during obstructive bladder, voiding is incomplete which infers that the micturition reflex is continually active, being accompanied by continuous release of muscarinic/purinergic agonists. Thus it is likely that during bladder obstruction, purinergic receptors are activated for prolonged periods. Since prolonged agonist signalling through GPCRs leads to receptor GRK/arrestin mediated desensitization, it is possible that GRK and arrestin expression like many other proteins (85,197,198) may change as a consequence either of the mechanical stretch, or in response to the increased GPCR activity seen during obstructive bladder. In support of this theory, GRK2 and GRK3 expression was significantly reduced in patients with obstructive bladder (89), which implies similar changes in GRK and arrestin expression may occur when rat DSMC are mechanically stretched to mimic obstructive bladder. Therefore, in an attempt to simulate the effects of bladder obstruction on isolated DSMC, cells were subjected to mechanical stretch (Figure 6-1) and the subsequent effects on GRK and arrestin expression examined by Western blotting as described below.

6.2. Aim

Extreme mechanical overload cells (mechanical stress) are assumed to be the cause producing both structural and functional alterations (129). The aim of this chapter was to investigate whether application of mechanical stretch to isolated DSMC affected the expression of GRK and arrestin proteins.

6.3. Methods

6.3.1. Bladder smooth muscle Preparation and culture

DSMC were isolated from rat bladder by enzymatic dissociation (2 mg of papain for 15 minutes at 37 C° following by 2 mg collagen type IA for 20 minutes at 37°C), then washed the bladder pieces with low $CaCl_2$ buffer containing (18 mg of bovine serum albumin (sigma) and 100 μM $CaCl_2$ in 20 mL of sterile dissection buffer). After washing the bladder pieces was vigorous mixed using a wide bore plastic Pasteur pipette (VWR International, Lutterworth, UK) to dissociate single detrusor smooth muscle cell from bladder tissue. Next, the mixture was strained through a sieve sterile cell strainer 40 μm nylon mesh (Fisher brand) into a fresh 50 mL Falcon tube to separate isolated cells from the remaining lumps of tissue, then centrifuged at 4°C for 5 min at 800 rpm. The pellet was re-suspended in medium 199 (Life Technologies, UK), supplemented with 20% foetal calf serum (FCS) and 100 IU/mL penicillin, 100 μg/mL amphotericin B (antifungal) and 100 μg/mL streptomycin were maintained at 37ºC and under 5% CO_2 in humidified conditions see section 2.1 for cell isolation. DSMC were plated and grown on a type I collagen-coated silicon membrane (Bioflex, from Dunn Labortechnik) for 3 days.

6.3.2. Mechanical cell stretching

Three days after DSMCs isolation the mechanical deformation of the smooth muscle cells was performed using a Flexercell Strain Unit FX-4000 (Flexcell McKeesport, PA). This machine is modified firstly from Banes et al., (2) and under computer control a vacuum (<15 to 20 kPa) was continuously applied to induce stretch silicone collagen culture plates. Prior to stretching DSMC cells were incubated for 24 h in serum free media maintained in a humidified incubator with 5% CO_2, 95% air atmosphere at 37°C. Biaxial strain was applied for 24 h at 10% or 30% stretch.

6.3.3. Protein assay and Western blotting analysis

After 24 h, 200 µL of the following lysis buffer (20 mM HEPES, pH 7.4, 200 mM NaCl, 10 mM EDTA, 1% Triton-X 100, 0.2 mg/mL benzamidine, 0.1 mg/mL leupeptin and 0.5 mM phenylmethylsulphonylfluoride) was added to control and stretched cells before they were scraped off the collagen membranes. Next cell lysates were cleared of insoluble material by centrifugation (14,000 rpm, 5 min, 4°C) and processed for a Bradford protein assay (1976). Cell lysates (40 µg) were then separated by 10% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. Western blot analysis was performed to evaluate the expression of GRK and arrestin proteins in DSMC, using appropriate primary antibodies (see Material and Methods for full details). Stretched cells and controls were processed at the same time and analysed identically. Immunoblot band intensities were quantified using the Syngene GeneGnome system with Gene Tools software.

6.4. Results

6.4.1. Application of mechanical stretch alters GRK protein expression in DSMC

Following 24 h of mechanical stretch the expression of GRKs 2, 3, 5 and 6 were assessed in DSMC cell lysates. Comparison of control (non-stretched) and stretched DSMC showed that GRK2 expression was significantly increased after application of 30% stretch (Figure 6-2). Although a similar trend was seen following application of 10% stretch the change was not significant (Figure 6-2). In contrast, GRK3 expression was reduced after application of 30%, yet unaffected by application of 10% stretch (Figure 6-3). Similarly, 10% stretch had no effect upon GRK5 expression, but 30% stretch resulted in a marked reduction in GRK5 expression, when compared to non-stretched DSMC (Figure 6-4). Finally, GRK6 expression was also reduced following cell stretching, however, this change was only significant in the 30% stretch treatment group (Figure 6-5).
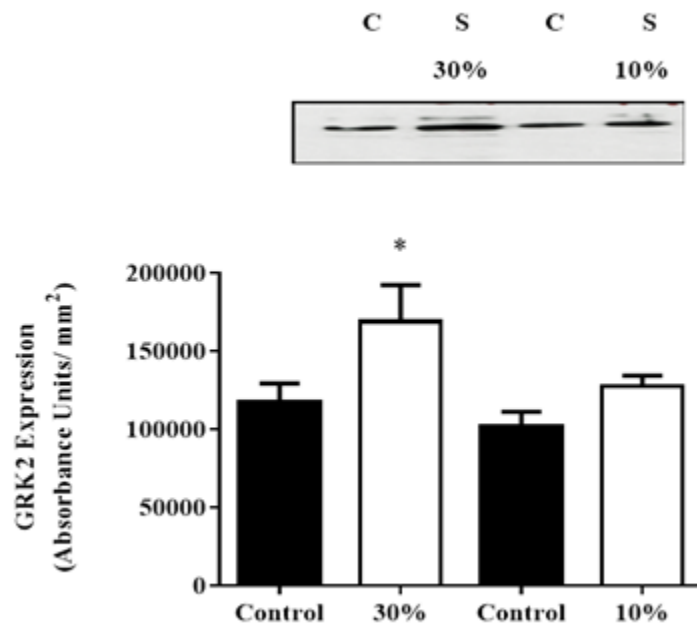
Figure 6-2: Effects of mechanical stretching on expression of GRK2 in DSMC.

DSMC were plated and grown on a type I collagen-coated silicon membrane and mechanically stretched for 24 h at 10% or 30%. Western blotting analysis was performed to determine relative GRK2 expression and the immunoblot band intensities were quantified using the Syngene GeneGnome system with Gene Tools software. Representative immunoblot (A) and cumulative data (B) (means ± SEM) indicate that GRK2 expression increases in DSMC after application of 30% stretch (*$p<0.05$ one-way ANOVA; Sidak's *post hoc* test; 30% stretch vs control, n $_=$15 cells from 5 separate bladder preparations).
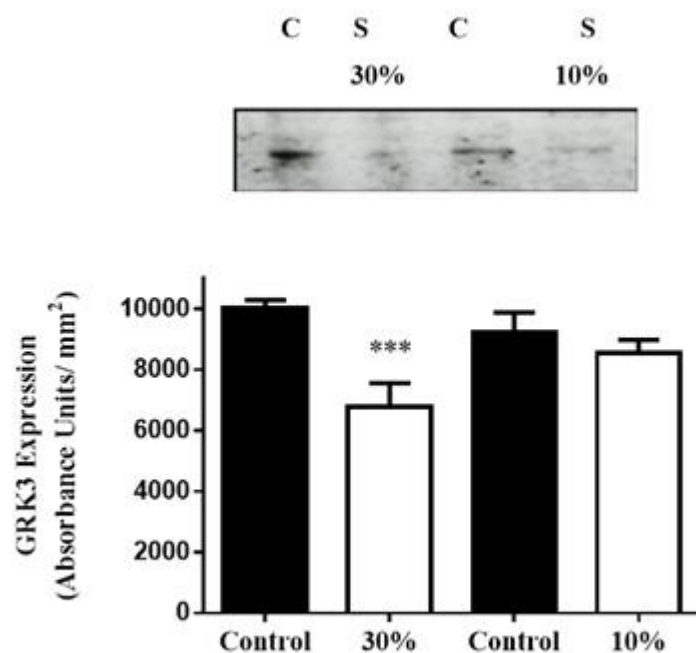
Figure 6-3: Effects of mechanical stretching on expression of GRK3 in DSMC.

DSMC were plated and grown on a type I collagen-coated silicon membrane and mechanically stretched for 24 h at 10% or 30%. Western blotting analysis was performed to determine relative GRK3 expression and the immunoblot band intensities were quantified using the Syngene GeneGnome system with Gene Tools software. Representative immunoblotting (A) and cumulative data (B) (means ± SEM) indicate that GRK3 expression decreases in DSMC after application of 30% stretch (***$p < 0.001$ one-way ANOVA; Sidak's *post hoc* test; 30% stretch vs control, n=19 cells from three different bladder preparations).
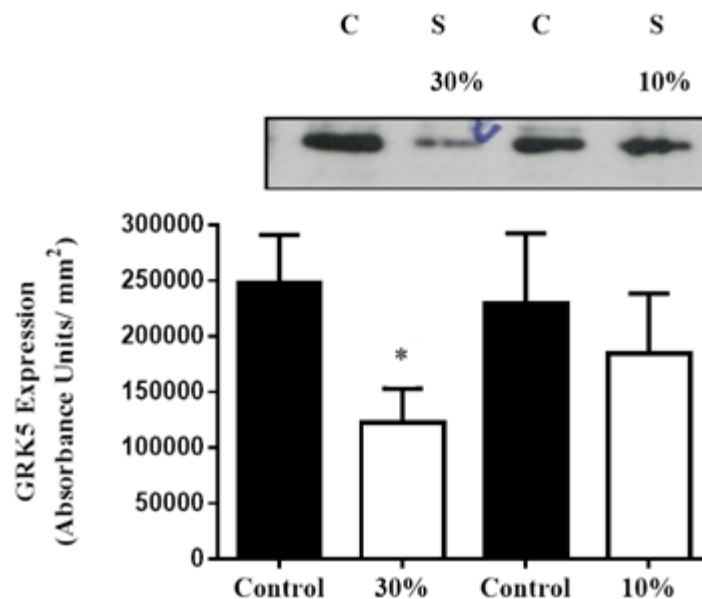
Figure 6-4: Effects of mechanical stretching on expression of GRK5 in DSMC.

DSMC were plated and grown on type I collagen-coated silicon membranes and mechanically stretched for 24 h at 10% or 30%. Western blotting analysis was performed to determine relative GRK5 expression and the immunoblot band intensities were quantified using the Syngene GeneGnome system with Gene Tools software. Representative immunoblot (A) and cumulative data (B) (means ± SEM) indicate that GRK5 expression decreases in DSMC after application of 30% stretch (*p<0.05 one-way ANOVA; Sidak's *post hoc* test; 30% stretch vs control, n = 20 cells from 3 different animals).
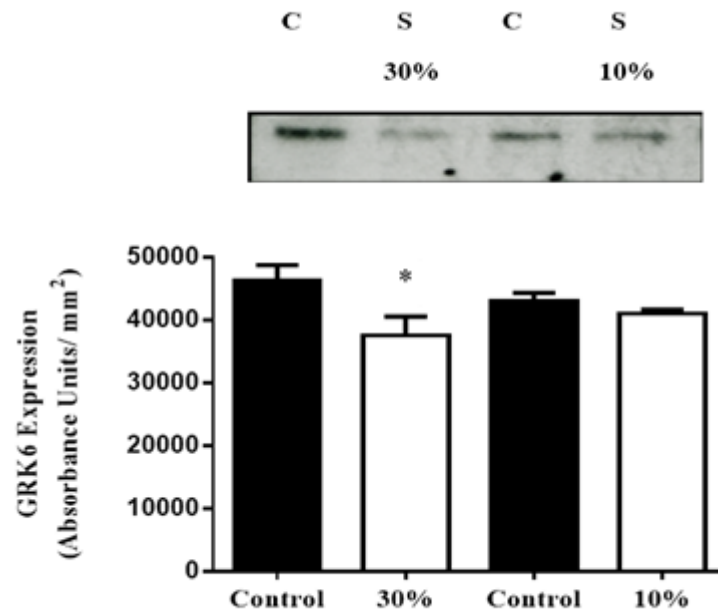
Figure 6-5: Effects of mechanical stretching on expression of GRK6 in DSMC.

Cells were plated and grown on a type I collagen-coated silicon membrane and mechanically stretched for 24 h at 10% or 30%. Western blotting analysis was performed to determine relative GRK6 expression, and the immunoblot band intensities were quantified using the Syngene GeneGnome system with Gene Tools software. Representative immunoblotting (A) and cumulative data (B) (means ± SEM) indicate that GRK6 expression decreases in DSMC after application of 30% stretch (*$p < 0.05$ one-way ANOVA; Sidak's *post hoc* test; 30% stretch vs control, n=16 cells from 3 different preparations).

6.4.2. Effects of mechanical stretch on arrestin protein expression in DSMC

After identifying the relevant changes to GRK expression in DSMC following the application of stretch for 24 h, the effects of cell strain on arrestin expression were determined by Western blotting. When compared to control non-stretched cells, arrestin2 expression appeared to decrease after the application of either 10% or 30% stretch; however the change were not significant (Figure 6-6 A). Again assessment of arrestin3 expression showed that arrestin3 protein levels were reduced in stretched cells, when compared to non-stretch controls, although the changes were again no quite significant (Figure 6-6 B).
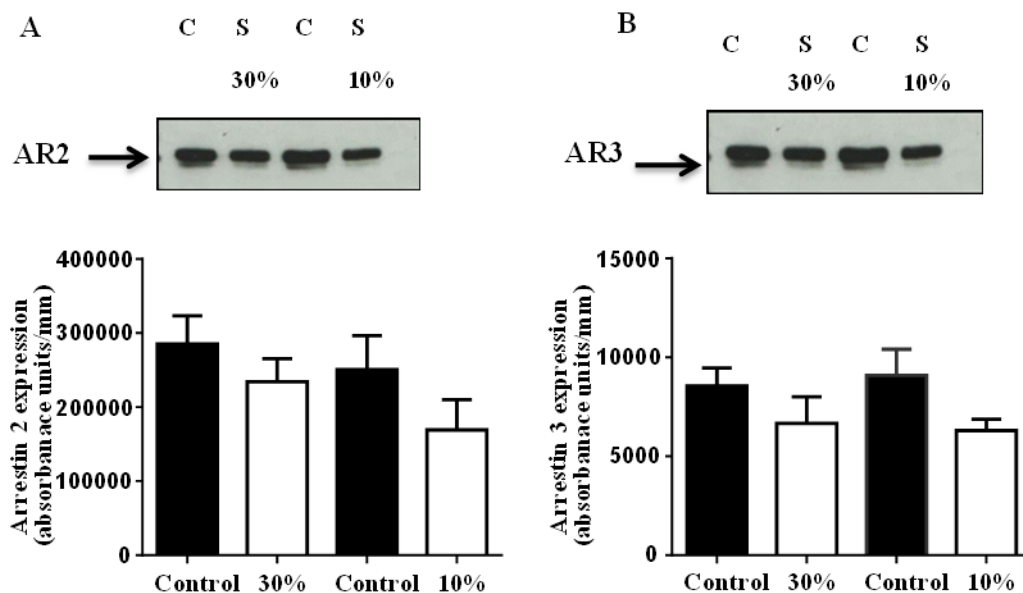
Figure 6-6: Effects of mechanical stretching on expression of arrestin2 and arrestin3 in DSMC.

DSMC were plated and grown on a type I collagen-coated silicon membrane and mechanically stretched for 24 h at 10% or 30%. Western blotting analysis was performed to determine relative arrestin protein expression, and the immunoblot band intensities were quantified using the Syngene GeneGnome system with Gene Tools software. Representative blotting (**A**) and cumulative data (**B**) (means $\pm$ SEM), n = 21 cells from 5 separate bladder preparations.

6.5. Discussion

Since in the obstructive bladder detrusor smooth muscle is continually stretched, this will persistently stimulate the nerve ending that sense the volume/size of the bladder. Thus neuronal feedback prompts the brain to initiate the voiding process (194), with micturition being initiated through increasing activity of the parasympathetic nervous system, resulting in increased acetylcholine release, which in turn activates M_3 muscarinic receptors to increase intracellular calcium and induce bladder contraction. As ATP is packaged with acetylcholine in parasympathetic nerve terminal vesicles, both neurotransmitters are co-released, implying that purinergic receptors are also likely to activated during this time and thus protentially play a role in bladder emptying. However during obstructive bladder, voiding is incomplete which infers that the micturition reflex is continually active, being accompanied by continuous release of muscarinic/purinergic agonists. Thus it is likely that during bladder obstruction, purinergic receptors are activated for prolonged periods. Since prolonged agonist signalling through GPCRs leads to receptor GRK/arrestin mediated desensitization (109,199), it is possible that GRK and arrestin expression like many other proteins (85,197,198) may change as a consequence either of the mechanical stretch, or in response to the increased GPCR activity seen during obstructive bladder. These findings may suggest that reduced GRK express would lead to enhanced receptor activity and possible increased bladder contractile effects, which could be beneficial in prolonging attempted bladder contraction. Therefore, the effects continual stretch on GRK and arrestin expression in DSMC was examined.

In this chapter our data confirm that physical challenge to DSMC increased GRK2 expression after application of 30% stretch for 24 h, but not after 10% stretch. Therefore, as we have shown that GRK2 regulates $P2Y_2$ receptor activity it is likely that obstructive bladder leads to increased desensitization of $P2Y_2$ receptor activity and thus reduced contractile force through $P2Y_2$/PLC signalling. Contrastingly, GRK3 expression was reduced, which suggests that during bladder obstruction $P2Y_1$ receptor activity would remain active for longer potentially promoting bladder emptying. Furthermore, continual stretch also decreased GRK6 expression, which considering previous reports that GRK6 can regulate M_3 receptor PLC signalling (185) may reflect a physiological to enhance DSMC contraction to acetylcholine during periods of bladder obstruction (185). In constrat to our findings, GRK2 and GRK3 expression was

significantly reduced in patients with obstructive bladder (89), which may reflect a chronic rather than acute effect of bladder stretch on DSMC phenotype.

6.6. Conclusion

Application of mechanical stretch to rat DSMC clearly has differential effects on GRK expression, enhancing GRK2 expression yet suppressing the expression of GRK3, GRK5 and GRK6. Interestingly, in pathophysiological conditions such as overactive or obstructive bladder disease, 60% of patient bladder samples show a significant atropine-resistant component to nerve induced contraction, which is attributed to co-release of ATP. This finding suggests that purinergic receptors are likely to play a central role in attempting to induce contraction and bladder voiding. Indeed, it is likely that mechanical stretch observed in obstructive bladder will lead to decreased GRK3 and increased GRK2 expression, which may result in enhanced $P2Y_1$ and reduced $P2Y_2$ receptor PLC activity. At present it is not obvious whether the likely differential changes in $P2Y_{1/2}$ receptor activity would help to promote bladder contraction and the overall outcome would depend on which P2Y receptor plays a more dominant role in inducing DSMC contraction.

Chapter 7

7. General discussion and future work

7.1. General discussion

Our data confirm that after 3 days in culture isolated rat DSMC express functional $P2Y_1$ and $P2Y_2$ receptors, but not P2X, $P2Y_4$ or $P2Y_6$ receptors. The profiles of ATP-stimulated $[Ca^{2+}]_i$ changes in the presence or absence of TNP were similar, these data indicate that DMSC may not express P2X receptors after two days in culture. Consequently, loss or decrease of P2X receptors in rat DSMC might be because culture procedures do not maintain the expression of P2X receptors.

In this study extra cellular nucleotides and purinergic agonists acting via P2Y receptor cause $G\alpha_q$-mediated activation of phospholipase C (PLC) and the production of inositol 1,4,5-trisphosphate (IP_3), leading to intracellular calcium release (79,88,202), which in smooth muscle leads to contraction. Consequently, it is likely that $P2Y_1$ and $P2Y_2$ receptors contribute to the control of detrusor smooth muscle contraction and thus bladder emptying. Ideally, it would have been interesting to examine the roles that $P2Y_1$ and $P2Y_2$ receptors play in the contraction of whole bladder, but that was beyond the scope of this thesis. Since the co-ordinated activity of receptor function is vital in regulating the contractile state of the bladder, regulation of receptor function may play an important role in the maintenance of continence. As little is known about how P2Y receptor activity is regulated in DSMC we will identify the mechanisms mediating this process, which should highlight processes that might be dis-regulated during incontinence. Consequently we know that the GPCR phosphorylation by GRKs increases receptor affinity for arrestin2 and or arrestin3, which can physically bind to GPCRs and suppress interaction between receptor and G proteins to inhibit signalling (94,108,109), indeed in the present study our data confirmed the response recovered gradually with increasing washout time suggesting an initial desensitization of P2Y receptor activity followed by re-sensitization of receptor responsiveness in DSMCs. GPCR desensitization is a fundamental process which occurs to the vast majority of receptors to prevent excessive or inappropriate signalling (94,171), and also plays a role in the regulation of signal duration, intensity and quality. Indeed, over activity of GPCR

signalling is likely to contribute to the unwanted dysregulation of bladder contractions observed in overactive bladder. Therefore, since uncontrolled overactive GPCR signalling to initiate inappropriate contractions may underlie the pathophysiology of overactive bladder, it is important to investigate the regulation of purinergic receptor contractile signalling in DSMC, accordingly GPCR desensitization is usually mediated by the action of GRK proteins, since that our data established the expression of GRKs, western blots data showing the expression of GRK2, 3, 5 and 6 in DSMCs and whole rat bladder. in present study analysis of $P2Y_2$ receptor desensitization in DSMC co-transfected with RFP and pcDNA3 (control), ^{D110A,K220R}GRK3, ^{K215R}GRK5, or ^{K215R}GRK6 showed that the changes in R2 responses compared with R1 were comparable to those observed in non-transfected cells show the presence of ^{D110A,K220R}GRK2 significantly attenuated reductions in the R2 response when compared with R1 suggesting that endogenous GRK2 activity plays a significant role in regulating UTP-stimulated $P2Y_2$-receptor-mediated PLC/Ca^{2+} signalling. These findings strongly suggest that GRK2 is a key mediator of UTP-induced $P2Y_2$ receptor desensitization,

These data agreeing with previous data confirmed the endogenous GRK2 depletion significantly attenuated $P2Y_2$ receptor desensitization (109). While the GRK3 activity plays a significant role in regulating ADP-stimulated $P2Y_1$-receptor-mediated PLC/Ca^{2+} signalling. These findings strongly suggest that GRK3 is a key mediator of ADP-induced $P2Y_1$ receptor desensitization. Following, to extend the data obtained in previous experiment using the catalytically inactive dominant-negative mutant (DNM) GRKs; we assessed the GRK knockdown using small interfering RNAs (siRNAs) on DSMC of rat bladder. DSMC were transfected with siRNAs designed to target GRK2, the result of the transfection with anti-GRK2 siRNA caused a marked reduction in GRK2 compare to the (NC) negative-control siRNA in cultured DSMC.

Since our data established that the GRK2 is a key mediator of UTP-induced $P2Y_2$ receptor desensitization and because of inefficiency of transfection techniques to deliver plasmids, or RNAi into primary cells, often results in low protein expression or reduced suppression of endogenous protein expression, and thus false negative data. Therefore, the discovery of cell permeable small molecule GRK inhibitors would enable examination of GRK/GPCR interactions in all cell types. Indeed, recent work, especially by the (Tesmer group) has identified a number of different potential GRK

inhibitors (180), including the selective serotonin reuptake inhibitor (SSRI) paroxetine and fluoxetine. This compound (SSRI) prompted us to examine whether this drug could enable us to explain a role for GRK2 in P2Y receptor desensitization in isolated DSMC. Since that the same experiment in our laboratory in ULTR these data suggests that paroxetine inhibits the desensitization PLC signalling of GPCRs that are regulated by GRK2, but not GRK6, in the present study our result suggesting that the paroxetine was able to prevent the desensitization of $P2Y_2$ receptor PLC activity in DSMC. These data highlighted the significant role to development next generation small molecule compounds with advanced effectiveness and efficiency concerning to GRK2 inhibition based on the mechanical structure properties of paroxetine.

In chapter five our data confirmed that the presence of both AR2 and AR3 proteins in the detrusor muscle cells and rat urinary bladders. In addition, the present data showed the effects of inhibiting endogenous arrestin2 and 3 in DSMC by using a molecular approach known as depletion siRNA, these data confirmed that were a marked reduction in arrestin2 and arrestin3 compare to the (NC) negative-control siRNA in cultured DSMC.

Selective depletion of arrestin3 markedly reversed ADP-induced decreases in the second responses which inhibit $P2Y_1$ receptor desensitization, as a result of that arrestin3 depletion enhanced ADP- stimulated $P2Y_1$ but not arrestin2, this did not achieve statistical significance, suggested that attenuation of the ADP signal is primarily effected by the arrestin3 isoform. Additionally, data shown inhibition of arrestin3 expression enhanced UTP-stimulated $P2Y_2$ receptor signalling.

Finally, following 24 h of mechanical stretch the expression of GRKs 2, 3, 5 and 6 were assessed in DSMC, our data in (non-stretched) and stretched DSMC showed that GRK2 expression was significantly increased after application of 30% stretch. While a similar trend was seen following application of 10% stretch the change was not significant. In contrast, GRK3 expression was reduced after application of 30%, yet unaffected by application of 10% stretch. Similarly, 10% stretch had no effect upon GRK5 expression, but 30% stretch resulted in a marked reduction in GRK5 expression, when compared to non-stretched DSMC. Finally, GRK6 expression was also reduced following cell stretching, however, this change was only significant in the 30% stretch treatment group. In our knowledge no study to date assessed the expression of GRKs and arrestin

proteins after exposure cells to mechanical stretch stress in bladder detrusor smooth muscle, In this study mechanical stretch stress cells are believed to change the expression of GRKs and arrestin proteins expression in DSMCs after exposure cells to mechanical stress for 30% and 10% strain for 24 hours. Other study shows that outlet obstruction significantly induced COX-2 expression in the bladder (200).

7.2. Future work

The basic science research of urinary bladder contractility continues to be an interesting subject for research in an attempt to improve the understanding the pathophysiology that underlies detrusor overactivity.

Firstly, the use of GRK2 inhibitors to investigate the urinary bladder contractility function using isolated bladder strip is a potential future topic requires further investigation. Nevertheless, designing different mechanical stretching protocols to stimulate bladder pathophysiological by stretching the DMSC cells in cyclic protocol to discover if this process induce the involuntary contraction of overactive bladder and subsequently investigate any changes on G protein coupled receptor kinases and arrestin proteins expression. Finally, the application of all these protocols, and investigating its effect on the G protein coupled receptor kinases and arrestin proteins, $P2Y_1$ and $P2Y_2$ expression and further research needs to be done to confirm these interesting findings in the relevant tissues or cells such as smooth muscles from patients with overactive bladder symptoms.

References:

(1) Haylen BT, de Ridder D, Freeman RM, Swift SE, Berghmans B, Lee J, et al. An International Urogynecological Association (IUGA)/International Continence Society (ICS) joint report on the terminology for female pelvic floor dysfunction. International Urogynecology Journal 2010 Jan;21 (1):5-26.

(2) Gormley EA, Lightner DJ, Burgio KL, Chai TC, Clemens JQ, Culkin DJ, et al. Diagnosis and treatment of overactive bladder (non-neurogenic) in adults: AUA/SUFU guideline. The Journal of Urology 2012 Dec;188 (6 Supplement):2455-2463.

(3) Abrams P, Kelleher CJ, Kerr LA, Rogers RG. Overactive bladder significantly affects quality of life. The American Journal of Managing Care 2000 Jul;6 (11 Supplement):S580-90.

(4) Wagner TH, Hu TW, Bentkover J, LeBlanc K, Stewart W, Corey R, et al. Health-related consequences of overactive bladder. The American Journal of Managing Care 2002 Dec;8 (19 Supplement):S598-607.

(5) Rovner ES, Wein AJ. Incidence and prevalence of overactive bladder. Current Urology Reports. 2002 Dec;3 (6):434-438.

(6) Milsom I, Abrams P, Cardozo L, Roberts RG, Thüroff J, Wein AJ. How widespread are the symptoms of an overactive bladder and how are they managed? A population-based prevalence study. BJU International 2001;87 (9):760.

(7) Irwin DE, Milsom I, Hunskaar S, Reilly K, Kopp Z, Herschorn S, et al. Population-based survey of urinary incontinence, overactive bladder, and other lower urinary tract symptoms in five countries: results of the EPIC study. European Urology 2006 Dec;50 (6):1306-14; discussion 1314-5.

(8) Stewart WF, Van Rooyen JB, Cundiff GW, Abrams P, Herzog AR, Corey R, et al. Prevalence and burden of overactive bladder in the United States. World Journal of Urology 2003 May;20 (6):327-336.

(9) Onukwugha E, Zuckerman IH, McNally D, Coyne KS, Vats V, Mullins CD. The total economic burden of overactive bladder in the United States: a disease-specific approach. American Journal of Managed Care 2009 Mar;15 (4 Suppl):S90-7.

(10) Andersson KE, Arner A. Urinary bladder contraction and relaxation: physiology and pathophysiology. Physiological Reviews 2004 Jul;84 (3):935-986.

(11) Ovalle WK, Nahirney PC, Netter FH. Netter's essential histology. Second ed. Philadelphia, PA: Elsevier/Saunders; 2013.

(12) Mokry J, Svihra J, Nosalova G, Kliment J. Mechanisms of reactivity of urinary bladder smooth muscle. Bratisl Lek Listy 2002;103 (9):279-282.

(13) Chai TC, Birder LA. Physiology and Pharmacology of the Bladder and Urethra. In: McDougal WS, Wein AJ, Kavoussi LR, Partin AW, Peters CA, editors. Campbell-Walsh Urology. 11th ed.: Elsevier Health Sciences; 2016. p. 1631.

(14) Alleemudder A, Ballaro A. The Physiology and pharmacology of the lower urinary tract. Surgery 2016;34 (7):326-332.

(15) Williams JP, Hashim H. New developments in the diagnosis and management of overactive bladder. Clinical Practice 2013;10 (1):79-87.

(16) Parsons BA, Drake MJ. Animal models in overactive bladder research. Handbook of Experimental Pharmacolology 2011; (202):15-43.

(17) Drake MJ, Mills IW, Gillespie JI. Model of peripheral autonomous modules and a myovesical plexus in normal and overactive bladder function. The Lancet 2001; 358:401-403.

(18) de Groat WC. A neurologic basis for the overactive bladder. Urology 1997 Dec;50 (6A Supplement):36-52; discussion 53-6.

(19) Rubenwolf PC, Georgopoulos NT, Clements LA, Feather S, Holland P, Thomas DF, et al. Expression and localisation of aquaporin water channels in human urothelium in situ and *in vitro*. European Urology 2009 Dec;56 (6):1013-1023.

(20) Birder LA, de Groat WC. Mechanisms of disease: involvement of the urothelium in bladder dysfunction. Nature Clinical Practice Urology. 2007 Jan;4(1):46-54.

(21) DiSanto ME, Wein AJ, Chacko S. Lower urinary tract physiology and pharmacology. Current Urology Reports 2000 Oct;1(3):227-234.

(22) Fry CH, Meng E, Young JS. The physiological function of lower urinary tract smooth muscle. Autonomic Neuroscience: basic & clinical 2010 Apr 19;154(1-2):3-13.

(23) Chopra B, Barrick SR, Meyers S, Beckel JM, Zeidel ML, Ford AP, et al. Expression and function of bradykinin B1 and B2 receptors in normal and inflamed rat urinary bladder urothelium. The Journal of Physiology 2005 Feb 1;562(Pt 3):859-871.

(24) Lee H, Koh BH, Peri LE, Sanders KM, Koh SD. Purinergic inhibitory regulation of murine detrusor muscles mediated by PDGFRalpha+ interstitial cells. The Journal of Physiology 2014 Mar 15;592(6):1283-1293.

(25) Hu P, Meyers S, Liang FX, Deng FM, Kachar B, Zeidel ML, et al. Role of membrane proteins in permeability barrier function: uroplakin ablation elevates urothelial permeability. American Journal of Physiology, Renal Physiology 2002 Dec;283(6):F1200-7.

(26) Birder LA, Ruan HZ, Chopra B, Xiang Z, Barrick S, Buffington CA, et al. Alterations in P2X and P2Y purinergic receptor expression in urinary bladder from normal cats and cats with interstitial cystitis. American Journal of Physiology, Renal Physiology 2004 Nov;287(5):F1084-91.

(27) Sui G, Fry CH, Montgomery B, Roberts M, Wu R, Wu C. Purinergic and muscarinic modulation of ATP release from the urothelium and its paracrine actions. American Journal of Physiology, Renal Physiology 2014 Feb 1;306(3):F286-98.

(28) Chopra B, Gever J, Barrick SR, Hanna-Mitchell AT, Beckel JM, Ford AP, et al. Expression and function of rat urothelial P2Y receptors. American Journal of Physiology, Renal Physiology 2008 Apr;294(4):F821-9.

(29) Tempest HV, Dixon AK, Turner WH, Elneil S, Sellers LA, Ferguson DR. P2X and P2X receptor expression in human bladder urothelium and changes in interstitial cystitis. BJU International 2004 Jun;93(9):1344-1348.

(30) Chess-Williams R. Muscarinic receptors of the urinary bladder: detrusor, urothelial and prejunctional. Auton Autacoid Pharmacol 2002 Jun;22(3):133-145.

(31) Beckel JM, Kanai A, Lee SJ, de Groat WC, Birder LA. Expression of functional nicotinic acetylcholine receptors in rat urinary bladder epithelial cells. Am J Physiol Renal Physiol 2006 Jan;290(1):F103-10.

(32) Beckel JM, Birder LA. Differential expression and function of nicotinic acetylcholine receptors in the urinary bladder epithelium of the rat. J Physiol 2012 Mar 15;590(6):1465-1480.

(33) Morris GE, Nelson CP, Standen NB, Challiss RA, Willets JM. Endothelin signalling in arterial smooth muscle is tightly regulated by G protein-coupled receptor kinase 2. Cardiovasc Res 2010 Feb 1;85(3):424-433.

(34) Willets JM, Challiss RA, Nahorski SR. Non-visual GRKs: are we seeing the whole picture? Trends Pharmacol Sci 2003 Dec;24(12):626-633.

(35) Webb RC. Smooth muscle contraction and relaxation. Adv Physiol Educ 2003 Dec;27(1-4):201-206.

(36) Fry CH, Gallegos CR, Montgomery BS. The actions of extracellular H^+ on the electrophysiological properties of isolated human detrusor smooth muscle cells. J Physiol 1994 Oct 1;480 (Part 1):71-80.

(37) Montgomery BS, Thomas PJ, Fry CH. The actions of extracellular magnesium on isolated human detrusor muscle function. Br J Urol 1992 Sep;70(3):262-268.

(38) Hashitani H, Brading AF. Electrical properties of detrusor smooth muscles from the pig and human urinary bladder. Br J Pharmacol 2003 Sep;140(1):146-158.

(39) Ambache N, Zar MA. Non-cholinergic transmission by post-ganglionic motor neurones in the mammalian bladder. J Physiol 1970 Oct;210(3):761-783.

(40) Yamazaki Y, Takeda H, Akahane M, Igawa Y, Nishizawa O, Ajisawa Y. Species differences in the distribution of β-adrenoceptor subtypes in bladder smooth muscle. Br J Pharmacol 1998;124(3):593-599.

(41) Anderson KE. Pharmacology of lower urinary tract smooth muscles and penile erectile tissues. Pharmacol Rev 1993 Sep;45(3):253-308.

(42) Anderson GF, Marks BH. Beta adrenoreceptors in the rabbit bladder detrusor muscle. J Pharmacol Exp Ther 1984 Feb;228(2):283-286.

(43) Andersson KE, Sjogren C. Aspects on the physiology and pharmacology of the bladder and urethra. Prog Neurobiol 1982;19(1-2):71-89.

(44) Yamaguchi O, Chapple CR. β3-Adrenoceptors in urinary bladder. Neurourol Urodyn 2007;26(6):752-756.

(45) Badawi J, Uecelehan H, Hatzinger M, Michel M, Haferkamp A, Bross S. Relaxant effects of β-adrenergic agonists on porcine and human detrusor muscle. Acta Physiologica 2005;185(2):151-159.

(46) Longhurst PA, Uvelius B. Pharmacological techniques for the *in vitro* study of the urinary bladder. J Pharmacol Toxicol Methods 2001 Mar-Apr;45(2):91-108.

(47) Sadananda P, Shang F, Liu L, Mansfield KJ, Burcher E. Release of ATP from rat urinary bladder mucosa: role of acid, vanilloids and stretch. Br J Pharmacol 2009 Dec;158(7):1655-1662.

(48) Mansfield KJ, Chandran JJ, Vaux KJ, Millard RJ, Christopoulos A, Mitchelson FJ, et al. Comparison of receptor binding characteristics of commonly used muscarinic antagonists in human bladder detrusor and mucosa. J Pharmacol Exp Ther 2009 Mar;328(3):893-899.

(49) Burnstock G. Discovery of purinergic signalling, the initial resistance and current explosion of interest. Br J Pharmacol 2012 Sep;167(2):238-255.

(50) Burnstock G. Physiology and pathophysiology of purinergic neurotransmission. Physiol Rev 2007 Apr;87(2):659-797.

(51) Moore KH, Ray FR, Barden JA. Loss of purinergic P2X(3) and P2X(5) receptor innervation in human detrusor from adults with urge incontinence. J Neurosci 2001 Sep 15;21(18):RC166.

(52) Elneil S, Skepper JN, Kidd EJ, Williamson JG, Ferguson DR. Distribution of P2X(1) and $P2X_3$ receptors in the rat and human urinary bladder. Pharmacology 2001;63(2):120-128.

(53) Rapp DE, Lyon MB, Bales GT, Cook SP. A role for the P2X receptor in urinary tract physiology and in the pathophysiology of urinary dysfunction. Eur Urol 2005 Aug;48(2):303-308.

(54) McMurray G, Dass N, Brading AF. Purinoceptor subtypes mediating contraction and relaxation of marmoset urinary bladder smooth muscle. Br J Pharmacol 1998 Apr;123(8):1579-1586.

(55) Ford AP, Gever JR, Nunn PA, Zhong Y, Cefalu JS, Dillon MP, et al. Purinoceptors as therapeutic targets for lower urinary tract dysfunction. Br J Pharmacol 2006 Feb;147 Supplement 2:S132-43.

(56) Burnstock G. Introduction and perspective, historical note. Front Cell Neurosci 2013 Nov 21;7:227.

(57) O'Reilly BA, Kosaka AH, Chang TK, Ford AP, Popert R, McMahon SB. A quantitative analysis of purinoceptor expression in the bladders of patients with symptomatic outlet obstruction. BJU Int 2001 May;87(7):617-622.

(58) Yu W, Robson SC, Hill WG. Expression and distribution of ectonucleotidases in mouse urinary bladder. PLoS One 2011 Apr 14;6(4):e18704.

(59) Burnstock G. Purinergic cotransmission. Exp Physiol 2009;94(1):20-24.

(60) Chapple CR, Rechberger T, Al-Shukri S, Meffan P, Everaert K, Huang M, et al. Randomized, double-blind placebo- and tolterodine-controlled trial of the once-daily antimuscarinic agent solifenacin in patients with symptomatic overactive bladder. BJU Int 2004 Feb;93(3):303-310.

(61) Speakman MJ, Kirby RS, Joyce A, Abrams P, Pocock R, British Association of Urological Surgeons. Guideline for the primary care management of male lower urinary tract symptoms. BJU Int 2004 May;93(7):985-990.

(62) Milani S, Djavan B. Lower urinary tract symptoms suggestive of benign prostatic hyperplasia: latest update on alpha-adrenoceptor antagonists. BJU Int 2005 Jun;95 Supplement 4:29-36.

(63) Soman AD, Gogtay JA. Lower urinary tract symptoms suggestive of benign prostatic hyperplasia: latest update on alpha(1)-adrenoceptor antagonists. BJU Int 2006 Jan;97(1):198.

(64) Fowler CJ. Intravesical treatment of overactive bladder. Urology 2000 May;55(5A Supplement):60-4; discussion 66.

(65) Saito M, Tabuchi F, Otsubo K, Miyagawa I. Treatment of overactive bladder with modified intravesical oxybutynin chloride. Neurourol Urodyn 2000;19(6):683-688.

(66) Burnstock G. P2X receptors in sensory neurones. Br J Anaesth 2000 Apr;84(4):476-488.

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

(68) Kinder RB, Restorick JM, Mundy AR. Vasoactive intestinal polypeptide in the hyper-reflexic neuropathic bladder. Br J Urol 1985 Jun;57(3):289-291.

(69) O'Reilly BA, Kosaka AH, Knight GF, Chang TK, Ford AP, Rymer JM, et al. P2X receptors and their role in female idiopathic detrusor instability. J Urol 2002 Jan;167(1):157-164.

(70) Palea S, Artibani W, Ostardo E, Trist DG, Pietra C. Evidence for purinergic neurotransmission in human urinary bladder affected by interstitial cystitis. J Urol 1993 Dec;150(6):2007-2012.

(71) Sigala S, Mirabella G, Peroni A, Pezzotti G, Simeone C, Spano P, et al. Differential gene expression of cholinergic muscarinic receptor subtypes in male and female normal human urinary bladder. Urology 2002 Oct;60(4):719-725.

(72) Caulfield MP, Birdsall NJ. International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. Pharmacol Rev 1998 Jun;50(2):279-290.

(73) Braverman A, Legos J, Young W, Luthin G, Ruggieri M. M2 receptors in genito-urinary smooth muscle pathology. Life Sci 1999;64(6-7):429-436.

(74) Berridge MJ. Inositol trisphosphate and calcium signalling. Nature 1993 Jan 28;361(6410):315-325.

(75) Defea K. Beta-arrestins and heterotrimeric G-proteins: collaborators and competitors in signal transduction. Br J Pharmacol 2008 Mar;153 Supplement 1:S298-309.

(76) Yeung CK, Yang SY, Hoebeke P. Lower Urinary Tract Conditions; Development and Assessment of Lower Urinary Tract Function in Children: McDougal WS, Wein AJ, Kavoussi LR, Partin AW, Peters CA, editors. Campbell-Walsh Urology. 11th ed.: Elsevier Health Sciences; 2016. p. 3121.

(77) Hall RA, Lefkowitz RJ. Regulation of G protein-coupled receptor signalling by scaffold proteins. Circ Res 2002 Oct 18;91(8):672-680.

(78) Millar RP, Newton CL. The year in G protein-coupled receptor research. Mol Endocrinol 2010 Jan;24(1):261-274.

(79) Stevens RC, Cherezov V, Katritch V, Abagyan R, Kuhn P, Rosen H, et al. The GPCR Network: a large-scale collaboration to determine human GPCR structure and function. Nat Rev Drug Discov 2013 Jan;12(1):25-34.

(80) Yeung CK, Yang SY, Hoebeke P. Lower Urinary Tract Conditions; Development and Assessment of Lower Urinary Tract Function in Children: McDougal WS, Wein AJ, Kavoussi LR, Partin AW, Peters CA, editors. Campbell-Walsh Urology. 11th ed.: Elsevier Health Sciences; 2016. p. 3121.

(81) Willets JM, Nash CA, Rainbow RD, Nelson CP, Challiss RA. Defining the roles of arrestin2 and arrestin3 in vasoconstrictor receptor desensitization in hypertension. Am J Physiol Cell Physiol 2015 Aug 1;309(3):C179-89.

(82) Erb L, Weisman GA. Coupling of P2Y receptors to G proteins and other signalling pathways. Wiley Interdiscip Rev Membr Transp Signal 2012 Nov-Dec;1(6):789-803.

(83) Robinson JD, Pitcher JA. G protein-coupled receptor kinase 2 (GRK2) is a Rho-activated scaffold protein for the ERK MAP kinase cascade. Cell Signal 2013 Dec;25(12):2831-2839.

(84) Lohse MJ, Hoffmann C. Arrestin interactions with G protein-coupled receptors. Handb Exp Pharmacol 2014;219:15-56.

(85) Furuya Y, Araki I, Kamiyama M, Zakoji H, Takihana Y, Takeda M. Decreased expression of G protein-coupled receptor kinases in the detrusor smooth muscle of human urinary bladder with outlet obstruction. Int J Urol 2006 Sep;13(9):1226-1232.

(86) Carman CV, Parent JL, Day PW, Pronin AN, Sternweis PM, Wedegaertner PB, et al. Selective regulation of Galpha(q/11) by an RGS domain in the G protein-coupled receptor kinase, GRK2. J Biol Chem 1999 Nov 26;274(48):34483-34492.

(87) Pitcher JA, Freedman NJ, Lefkowitz RJ. G protein–coupled receptor kinases. Annu Rev Biochem. 1998;67:653-92

(88) Lodowski DT, Pitcher JA, Capel WD, Lefkowitz RJ, Tesmer JJ. Keeping G proteins at bay: a complex between G protein-coupled receptor kinase 2 and Gbetagamma. Science 2003 May 23;300(5623):1256-1262.

(89) Eichmann T, Lorenz K, Hoffmann M, Brockmann J, Krasel C, Lohse MJ, et al. The amino-terminal domain of G-protein-coupled receptor kinase 2 is a regulatory Gbeta gamma binding site. J Biol Chem 2003 Mar 7;278(10):8052-8057.

(90) Pronin AN, Carman CV, Benovic JL. Structure-function analysis of G protein-coupled receptor kinase-5. Role of the carboxyl terminus in kinase regulation. J Biol Chem 1998 Nov 20;273(47):31510-31518.

(91) Penela P, Murga C, Ribas C, Tutor AS, Peregrin S, Mayor F,Jr. Mechanisms of regulation of G protein-coupled receptor kinases (GRKs) and cardiovascular disease. Cardiovasc Res 2006 Jan;69(1):46-56.

(92) Freedman NJ, Lefkowitz RJ. Desensitization of G protein-coupled receptors. Recent Prog Horm Res 1996;51:319-51; discussion 352-3.

(93) Ferguson SS. Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signalling. Pharmacol Rev 2001 Mar;53(1):1-24.

(94) Murakami A, Yajima T, Sakuma H, McLaren MJ, Inana G. X-arrestin: a new retinal arrestin mapping to the X chromosome. FEBS Lett 1993 Nov 15;334(2):203-209.

(95) Craft CM, Whitmore DH, Wiechmann AF. Cone arrestin identified by targeting expression of a functional family. J Biol Chem 1994 Feb 11;269(6):4613-4619.

(96) Shinohara T, Dietzschold B, Craft CM, Wistow G, Early JJ, Donoso LA, et al. Primary and secondary structure of bovine retinal S antigen (48-kDa protein). Proc Natl Acad Sci U S A 1987 Oct;84(20):6975-6979.

(97) Yamaki K, Takahashi Y, Sakuragi S, Matsubara K. Molecular cloning of the S-antigen cDNA from bovine retina. Biochem Biophys Res Commun 1987 Feb 13;142(3):904-910.

(98) Lohse MJ, Benovic JL, Codina J, Caron MG, Lefkowitz RJ. beta-Arrestin: a protein that regulates beta-adrenergic receptor function. Science 1990 Jun 22;248(4962):1547-1550.

(99) Attramadal H, Arriza JL, Aoki C, Dawson TM, Codina J, Kwatra MM, et al. Beta-arrestin2, a novel member of the arrestin/beta-arrestin gene family. J Biol Chem 1992 Sep 5;267(25):17882-17890.

(100) Gurevich VV, Gurevich EV. Analyzing the roles of multi-functional proteins in cells: The case of arrestins and GRKs. Crit Rev Biochem Mol Biol 2015;50(5):440-452.

(101) Kolch W. Coordinating ERK/MAPK signalling through scaffolds and inhibitors. Nat Rev Mol Cell Biol 2005 Nov;6(11):827-837.

(102) Magalhaes AC, Dunn H, Ferguson SS. Regulation of GPCR activity, trafficking and localization by GPCR-interacting proteins. Br J Pharmacol 2012 Mar;165(6):1717-1736.

(103) Ferguson DR, Kennedy I, Burton TJ. ATP is released from rabbit urinary bladder epithelial cells by hydrostatic pressure changes--a possible sensory mechanism? J Physiol 1997 Dec 1;505 (Part 2):503-511.

(104) Smith NJ, Luttrell LM. Signal switching, crosstalk, and arrestin scaffolds: novel G protein-coupled receptor signalling in cardiovascular disease. Hypertension 2006 Aug;48(2):173-179.

(105) Luttrell LM, Lefkowitz RJ. The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. J Cell Sci 2002 Feb 1;115(Part 3):455-465.

(106) Tilley DG. G protein-dependent and G protein-independent signalling pathways and their impact on cardiac function. Circ Res 2011 Jul 8;109(2):217-230.

(107) Morris GE, Nelson CP, Brighton PJ, Standen NB, Challiss RA, Willets JM. Arrestins 2 and 3 differentially regulate ETA and P2Y2 receptor-mediated cell signalling and migration in arterial smooth muscle. Am J Physiol Cell Physiol 2012 Mar 1;302(5):C723-34.

(108) Morris GE, Nelson CP, Everitt D, Brighton PJ, Standen NB, Challiss RA, et al. G protein-coupled receptor kinase 2 and arrestin2 regulate arterial smooth muscle P2Y-purinoceptor signalling. Cardiovasc Res 2011 Jan 1;89(1):193-203.

(109) Willis-Gray MG, Dieter AA, Geller EJ. Evaluation and management of overactive bladder: strategies for optimizing care. Res Rep Urol 2016 Jul 27;8:113-122.

(110) Lekskulchai O, Dietz HP. Detrusor wall thickness as a test for detrusor overactivity in women. Ultrasound Obstetric and Gynecology 2008;32:535-539.

(111) Manieri C, Carter SS, Romano G, Trucchi A, Valenti M, Tubaro A. The diagnosis of bladder outlet obstruction in men by ultrasound measurement of bladder wall thickness. The Journal of urology 1998;159(3):761-765.

(112) Ghoniem G, Faruqui N, Elmissiry M, Mahdy A, Abdelwahab H, Oommen M, et al. Differential profile analysis of urinary cytokines in patients with overactive bladder. Int Urogynecol J 2011 Aug;22(8):953-961.

(113) Yamaguchi O, Nishizawa O, Takeda M, Yokoyama O, Homma Y, Kakizaki H, et al. Clinical guidelines for overactive bladder. Int J Urol 2009 Feb;16(2):126-142.

(114) Gormley EA, Lightner DJ, Faraday M, Vasavada SP, American Urological Association, Society of Urodynamics, Female Pelvic Medicine. Diagnosis and treatment of overactive bladder (non-neurogenic) in adults: AUA/SUFU guideline amendment. J Urol 2015 May;193(5):1572-1580.

(115) National Institute for Health and Care Excellence Guidelines. Urinary Incontinence in women: Management (A clinical guideline). 2013; Available at: https://www.nice.org.uk/guidance/cg171. Accessed 04/September, 2017.

(116) Scottish Intercollegiate Guidelines Network. Management of incontinence in primary care: A national clinical guidelie (SIGN Guideline No 79). 2004; Available at: http://www.stroke.scot.nhs.uk/docs/sign79.pdf. Accessed 09/04, 2017.

(117) Tomlinson BU, Dougherty MC, Pendergast JF, Boyington AR, Coffman MA, Pickens SM. Dietary caffeine, fluid intake and urinary incontinence in older rural women. Int Urogynecol J Pelvic Floor Dysfunct 1999;10(1):22-28.

(118) Dowd TT, Campbell JM, Jones JA. Fluid intake and urinary incontinence in older community-dwelling women. J Community Health Nurs 1996;13(3):179-186.

(119) Hashim H, Abrams P. How should patients with an overactive bladder manipulate their fluid intake? BJU Int 2008 Jul;102(1):62-66.

(120) Vella VL, Jaffe W, Lidicker J, Meilahn J, Dandolu V. Prevalence of urinary symptoms in morbidly obese women and changes after bariatric surgery. J Reprod Med 2009 Oct;54(10):597-602.

(121) Nygaard IE, Kreder KJ, Lepic MM, Fountain KA, Rhomberg AT. Efficacy of pelvic floor muscle exercises in women with stress, urge, and mixed urinary incontinence. Am J Obstet Gynecol 1996 Jan;174(Part 1):120-125.

(122) Dattilo J. A long-term study of patient outcomes with pelvic muscle re-education for urinary incontinence. J Wound Ostomy Continence Nurs 2001 Jul;28(4):199-205.

(123) Haeusler G, Leitich H, van Trotsenburg M, Kaider A, Tempfer CB. Drug therapy of urinary urge incontinence: a systematic review. Obstet Gynecol 2002 Nov;100(Part 1):1003-1016.

(124) Diokno AC, Appell RA, Sand PK, Dmochowski RR, Gburek BM, Klimberg IW, et al. Prospective, randomized, double-blind study of the efficacy and tolerability of the extended-release formulations of oxybutynin and tolterodine for overactive bladder: results of the OPERA trial. Mayo Clin Proc 2003 Jun;78(6):687-695.

(125) Cardozo L, Chapple CR, Toozs-Hobson P, Grosse-Freese M, Bulitta M, Lehmacher W, et al. Efficacy of trospium chloride in patients with detrusor instability: a placebo-controlled, randomized, double-blind, multicentre clinical trial. BJU Int 2000 Apr;85(6):659-664.

(126) Tyagi P, Tyagi V. Mirabegron, a beta(3)-adrenoceptor agonist for the potential treatment of urinary frequency, urinary incontinence or urgency associated with overactive bladder. IDrugs 2010 Oct;13(10):713-722.

(127) Wu C, Sui G, Fry CH. The role of the L-type Ca(2+) channel in refilling functional intracellular Ca(2+) stores in guinea-pig detrusor smooth muscle. J Physiol 2002 Jan 15;538(Part 2):357-369.

(128) Tomoda T, Zhu HL, Iwasa K, Aishima M, Shibata A, Seki N, et al. Effects of flavoxate hydrochloride on voltage-dependent Ba2+ currents in human detrusor myocytes at different experimental temperatures. Naunyn Schmiedebergs Arch Pharmacol 2007 Nov;376(3):195-203.

(129) Tomoda T, Aishima M, Takano N, Nakano T, Seki N, Yonemitsu Y, et al. The effects of flavoxate hydrochloride on voltage-dependent L-type Ca2+ currents in human urinary bladder. Br J Pharmacol 2005 Sep;146(1):25-32.

(130) Brubaker L, Richter HE, Visco A, Mahajan S, Nygaard I, Braun TM, et al. Refractory idiopathic urge urinary incontinence and botulinum A injection. J Urol 2008 Jul;180(1):217-222.

(131) Nitti VW, Dmochowski R, Herschorn S, Sand P, Thompson C, Nardo C, et al. OnabotulinumtoxinA for the Treatment of Patients with Overactive Bladder and Urinary Incontinence: Results of a Phase 3, Randomized, Placebo Controlled Trial. J Urol 2017 Feb;197(Supplement 2):S216-S223.

(132) Ram-Liebig G, Ravens U, Balana B, Haase M, Baretton G, Wirth MP. New approaches in the modulation of bladder smooth muscle cells on viable detrusor constructs. World J Urol 2006;24(4):429-437.

(133) Willets JM, Brighton PJ, Mistry R, Morris GE, Konje JC, Challiss RA. Regulation of oxytocin receptor responsiveness by G protein-coupled receptor kinase 6 in human myometrial smooth muscle. Mol Endocrinol 2009 Aug;23(8):1272-1280.

(134) Gur S, Kadowitz PJ, Hellstrom WJ. Purinergic (P2) receptor control of lower genitourinary tract function and new avenues for drug action: an overview. Curr Pharm Des 2007;13(31):3236-3244.

(135) Wu C, Sui G, Fry CH. The role of the L-type Ca(2+) channel in refilling functional intracellular Ca(2+) stores in guinea-pig detrusor smooth muscle. J Physiol 2002 Jan 15;538(Part 2):357-369.

(136) Galvin DJ, Watson RW, Gillespie JI, Brady H, Fitzpatrick JM. Mechanical stretch regulates cell survival in human bladder smooth muscle cells *in vitro*. Am J Physiol Renal Physiol 2002 Dec;283(6):F1192-9.

(137) Vial C, Evans RJ. P2X receptor expression in mouse urinary bladder and the requirement of P2X(1) receptors for functional P2X receptor responses in the mouse urinary bladder smooth muscle. Br J Pharmacol 2000 Dec;131(7):1489-1495.

(138) Boland B, Himpens B, Paques C, Casteels R, Gillis JM. ATP induced-relaxation in the mouse bladder smooth muscle. Br J Pharmacol 1993 Mar;108(3):749-753.

(139) Burnstock G. Do some nerve cells release more than one transmitter? Neuroscience 1976 Aug;1(4):239-248.

(140) Burnstock G, Cocks T, Crowe R, Kasakov L. Purinergic innervation of the guinea-pig urinary bladder. Br J Pharmacol 1978 May;63(1):125-138.

(141) Lewis CJ, Surprenant A, Evans RJ. 2',3'-O-(2,4,6- trinitrophenyl) adenosine 5'-triphosphate (TNP-ATP)--a nanomolar affinity antagonist at rat mesenteric artery P2X receptor ion channels. Br J Pharmacol 1998 Aug;124(7):1463-1466.

(142) Ray FR, Moore KH, Hansen MA, Barden JA. Loss of purinergic P2X receptor innervation in human detrusor and subepithelium from adults with sensory urgency. Cell Tissue Res 2003 Dec;314(3):351-359.

(143) Bo X, Zhang Y, Nassar M, Burnstock G, Schoepfer R. A P2X purinoceptor cDNA conferring a novel pharmacological profile. FEBS Lett 1995 Nov 13;375(1-2):129-133.

(144) Moore KH, Ray FR, Barden JA. Loss of purinergic P2X(3) and P2X(5) receptor innervation in human detrusor from adults with urge incontinence. J Neurosci 2001 Sep 15;21(18):RC166.

(145) Burnstock G. Development and perspectives of the purinoceptor concept. J Auton Pharmacol 1996 Dec;16(6):295-302.

(146) Brake AJ, Wagenbach MJ, Julius D. New structural motif for ligand-gated ion channels defined by an ionotropic ATP receptor. Nature 1994 Oct 6;371(6497):519-523.

(147) Valera S, Hussy N, Evans RJ, Adami N, North RA, Surprenant A, et al. A new class of ligand-gated ion channel defined by P2x receptor for extracellular ATP. Nature 1994 Oct 6;371(6497):516-519.

(148) Hansen MA, Dutton JL, Balcar VJ, Barden JA, Bennett MR. P2X (purinergic) receptor distributions in rat blood vessels. J Auton Nerv Syst 1999 Feb 15;75(2-3):147-155.

(149) Lee HY, Bardini M, Burnstock G. Distribution of P2X receptors in the urinary bladder and the ureter of the rat. J Urol 2000 Jun;163(6):2002-2007.

(150) Erlinge D, Burnstock G. P2 receptors in cardiovascular regulation and disease. Purinergic Signal 2008 Mar;4(1):1-20.

(151) Suzuki H, Kokubun S. Subtypes of purinoceptors in rat and dog urinary bladder smooth muscles. Br J Pharmacol 1994 May;112(1):117-122.

(152) Palea S, Corsi M, Pietra W, Artibani A, Calpista A, Gaviraghi G, et al. ADP/3S induces contraction of the human isolated urinary bladder through a purinoceptor subtype different from P2X and P2Y. Journal of Pharmacology and Experimental Therapeutics 1994;269:193197.

(153) Heppner TJ, Bonev AD, Nelson MT. Elementary purinergic Ca2+ transients evoked by nerve stimulation in rat urinary bladder smooth muscle. J Physiol 2005 Apr 1;564(Pt 1):201-212.

(154) Phaneuf S, Beaudoin AR. Calcium binding properties of purified zymogen granule membrane of pig pancreas. Evidence for calcium binding proteins. Biochim Biophys Acta 1986 Apr 14;856(2):348-356.

(155) Kenakin TP, Pike NB. An *in vitro* analysis of purine-mediated renal vasoconstriction in rat isolated kidney. Br J Pharmacol 1987 Feb;90(2):373-381.

(156) Bailey SJ, Hickman D, Hourani SM. Characterization of the P1-purinoceptors mediating contraction of the rat colon muscularis mucosae. Br J Pharmacol 1992 Feb;105(2):400-404.

(157) Burnstock G, Kennedy C. Is there a basis for distinguishing two types of P2-purinoceptor? Gen Pharmacol 1985;16(5):433-440.

(158) Bailey SJ, Hourani SM. A study of the purinoceptors mediating contraction in the rat colon. Br J Pharmacol 1990 Aug;100(4):753-756.

(159) Wiklund NP, Gustafsson LE. Indications for P2-purinoceptor subtypes in guinea pig smooth muscle. Eur J Pharmacol 1988 Apr 13;148(3):361-370.

(160) Burnstock G, Warland JJ. P2-purinoceptors of two subtypes in the rabbit mesenteric artery: reactive blue 2 selectively inhibits responses mediated via the P2Y- but not the P2x-purinoceptor. Br J Pharmacol 1987 Feb;90(2):383-391.

(161) Govindan S, Taylor EJ, Taylor CW. Ca(2+) signalling by P2Y receptors in cultured rat aortic smooth muscle cells. Br J Pharmacol 2010 Aug;160(8):1953-1962.

(162) Chhatriwala M, Ravi RG, Patel RI, Boyer JL, Jacobson KA, Harden TK. Induction of novel agonist selectivity for the ADP-activated P2Y1 receptor versus the ADP-activated P2Y12 and P2Y13 receptors by conformational constraint of an ADP analog. J Pharmacol Exp Ther 2004 Dec;311(3):1038-1043.

(163) Boyer JL, Delaney SM, Villanueva D, Harden TK. A molecularly identified P2Y receptor simultaneously activates phospholipase C and inhibits adenylyl cyclase and is nonselectively activated by all nucleoside triphosphates. Mol Pharmacol 2000 Apr;57(4):805-810.

(164) Waldo GL, Corbitt J, Boyer JL, Ravi G, Kim HS, Ji XD, et al. Quantitation of the P2Y(1) receptor with a high affinity radiolabeled antagonist. Mol Pharmacol 2002 Nov;62(5):1249-1257.

(165) Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, et al. International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. Pharmacol Rev 2006 Sep;58(3):281-341.

(166) Kemp PA, Sugar RA, Jackson AD. Nucleotide-mediated mucin secretion from differentiated human bronchial epithelial cells. Am J Respir Cell Mol Biol 2004 Oct;31(4):446-455.

(167) Rajagopal M, Kathpalia PP, Thomas SV, Pao AC. Activation of P2Y1 and P2Y2 receptors induces chloride secretion via calcium-activated chloride channels in kidney inner medullary collecting duct cells. Am J Physiol Renal Physiol 2011 Sep;301(3):F544-53.

(168) Sui GP, Wu C, Fry CH. Characterization of the purinergic receptor subtype on guinea-pig suburothelial myofibroblasts. BJU Int 2006 Jun;97(6):1327-1331.

(169) Willets JM, Challiss RA, Kelly E, Nahorski SR. G protein-coupled receptor kinases 3 and 6 use different pathways to desensitize the endogenous M3 muscarinic acetylcholine receptor in human SH-SY5Y cells. Mol Pharmacol 2001 Aug;60(2):321-330.

(170) Willets J, Kelly E. Desensitization of endogenously expressed δ-opioid receptors: no evidence for involvement of G protein-coupled receptor kinase 2. Eur J Pharmacol 2001;431(2):133-141.

(171) Mundell SJ, Benovic JL, Kelly E. A dominant negative mutant of the G protein-coupled receptor kinase 2 selectively attenuates adenosine A2 receptor desensitization. Mol Pharmacol 1997 Jun;51(6):991-998.

(172) Willets JM, Taylor AH, Shaw H, Konje JC, Challiss RA. Selective regulation of H1 histamine receptor signalling by G protein-coupled receptor kinase 2 in uterine smooth muscle cells. Mol Endocrinol 2008 Aug;22(8):1893-1907.

(173) Sterne-Marr R, Tesmer JJ, Day PW, Stracquatanio RP, Cilente JA, O'Connor KE, et al. G protein-coupled receptor Kinase 2/G alpha q/11 interaction. A novel surface on a regulator of G protein signalling homology domain for binding G alpha subunits. J Biol Chem 2003 Feb 21;278(8):6050-6058.

(174) Willets JM, Nahorski SR, Challiss RA. Roles of phosphorylation-dependent and -independent mechanisms in the regulation of M1 muscarinic acetylcholine receptors by G protein-coupled receptor kinase 2 in hippocampal neurons. J Biol Chem 2005 May 13;280(19):18950-18958.

(175) Willets JM, Nash MS, Challiss RA, Nahorski SR. Imaging of muscarinic acetylcholine receptor signalling in hippocampal neurons: evidence for phosphorylation-dependent and -independent regulation by G-protein-coupled receptor kinases. J Neurosci 2004 Apr 28;24(17):4157-4162.

(176) Willets JM, Mistry R, Nahorski SR, Challiss RA. Specificity of g protein-coupled receptor kinase 6-mediated phosphorylation and regulation of single-cell m3 muscarinic acetylcholine receptor signalling. Mol Pharmacol 2003 Nov;64(5):1059-1068.

(177) Brighton PJ, Rana S, Challiss RJ, Konje JC, Willets JM. Arrestins differentially regulate histamine- and oxytocin-evoked phospholipase C and mitogen-activated protein kinase signalling in myometrial cells. Br J Pharmacol 2011 Apr;162(7):1603-1617.

(178) Schumacher SM, Gao E, Zhu W, Chen X, Chuprun JK, Feldman AM, et al. Paroxetine-mediated GRK2 inhibition reverses cardiac dysfunction and remodeling after myocardial infarction. Sci Transl Med 2015 Mar 4;7(277):277ra31.

(179) Thal DM, Homan KT, Chen J, Wu EK, Hinkle PM, Huang ZM, et al. Paroxetine is a direct inhibitor of g protein-coupled receptor kinase 2 and increases myocardial contractility. ACS Chem Biol 2012 Nov 16;7(11):1830-1839.

(180) Owens JM, Knight DL, Nemeroff CB. Second generation SSRIS: human monoamine transporter binding profile of escitalopram and R-fluoxetine. Encephale 2002 Jul-Aug;28(4):350-355.

(181) Homan KT, Wu E, Wilson MW, Singh P, Larsen SD, Tesmer JJ. Structural and functional analysis of g protein-coupled receptor kinase inhibition by paroxetine and a rationally designed analog. Mol Pharmacol 2014 Feb;85(2):237-248.

(182) Hata JA, Williams ML, Koch WJ. Genetic manipulation of myocardial beta-adrenergic receptor activation and desensitization. J Mol Cell Cardiol 2004 Jul;37(1):11-21.

(183) Liu R, Gutierrez AM, Ring A, Persson AE. Nitric oxide induces resensitization of P2Y nucleotide receptors in cultured rat mesangial cells. J Am Soc Nephrol 2002 Feb;13(2):313-321.

(184) Obara K, Arai K, Tomita Y, Hatano A, Takahashi K. G-protein coupled receptor kinase 2 and 3 expression in human detrusor cultured smooth muscle cells. Urol Res 2001 Oct;29(5):325-329.

(185) Willets JM, Challiss RA, Nahorski SR. Endogenous G protein-coupled receptor kinase 6 Regulates M3 muscarinic acetylcholine receptor phosphorylation and desensitization in human SH-SY5Y neuroblastoma cells. J Biol Chem 2002 May 3;277(18):15523-15529.

(186) Guo S, Carter RL, Grisanti LA, Koch WJ, Tilley DG. Impact of paroxetine on proximal beta-adrenergic receptor signalling. Cell Signal 2017 Oct;38:127-133.

(187) Ahn S, Wei H, Garrison TR, Lefkowitz RJ. Reciprocal regulation of angiotensin receptor-activated extracellular signal-regulated kinases by beta-arrestins 1 and 2. J Biol Chem 2004 Feb 27;279(9):7807-7811.

(188) Hoffmann C, Ziegler N, Reiner S, Krasel C, Lohse MJ. Agonist-selective, receptor-specific interaction of human P2Y receptors with beta-arrestin-1 and -2. J Biol Chem 2008 Nov 7;283(45):30933-30941.

(189) Han CC, Ma Y, Li Y, Wang Y, Wei W. Regulatory effects of GRK2 on GPCRs and non-GPCRs and possible use as a drug target (Review). Int J Mol Med 2016 Oct;38(4):987-994.

(190) Turner DA, Shaw C, McGrother CW, Dallosso HM, Cooper NJ, MRC Incontinence Team. The cost of clinically significant urinary storage symptoms for community dwelling adults in the UK. BJU Int 2004 Jun;93(9):1246-1252.

(191) Smith TH, Sim-Selley LJ, Selley DE. Cannabinoid CB1 receptor-interacting proteins: novel targets for central nervous system drug discovery? Br J Pharmacol 2010 Jun;160(3):454-466.

(192) Chaqour B, Yang R, Sha Q. Mechanical stretch modulates the promoter activity of the profibrotic factor CCN2 through increased actin polymerization and NF-kappaB activation. J Biol Chem 2006 Jul 21;281(29):20608-20622.

(193) Adam RM, Eaton SH, Estrada C, Nimgaonkar A, Shih SC, Smith LE, et al. Mechanical stretch is a highly selective regulator of gene expression in human bladder smooth muscle cells. Physiol Genomics 2004 Dec 15;20(1):36-44.

(194) Boopathi E, Gomes C, Zderic SA, Malkowicz B, Chakrabarti R, Patel DP, et al. Mechanical stretch upregulates proteins involved in Ca2+ sensitization in urinary bladder smooth muscle hypertrophy. Am J Physiol Cell Physiol 2014 Sep 15;307(6):C542-53.

(195) Yamaguchi O. Response of bladder smooth muscle cells to obstruction: signal transduction and the role of mechanosensors. Urology 2004;63(3):11-16.

(196) Metaye T, Perdrisot R, Kraimps JL. GRKs and arrestins: the therapeutic pathway? Med Sci (Paris) 2006 May;22(5):537-543.

(197) Vroon A, Heijnen CJ, Kavelaars A. GRKs and arrestins: regulators of migration and inflammation. J Leukoc Biol 2006 Dec;80(6):1214-1221.

(198) Otero M, Garrad RC, Velazquez B, Hernandez-Perez MG, Camden JM, Erb L, et al. Mechanisms of agonist-dependent and -independent desensitization of a recombinant P2Y2 nucleotide receptor. Mol Cell Biochem 2000 Feb;205(1-2):115-123.

(199) Park JM, Yang T, Arend LJ, Schnermann JB, Peters CA, Freeman MR, et al. Obstruction stimulates COX-2 expression in bladder smooth muscle cells via increased mechanical stretch. Am J Physiol 1999 Jan;276 (Part 2):F129-36.

(200) Wazir R, Luo DY, Dai Y, Yue X, Tian Y, Wang KJ. Expression and proliferation profiles of PKC, JNK and p38MAPK in physiologically stretched human bladder smooth muscle cells. Biochem Biophys Res Commun 2013 Aug 30;438(3):479-482.

(201) Lymperopoulos A, Negussie S. betaArrestins in Cardiac G Protein-Coupled Receptor Signalling and Function: Partners in Crime or "Good Cop, Bad Cop"? Int J Mol Sci 2013 Dec 18;14(12):24726-24741.