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)

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Professor Douglas Tincello
I. Abstract

ATP released from parasympathetic nerves can mediate 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

First, I would like to thank my supervisors Dr Jonathon Willets (Department of molecular and cell biology, University of Leicester) and Professor Douglas G. Tincello (Department of Health Sciences, University of Leicester) for their support that helped me produce this work. In particular, I am grateful to Dr Willets for his endless support and help through my PhD and his advice on research was invaluable.

I would like to thank my committee member Dr Richard Rainbow for his help in collecting the urinary bladder samples from his laboratory. I would also like to thank Shashi Rana for her assistant and help.
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)

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

I. Abstract ................................................................................................................................. 2
II. Acknowledgements ............................................................................................................... 3
III. Dedication ........................................................................................................................... 4
IV. Presentations ...................................................................................................................... 5
V. Table of contents ................................................................................................................. 6
VI. List of figures ..................................................................................................................... 12
VII. List of Tables ..................................................................................................................... 15
VIII. List of abbreviation ......................................................................................................... 16

Chapter 1 .................................................................................................................................. 19
1. General Introduction: ......................................................................................................... 20
   1.1. Overactive Urinary Bladder .......................................................................................... 20
   1.2. Different types of urinary incontinence ....................................................................... 20
   1.3. Prevalence and burdens of OAB .................................................................................. 21
      1.3.1. Prevalence ............................................................................................................... 21
      1.3.2. Burdens related to OAB and its cost ...................................................................... 22
   1.1. Diagnosis of overactive bladder ................................................................................... 23
      1.1.1. History .................................................................................................................... 23
      1.1.2. Examination .......................................................................................................... 23
      1.1.3. Investigation .......................................................................................................... 23
         1.1.3.1. Urine analysis ...................................................................................................... 23
         1.1.3.2. Bladder diaries .................................................................................................... 24
         1.1.3.3. Urodynamic studies ............................................................................................ 24
            1.1.3.3.1. Uroflowmetry ............................................................................................... 24
            1.1.3.3.2. Post-void residual urine volume ...................................................................... 24
         1.1.3.4. Ultrasound scan (USS) ...................................................................................... 24
         1.1.3.5. Magnetic resonance (MRI) ................................................................................. 25
         1.1.3.6. Biomarkers ........................................................................................................ 25
            1.1.3.6.1. Nerve growth factors (NGF) ......................................................................... 25
            1.1.3.6.2. Cytokines ...................................................................................................... 25
   1.2. Anatomy of the urinary bladder .................................................................................... 26
   1.3. Innervation of the urinary bladder ................................................................................. 28
   1.4. Histology of the urinary bladder .................................................................................... 30
   1.5. Physiology of the urinary bladder ................................................................................... 31
Chapter 2

2. General Methods

2.1. Materials & Methods

2.1.1. Source of materials and instruments

2.1.2. Bladder smooth muscle cells isolation and culture

2.1.3. Cell maintenance and culture

2.1.4. Characterization of smooth muscle

2.1.5. Immunocytochemistry of smooth muscle

2.1.6. Cell counting
3. Characterisation of purinergic receptor subtype expression and activity in rat detrusor smooth muscle

3.1. Introduction

3.2. Aims

3.3. Method

3.4. Data analysis

3.5. Results

3.5.1. Characterization of DSMC cultures

3.5.2. Immunocytochemistry of smooth muscle

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
4.1. Introduction ........................................................................................................... 110
4.2. Aims ...................................................................................................................... 112
4.3. Methods .............................................................................................................. 113
    4.3.1. Single cell confocal imaging techniques to study P2Y receptor desensitization 113
    4.3.2. Western Blot Analysis .................................................................................. 113
    4.3.3. Inhibiting Endogenous GRK Activities on DSMC by dominant-negative GRK mutants 114
4.4. Data analysis ....................................................................................................... 114
4.5. Results ............................................................................................................... 115
    4.5.1. Single-Cell Ca\(^{2+}\) Imaging and Receptor Desensitization Studies .............. 115
        4.5.1.1. Desensitization and re-sensitization kinetics of P2YR-stimulated PLC signalling in DSMC ................................................................................................................................. 115
        4.5.1.2. Desensitization and re-sensitization of P2Y\(_1\) PLC signalling mediated by ADP in isolated DSMC .................................................................................................................. 117
        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 ... 120
    4.5.2. Identification of GRK protein expression in DSMC and whole bladder ......... 122
4.5.3. Effects of dominant-negative GRKs expression on P2Y_{1}-receptor PLC signalling in DSMC .................................................................124

4.5.4. G protein-coupled receptor kinase 2 regulates P2Y_{2}-purinoceptor mediated PLC signalling in DSMC .........................................................................................126

4.5.4.1. Effects of dominant-negative GRKs expression on P2Y_{2} receptor PLC signalling in DSMC ..............................................................126

4.5.4.2. Effects of inhibiting endogenous GRK2 activities in DSMC by depletion siRNA ......................................................................................126

4.5.5. Characterising the selectivity of the putative GRK2 inhibitor paroxetine to inhibit GRK2 mediated receptor desensitization .......................................129

4.5.6. Does the putative GRK2 inhibitor paroxetine prevent the desensitization of P2Y receptor PLC activity in DSMC? .................................................................133

4.6. Discussion ..................................................................................................................136

4.7. Conclusion ................................................................................................................140

Chapter 5 ..................................................................................................................141

5. Characterising the roles that arrestin proteins play in the regulation P2Y-purinoceptor PLC signalling in DSMCs .................................................................141

5.1. Introduction .................................................................................................................142

5.2. Aims ...........................................................................................................................144

5.3. Methods .....................................................................................................................145

5.3.1. Cell maintenance and culture ..............................................................................145

5.3.2. Knockdown of endogenous arrestin2 and arrestin3 in DSMC .........................145

5.3.3. Single cell imaging and receptor desensitization studies .................................146

5.4. Results .......................................................................................................................147

5.4.1. Expression of arrestin2 and arrestin3 proteins in DSMCs and whole bladder ...147

5.4.2. Effects of depletion of the siRNA on endogenous arrestin2 and 3 in DSMC ....148

5.4.3. Effects of arrestin2 and arrestin3 depletion on ADP-Stimulated PLC Signalling and P2Y_{1}-receptor desensitization in DSMC ........................................150

5.4.4. Effects of isoform-specific arrestin knockdown on UTP-Stimulated P2Y_{2} receptors signalling ......................................................................................152

5.4.5. Differential effects of arrestin proteins depletion on P2Y receptors and stimulated PLC signalling by measuring intracellular calcium [Ca^{2+}] ..........154

5.5. Discussion .................................................................................................................156

5.6. Conclusion .................................................................................................................158

Chapter 6 ..................................................................................................................159

6. Characterising the effects of mechanical stretch on GRK and arrestin protein expression in isolated DSMC .................................................................159

6.1. Introduction .................................................................................................................160
6.2. Aim................................................................................................................................. 163
6.3. Methods.......................................................................................................................... 164
  6.3.1. Bladder smooth muscle Preparation and culture....................................................... 164
  6.3.2. Mechanical cell stretching......................................................................................... 164
  6.3.3. Protein assay and Western blotting analysis ............................................................. 165
6.4. Results................................................................................................................................ 166
  6.4.1. Application of mechanical stretch alters GRK protein expression in DSMC .... 166
  6.4.2. Effects of mechanical stretch on arrestin protein expression in DSMC............. 171
6.5. Discussion....................................................................................................................... 172
6.6. Conclusion....................................................................................................................... 173

Chapter 7 ................................................................................................................................. 174
7. General discussion and future work................................................................................... 174
  7.1. General discussion........................................................................................................ 174
  7.2. Future work................................................................................................................... 178

References:............................................................................................................................... 179
VI. List of figures

Figure 1-1: Anatomy of a female urinary bladder .............................................. 27
Figure 1-2: Nerve supply of urinary bladder modified from www.wikipedia.com...... 29
Figure 1-3: Histology of Urinary Bladder wall. ................................................... 30
Figure 1-4: Micturition cycle control pathways: .................................................. 32
Figure 1-5: Signalling pathways in detrusor muscle cells and the contraction of urinary
bladder (26). ........................................................................................................... 35
Figure 1-6: Diagram illustrations the distribution of α-adrenergic, β-adrenergic and
muscarinic cholinergic receptors in urinary bladder (This Adapted from Caine, 1984). 37
Figure 1-7: The roles of ATP and purinergic receptors in the micturition process (This
figure adapted from Ford et al 2006 (59)) ............................................................... 39
Figure 1-8: The hypothesis for purinergic mechanosensory transduction in tubes and
sacs (ureter and urinary bladder). ........................................................................... 41
Figure 1-9: G-Protein coupled receptor kinase family (figure adapted from (38)). .... 46
Figure 1-10: Intracellular signalling of GPCR in the smooth muscle ...................... 48
Figure 1-11: Roles of β-arrestins in receptor desensitization .................................. 51
Figure 2-1: Wister rat whole urinary bladder and DSMC photographed at different
stages......................................................................................................................... 63
Figure 2-2: Cell counting using haemocytometer method...................................... 66
Figure 2-3: The picture shows confocal imaging .................................................... 71
Figure 2-4: Laser scanning confocal inverted microscope using (X60 oil immersion
objective). ............................................................................................................... 72
Figure 2-5: Detrusor smooth muscles collected from four different animals at different
dates ...................................................................................................................... 74
Figure 3-1: Characterization of bladder detrusor smooth muscle cells (DSMC). ....... 85
Figure 3-2: Immunocytochemical pictures of bladder smooth muscle cell ............... 86
Figure 3-3: Representative traces showing changes in intracellular Ca²⁺ levels ....... 88
Figure 3-4: ATP- and UTP-stimulated -concentration-response curves .................... 90
Figure 3-5: Representative traces showing changes in intracellular Ca²⁺ levels ....... 92
Figure 3-6: Concentration-dependent curves showing [Ca²⁺], elevation in response to
ATP, UTP and ADP in DSMC. ............................................................................... 94
Figure 3-7: Calcium response curves of P2Y₁ receptor challenged with MRS2365 ..... 95
Figure 3-8: Calcium response curves of P2Y<sub>2</sub> receptor challenged with antagonists (AR-C118925XX) in the presences of UTP. ................................................................. 96
Figure 3-9: Effects of the non-selective P2X antagonist TNP on ATP-stimulated calcium signals.................................................................................................................. 98
Figure 3-10: The effects of the non-selective P2X receptor agonist on [Ca<sup>2+</sup>]<sub>i</sub> in Fluo4-AM loaded DSMC ................................................................................................. 99
Figure 3-11: Pharmacological profile of P2Y receptor activity in DSMC. .................. 101
Figure 3-12: Effects of the P2Y<sub>1</sub> selective antagonist MRS2279 on ADP stimulated calcium signalling in isolated DSMC .................................................................................. 102
Figure 4-1: Assessment of P2Y receptor desensitization and re-sensitization in DSMC. ............................................................................................................................................. 116
Figure 4-2: Desensitization and re-sensitization kinetics of P2Y<sub>1</sub> receptor stimulated [Ca<sup>2+</sup>]<sub>i</sub> signalling following ADP challenge. ................................................................. 118
Figure 4-3: Assessment of P2Y<sub>1</sub> receptor desensitization and re-sensitization in DSMC....................................................................................................................................... 119
Figure 4-4: Desensitization and re-sensitization of P2Y<sub>2</sub> receptor-stimulated Ca<sup>2+</sup> signalling in DSMC ........................................................................................................ 121
Figure 4-5: Western blots showing protein expression for GRK2 and GRK5 in cultured DSMC and whole bladder.................................................................................................................................... 122
Figure 4-6: Western blots showing protein expression for GRK 3, 5 and 6 in cultured DSMC and whole bladder ................................................................................................................................... 123
Figure 4-7: GRK3 inhibition decreases P2Y<sub>1</sub>-receptor desensitization induced by ADP. ........................................................................................................................................... 125
Figure 4-8: GRK2 inhibition decreases P2Y<sub>2</sub>-receptor desensitization induced by UTP. ........................................................................................................................................... 127
Figure 4-9: Anti-GRK2 siRNA causes endogenous GRK2 protein depletion. .......... 128
Figure 4-11: Paroxetine but not fluoxetine prevents histamine H<sub>1</sub> receptor desensitization in ULTR cells ...................................................................................................................... 131
Figure 4-10: Paroxetine and fluoxetine do not affect the desensitization oxytocin receptor PLC activity in ULTR cells .................................................................................................... 132
Figure 4-12: Paroxetine but not fluoxetine inhibits the desensitization of P2Y<sub>2</sub> receptor PLC activity in DSMC ........................................................................................................... 134
Figure 4-13: Assessment of the desensitization of P2Y<sub>1</sub> receptor PLC activity in DSMC by paroxetine and fluoxetine........................................................................................................... 135
Figure 5-1: Arrestin 2 and arrestin 3 are expressed in rat bladder and DMSC........... 147
Figure 5-2: siRNA-mediated depletion of endogenous arrestin2 and arrestin3. ........ 149
Figure 5-3: Arrestin knockdown prevents P2Y$_1$ receptor desensitization signalling. .. 151
Figure 5-4: Arrestin knockdown prevents desensitization of P2Y$_2$ receptor PLC
signalling................................................................................................................. 153
Figure 5-5: Arrestin depletion attenuates the desensitization of P2Y$_2$ receptor stimulated
$[Ca^{2+}]_i$, signalling in DSMC................................................................................. 155
Figure 5-6: G-protein-coupled receptor (GPCR) desensitization, internalization and
down-regulation mechanisms (Figure adapted from Smith et al 2010 (192). .......... 157
Figure 6-1: Flow diagram showing the protocol for isolation and mechanical stretching
of DSMC.................................................................................................................... 161
Figure 6-2: Effects of mechanical stretching on expression of GRK2 in DSMC........ 167
Figure 6-3: Effects of mechanical stretching on expression of GRK3 in DSMC........ 168
Figure 6-4: Effects of mechanical stretching on expression of GRK5 in DSMC........ 169
Figure 6-5: Effects of mechanical stretching on expression of GRK6 in DSMC........ 170
Figure 6-6: Effects of mechanical stretching on expression of arrestin2 and arrestin3 in
DSMC....................................................................................................................... 171
VII. List of Tables

Table 2-1: Antibodies, dilutions used for Western Blot and their suppliers.................. 69
### VIII. List of abbreviation

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>Ach</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>A1CT</td>
<td>Antiarrestin 2 antibody</td>
</tr>
<tr>
<td>ASMC</td>
<td>Aortic smooth muscle cells</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>BPH</td>
<td>Benign prostate hyperplasia</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>βAR</td>
<td>Beta arrestin</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>Calcium ions</td>
</tr>
<tr>
<td>Ca^{2+},i</td>
<td>Intracellular calcium</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CaCl_{2}</td>
<td>Calcium Chloride</td>
</tr>
<tr>
<td>CICR</td>
<td>Calcium induced calcium release</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CO_{2}</td>
<td>Carbone Dioxide</td>
</tr>
<tr>
<td>CLSM</td>
<td>Confocal laser scanning microscopy</td>
</tr>
<tr>
<td>DSM</td>
<td>Detrusor smooth muscles</td>
</tr>
<tr>
<td>DSMC</td>
<td>Detrusor smooth muscle cell</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DAB</td>
<td>3, 3'-diaminobenzidine</td>
</tr>
<tr>
<td>DDT</td>
<td>Dichlorodiphenyltrichloroethane</td>
</tr>
<tr>
<td>D-PBS</td>
<td>Dulbecco’s phosphate buffer saline</td>
</tr>
<tr>
<td>DS</td>
<td>Dissection Solution</td>
</tr>
<tr>
<td>DNM</td>
<td>Dominant negative</td>
</tr>
<tr>
<td>DNM GRKs</td>
<td>Dominant-negative mutant GRKs</td>
</tr>
<tr>
<td>Em</td>
<td>Emission</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescent</td>
</tr>
<tr>
<td>ETA</td>
<td>Endothelin type A</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Fluo 4-AM</td>
<td>Fluo-4-acetoxymethyl ester</td>
</tr>
<tr>
<td>EC50</td>
<td>Half maximal effective concentration</td>
</tr>
<tr>
<td>eGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>eGFP-PH</td>
<td>Enhanced green fluorescent protein- Pleckstrin homology</td>
</tr>
<tr>
<td>F</td>
<td>Fluorescence</td>
</tr>
<tr>
<td>F0</td>
<td>Basal fluorescence</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>Fluo4-AM</td>
<td>Fluo-4-acetoxymethyl ester</td>
</tr>
<tr>
<td>GEFs</td>
<td>Guanine nucleotide exchange factors</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptors</td>
</tr>
<tr>
<td>GRK</td>
<td>G protein-coupled receptors Kinases</td>
</tr>
<tr>
<td>H_{2}O_{2}</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>H</td>
<td>Hour</td>
</tr>
<tr>
<td>H_{1}</td>
<td>Histamine receptor</td>
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</tbody>
</table>
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
mm²  Millimetre square
µm  Micro molar
µg  Micro gram
nM  Nano meter
NaH2PO4  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 fluorescent protein
Rmax  Maximum response
RGS  Regulator of G protein signalling
RNA  Ribonucleic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
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</tr>
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<tbody>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolution per minute</td>
</tr>
<tr>
<td>RTX</td>
<td>Resiniferatoxin</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small/short interfering RNA</td>
</tr>
<tr>
<td>Sec</td>
<td>Seconds</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic Reticulum</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS- Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SSRI</td>
<td>Selective Serotonin reuptake inhibitor</td>
</tr>
<tr>
<td>SIGN</td>
<td>Scottish intercollegiate guidelines network</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tric buffered saline with Tween 20</td>
</tr>
<tr>
<td>TNP-ATP</td>
<td>2’, 3’-O-trinitrophenyl-Adenosine triphosphate</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>UTI</td>
<td>Urinary Tract Infection</td>
</tr>
<tr>
<td>USS</td>
<td>Ultrasound scan</td>
</tr>
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<td>UTP</td>
<td>Uridine triphosphate</td>
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<td>Uridine Triphosphate</td>
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<td>ULTR</td>
<td>Uterine smooth muscle cell lines</td>
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<tr>
<td>v/v</td>
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</tr>
<tr>
<td>w/v</td>
<td>Weight/volume</td>
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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

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 pudendal 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.
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).

![Micturition cycle control pathways](image_url)

**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 M₃-receptors that are coupled to Gαq/11, a member of the G-protein superfamily. Recruitment of Gαq/11 to the agonist-bound M₃-receptor activates phospholipase C (PLC), leading to the generation of inositol 1, 4, 5-triphosphate (IP₃) and diacylglycerol (DAG). Liberation of intracellular Ca²⁺ from stores is triggered by IP₃ interacting with its IP₃ receptor in the sarcoplasmic reticulum. Increasing intracellular [Ca²⁺] and DAG levels which stimulates protein kinase C (PKC) activity (37). M₃ 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) M₃ receptor and the P₂X₁-ATP receptor. The M₃ receptor initiates the formation of inositol triphosphate (IP₃) and diacylglycerol (DAG) from membrane phospholipids (PIP₂) by the action of phospholipase-C (PLC). IP₃ initiates Ca²⁺ release from intracellular stores (SR). Ca²⁺ binds to calmodulin (CaM) to activate myosin light chain (MLC) kinase and phosphorylate and activate myosin to bind to actin. M₃ 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 P₂X₁ receptor ion channel and the L-type Ca²⁺ channel.
Detrusor 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 \([\text{Ca}^{2+}]_i\).\(^{(39)}\). 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 \((40-42)\). Opening of Ca\(^{2+}\) channels is a vital event in depolarization-mediated changes in \([\text{Ca}^{2+}]_i\), 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 filaments of \(\alpha\)- and \(\beta\)-actin are attached to the cell membrane on the dense bodies, to arrange for binding locations for the myosin thick filaments. 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.

![Diagram illustrating the distribution of α-adrenergic, β-adrenergic, and muscarinic cholinergic receptors in the urinary bladder.](image)

**Figure 1-6: Diagram illustrating the distribution of α-adrenergic, β-adrenergic, and muscarinic cholinergic receptors in the 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 the urinary bladder and a small number of β-adrenergic receptors. Cholinergic muscarinic receptors are found throughout 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 $\beta$-adrenoreceptors results in relaxation of smooth muscle cells (44,45), and activation of $\alpha$-adrenoceptors leads to contraction of smooth muscle (44,46). The expression of $\alpha$- and $\beta$-adrenoceptors differs in the bladder wall; $\beta$-adrenoceptors have been shown to dominate over $\alpha$-adrenoceptors (47). There are three forms of $\beta$-adrenoceptors present in the detrusor smooth muscle cells; $\beta_1$, $\beta_2$, and $\beta_3$ with $\beta_3$ being the most abundant (48). The $\beta$-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 $\alpha$-adrenoceptors, and little number of $\beta$-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 $\text{Na}^+$ and $\text{Ca}^{2+}$ current, that activates L-type $\text{Ca}^{2+}$ channels to produce an action potential and consequently cause an increase in intracellular calcium [$\text{Ca}^{2+}]_i$ to induce smooth muscle contraction (54). There are eight subtypes of P2Y receptors (P2Y$_{1, 2, 4, 6, 11, 12, 13, \text{ and } 14}$), 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 Gβγ 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 Gβγ 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).

![Diagram of the roles of ATP and purinergic receptors in the micturition process](image)

*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 P2X₁ and muscarinic (M₃) receptors, correspondingly to cause detrusor smooth muscle contraction (59).
The presence of P2Y2 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, P2Y1 receptor subtype expression is increased in obstructed bladder (61). Previous data indicate that ATP acts on P2X2 and P2X3 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\textsubscript{3} and/or P2X\textsubscript{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<sub>1</sub>-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 nocturia, 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<sub>1</sub>-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-
The muscarinic receptors mainly found in the human bladder are of the M\textsubscript{2} (80\%) and M\textsubscript{3} (20\%) subtypes (75) but M\textsubscript{1}, M\textsubscript{4} and M\textsubscript{5} subtypes have also been identified, all of which are coupled to G proteins (76). In humans, the M\textsubscript{3} receptors are assumed to be the most important for inducing detrusor contraction (34). The function of M\textsubscript{2} receptors has not yet been clarified and their action may increase in some pathological conditions (77). M\textsubscript{1}, M\textsubscript{5}, and M\textsubscript{5} receptors couple with heterotrimeric G\textsubscript{aq/11}, activating phospholipase-C (PLC) (71) Activation of this enzyme generates the second messengers IP\textsubscript{3} and diacylglycerol (DAG) from membrane phosphoinositides (PIP\textsubscript{2}). IP\textsubscript{3} binds to receptors on the sarcoplasmic reticulum and releases intracellular Ca\textsuperscript{2+} into the sarcoplasm (78) (Figure 7). The M\textsubscript{2} and M\textsubscript{4} receptors are coupled to a pertussis toxin sensitive G\textsubscript{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\textsubscript{3}-receptors that are coupled to G\textsubscript{aq/11}, a member of the G-protein superfamily. Recruitment of G\textsubscript{aq/11} to the agonist-bound M\textsubscript{3}-receptor activates phospholipase C (PLC), leading to the generation of inositol 1, 4, 5-triphosphate (IP\textsubscript{3}) and diacylglycerol (DAG). Liberation of intracellular Ca\textsuperscript{2+} from stores is trigged by IP\textsubscript{3} interacting with its IP\textsubscript{3} receptor in the sarcoplasmic reticulum. Increasing intracellular [Ca\textsuperscript{2+}], and DAG levels stimulates protein kinase C (PKC) activity (37) (Figure 1-11). The M\textsubscript{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 Gα_{5/11} 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 Gα_q/11 but not to Gα_s, Gα_i or Gα_{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 Gβγ and phosphatidylinositol 4,5-bisphosphate (PIP2) binding (91). In addition, the N-terminal RGS-like domain has been shown to bind to stimulated GTP-bound Gaq/11 (90), so not merely inhibit the interact of Gaq/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 initiation of multiple intracellular signalling routes, including second messenger pathways controlled by adenyl cyclases 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 M3/ P2Y receptors to associate with heterotrimeric Gαq-mediated activation of phospholipase C (PLC) and the production of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol, leading to intracellular Ca2+ release and activation of protein kinase C, respectively and promoting bladder contraction. While the smooth muscle relaxation occur when the agonist associate with heterotrimeric Gι/ι0, 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).
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 Gαq-mediated activation of phospholipase C (PLC) and the production of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol, leading to intracellular Ca2+ 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 (β₁, β₂, and β₃ receptors), stimulation of these receptors leads to detrusor smooth muscle relaxation (10). Mirabegron is β₃ 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 Ca²⁺ 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 of the lower urinary tract is partially mediated by purinergic signalling. Purinergic receptor activated 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- H2O2, 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, Na₂HPO₄ 0.44 mM, NaH₂PO₄ 0.42 mM, Glucose 10 mM, HEPES 10 mM, MgCl₂ 1 mM) was prepared without CaCl₂. 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 CaCl₂ buffer containing (18 mg of bovine serum albumin and 100 µM CaCl₂ 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 CaCl₂ 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 Ca²⁺ solution and washed twice with 5 mL of low Ca²⁺ solution without BSA to remove the BSA. Finally, the minced tissue was washed with 5 mL of dissection solution without CaCl₂.

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 CaCl₂) 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% CO₂ 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](image)

**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 37°C in 5% CO₂ 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 37°C in 5% CO₂ 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% CO₂) 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 which 20 µL of suspension cells were placed in the haemocytometer and cells were counted in each 2 x 1 mm² 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 $10^{3}$, 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

<table>
<thead>
<tr>
<th>Target protein</th>
<th>Primary antibody dilution</th>
<th>Secondary antibody</th>
<th>suppliers</th>
<th>Secondary antibody dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRK2</td>
<td>1 : 1000</td>
<td>Anti-rabbit, Anti-biotin</td>
<td>Santa Cruz</td>
<td>1 : 1000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 : 2000</td>
</tr>
<tr>
<td>GRK3</td>
<td>1 : 1000</td>
<td>Anti-rabbit, Anti-biotin</td>
<td>Santa Cruz</td>
<td>1 : 1000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 : 2000</td>
</tr>
<tr>
<td>GRK5</td>
<td>1 : 500</td>
<td>Anti-rabbit, Anti-biotin</td>
<td>Santa Cruz</td>
<td>1 : 1000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 : 2000</td>
</tr>
<tr>
<td>GRK6</td>
<td>1 : 1000</td>
<td>Anti-rabbit, Anti-biotin</td>
<td>Santa Cruz</td>
<td>1 : 3000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 : 2000</td>
</tr>
<tr>
<td>Arrestin 2/3 (A1CT)</td>
<td>1:1000</td>
<td>Anti-rabbit</td>
<td>Lefkowitz laboratory</td>
<td>1: 1000</td>
</tr>
<tr>
<td>α-actin</td>
<td>1 :1000</td>
<td>Goat anti-Mouse</td>
<td>Santa Cruz</td>
<td>1:400</td>
</tr>
<tr>
<td>calponin</td>
<td>1 :10000</td>
<td>Goat anti-Mouse</td>
<td>Santa Cruz</td>
<td>1:400</td>
</tr>
</tbody>
</table>
2.1.8. Measurement of intracellular $[\text{Ca}^{2+}]_i$ 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 pinhole, 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).

![Image of laser scanning confocal inverted microscope](image_url)

**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\textsuperscript{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\textsuperscript{2+} sensitive dye (4 µM Fluo4-AM at room temperature for 1 h). Cells were washed with Kerbs buffer prior to challenge. Selective P2Y\textsubscript{2/4/6}-receptor agonists (Tocris, UK), then the antagonist was added and the changes in intracellular Ca\textsuperscript{2+} levels, \([\text{Ca}^{2+}]_i\), 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: \textsuperscript{D110A,K220R}GRK, \textsuperscript{D110A,K220R}GRK3, \textsuperscript{K215R}GRK5, \textsuperscript{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 Amaza 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’-GCAGGUACCUCAGAUCCTtt-3’), anti-arrestin2 (5’-GCCACUGACUGCACAAAtt-3’) or anti-arrestin3 (5’-GCCUUCUGUGCCAUAUAtt-3’) siRNAs (Applied Biosystems, UK) at a final concentration of 10 nM, were added as required. Next cells were transferred to Amaza-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% CO₂ 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

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>137 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>5.4 mM</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>0.44 mM</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$</td>
<td>0.42 mM</td>
</tr>
<tr>
<td>Glucose</td>
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</tr>
<tr>
<td>HEPES</td>
<td>10 mM</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>1 mM</td>
</tr>
</tbody>
</table>

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

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<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled H$_2$O</td>
<td>7.9 mL</td>
<td></td>
</tr>
<tr>
<td>Acrylamide</td>
<td>6.7 mL</td>
<td></td>
</tr>
<tr>
<td>1.5M Tris HCl</td>
<td>5 mL</td>
<td></td>
</tr>
<tr>
<td>SDS</td>
<td>200 μL</td>
<td>Do not add AMPS or TEMED until immediately before use</td>
</tr>
<tr>
<td>10% AMPS</td>
<td>200 μL</td>
<td></td>
</tr>
<tr>
<td>TEMED</td>
<td>8 μL</td>
<td>In metal yellow cupboard</td>
</tr>
</tbody>
</table>

ml=millilitre, μl=microlitre

2.1.12.3. Stacking gel composition (per 2 gels)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled H$_2$O</td>
<td>5.5 mL</td>
<td></td>
</tr>
<tr>
<td>Acrylamide</td>
<td>1.3 mL</td>
<td>On bench shelf</td>
</tr>
<tr>
<td>1M Tris</td>
<td>1 mL</td>
<td>Do not confuse with 1.5M</td>
</tr>
<tr>
<td>SDS</td>
<td>80 μL</td>
<td>On bench shelf</td>
</tr>
<tr>
<td>10% AMPS</td>
<td>80 μL</td>
<td>Make when required (see point 3)</td>
</tr>
<tr>
<td>TEMED</td>
<td>8 μL</td>
<td>In metal yellow cupboard</td>
</tr>
</tbody>
</table>

M=molar, ml=millilitre, μl=microliter
2.1.12.4. Running buffer

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>10x TGS running buffer</td>
<td>100 mL</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>900 mL</td>
</tr>
</tbody>
</table>

Running buffer was supplied as a 10X concentrate, which was diluted to 10X working stock in distilled water (dH₂O) TGS is 25mM Tris, 192mM glycine and 0.1% SDS

2.1.12.5. Semi-dry blotting buffer

<p>| | |</p>
<table>
<thead>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled H₂O</td>
<td>600 mL</td>
</tr>
<tr>
<td>Tris-Base</td>
<td>48 mM</td>
</tr>
<tr>
<td>Glycine</td>
<td>39 mM</td>
</tr>
<tr>
<td>SDS</td>
<td>0.037 %</td>
</tr>
<tr>
<td>Methanol</td>
<td>20 %</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>Make up to 1 L</td>
</tr>
</tbody>
</table>

L=litre, mL=millilitre, g=gram

2.1.12.6. TBS-T

<p>| | |</p>
<table>
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</thead>
<tbody>
<tr>
<td>5M NaCl</td>
<td>60mL</td>
</tr>
<tr>
<td>1M Tris</td>
<td>20mL</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>1950mL</td>
</tr>
<tr>
<td>pH adjusted to 7.5</td>
<td>by slowly adding 1M NaOH</td>
</tr>
<tr>
<td>Tween 20</td>
<td>1mL</td>
</tr>
</tbody>
</table>

M=molar, mL=millilitre

2.1.12.7. Krebs-Henseleit buffer

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<table>
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</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>134 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>6 mM</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1 mM</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.2 mM</td>
</tr>
<tr>
<td>Glucose</td>
<td>10 mM</td>
</tr>
<tr>
<td>HEPES</td>
<td>10 mM</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>1.3 mM</td>
</tr>
<tr>
<td>pH 7.4</td>
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mM=millimolar
2.1.12.8. Lysis Buffer

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<tbody>
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</tr>
<tr>
<td>HEPES</td>
<td>20 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>200 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>10 mM</td>
</tr>
<tr>
<td>Triton-X 100</td>
<td>1%</td>
</tr>
<tr>
<td>Benzamidine</td>
<td>0.2 mg/mL</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>0.1 mg/mL</td>
</tr>
<tr>
<td>Phenylmethylsulphonylfluoride</td>
<td>0.5 mM</td>
</tr>
</tbody>
</table>

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 °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).
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\textsuperscript{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\textsuperscript{2+} and therefore induce contraction. Consequently we examined whether DSMC respond to the purinergic agonists ATP and UTP. DSMC loaded with the Ca\textsuperscript{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\textsuperscript{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\textsuperscript{2+}], suggesting the presence of P2Y and/or P2X receptors (Figure 3-3).
Figure 3-3: Representative traces showing changes in intracellular Ca\textsuperscript{2+} levels

When DSMC loaded with the Ca\textsuperscript{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\textsuperscript{2+}], 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.
**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\textsuperscript{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\textsuperscript{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\textsubscript{50} values of 12.5 µM for UTP).
Figure 3-5: 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 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 P2Y1 receptor agonist MRS2365-mediated elevation of \([\text{Ca}^{2+}]_i\)

As ADP could stimulate 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 EC50 value of 9.4 µM for MRS2365 (Figure 3-7).

![Figure 3-7: Calcium response curves of P2Y1 receptor challenged with MRS2365](image)

DSMC seeded into 96 well plates were loaded with Fluo-4-AM (4 µM, for 1 h) before challenge with various concentration of the P2Y1 selective agonist MRS2365 (0.1 nM to 100 µM). \([\text{Ca}^{2+}]_i\) changes were monitored using a microplate reader (NOVOstar) imaging system. Florescence changes were measured as the maximum \([\text{Ca}^{2+}]_i\) response to agonist minus the basal \([\text{Ca}^{2+}]_i\) 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 \( [\text{Ca}^{2+}]_i \) 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) compared to first (R1). Data represent means ± SEM \([\text{Ca}^{2+}]_i\) 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 $[\text{Ca}^{2+}]_i$ changes in DSMC

To further examine whether cultured rat DSMC expressed functional P2X receptors, cells were challenged with the non-selective P2X receptor agonist $\alpha,\beta$-methylene adenosine 5'-triphosphate. DSMC were loaded with calcium dye Flou-4 and initially challenged with $\alpha,\beta$-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 $\alpha,\beta$-methylene adenosine 5'-triphosphate failed to promote changes of $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_i$ in Fluo4-AM loaded DSMC

(A) Representative trace showing the effects of the non-selective P2X receptor agonist $\alpha,\beta$-methylene adenosine 5'-triphosphate (20 μM, 30 sec) on $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_i$ signals after stimulation with ADP (*p<0.05, Wilcoxon test; when compared to $\alpha,\beta$-methylene adenosine 5'-triphosphate treatment). Data are expressed as means ± SEM $[\text{Ca}^{2+}]_i$ 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\textsuperscript{2+} signalling in isolated rat DSMC

Previous reports suggest that rat DSMC express a number of different P2Y receptors, including P2Y\textsubscript{1}, P2Y\textsubscript{2}, P2Y\textsubscript{4} and P2Y\textsubscript{6}, which may contribute to the detected Ca\textsuperscript{2+} signals (32). To investigate which P2Y receptor subtypes predominantly mediate PLC/Ca\textsuperscript{2+} signalling in DSMC, cells were loaded with the Ca\textsuperscript{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\textsubscript{1} receptor (100 nM) for 30 sec (Figure 3-11). DSMC also were challenged with the P2Y\textsubscript{4} and P2Y\textsubscript{6} receptors selective agonists (MRS4062 100 nM) and (MRS2693 10 nM), respectively. Our data suggested that MRS2365 (P2Y\textsubscript{1} receptor agonist) evokes Ca\textsuperscript{2+} signalling, yet addition of MRS4062 and MRS2693 did not produce any [Ca\textsuperscript{2+}]\textsubscript{i} signals in cultured rat DSMC. Indicating that the presence of P2Y\textsubscript{1} but not P2Y\textsubscript{4} or P2Y\textsubscript{6} receptors. It is worth noting that stimulation of DSMC with very high concentrations of the selective P2Y\textsubscript{4} (MRS4062; 10 μM) or P2Y\textsubscript{6} (MRS2693; 10 μM) receptor agonists produced increased [Ca\textsuperscript{2+}]\textsubscript{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\textsubscript{1} receptors and absence of P2Y\textsubscript{4} and P2Y\textsubscript{6} in our DSMC cultures.
Figure 3-11: Pharmacological profile of P2Y receptor activity in DSMC.

(A) Representative trace showing the [Ca\textsuperscript{2+}]\textsubscript{i} changes in Fluo4-AM loaded cells, following challenge with either the P2Y\textsubscript{1} (MRS2365, 100 nM), P2Y\textsubscript{4} (MRS4062, 100 nM) and or P2Y\textsubscript{6} (MRS2693, 10 nM), selective agonists. (B) Cumulative data shown as means ± SEM [Ca\textsuperscript{2+}]\textsubscript{i} changes from 20-30 cells from three separate preparation animals. Significant changes in [Ca\textsuperscript{2+}]\textsubscript{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\textsuperscript{2+}]\textsubscript{i} when DSMC were exposed to high concentrations of (C) MRS4062 (10 μM) or (D) MRS2693 (10 μM).
3.5.12. Effects of the P2Y₁ selective antagonist MRS2279 on ADP–stimulated calcium signalling in isolated DSMC

MRS2279 (2-chloro-\(N^6\)-methyl-(\(N\))-methanocarba-2’-deoxyadenosine-3’,5’ bisphosphate) is a selective high affinity competitive antagonist of the P2Y₁ receptor. In order to confirm the presence of P2Y₁ 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 P2Y₁ selective antagonist MRS2279 (20 μM) during the washout period, our result suggested that DSMCs do indeed express endogenous P2Y₁ receptors.

![Figure 3-12: Effects of the P2Y₁ selective antagonist MRS2279 on ADP stimulated calcium signalling in isolated DSMC.](image)

Representative traces showing the \([\text{Ca}^{2+}]_\text{i}\) 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 P2Y₁ 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 \([\text{Ca}^{2+}]_\text{i}\) 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 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-
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: P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12 and P2Y13 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 P2Y1 and P2Y2 receptors, but not expression of P2X, P2Y4 or P2Y6 in cultured rat DSMC. Nevertheless, previous studies reported the expression of P2Y1, P2Y2, and P2Y4 in the urothelium of cat (30), and P2Y6 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 P2Y2, UDP P2Y6 and ADP P2Y12 receptors), endothelium (ADP P2Y1, UTP/ATP P2Y2, UDP P2Y6), myocardium (ATP/UTP P2Y2, UDP P2Y6, ATP P2Y11), and platelets (ADP P2Y1, ADP P2Y12) (166). Von Kugelgen (2006) and Abbracchio et al (2006) confirmed the expression of P2Y2,4,6 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 P2Y1,11 in the same tissues (169). Immunocytochemical studies, provided evidence for the expression of P2Y2 and possibly P2Y4 receptor staining in the normal rat urinary bladder. However, P2Y2 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 P2Y2 (32) and to a lesser extent P2Y4 (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 P2Y1 receptors which evoked an increase of [Ca^{2+}]_i signals in DSMC.

To confirm the presence of P2Y1 receptor in rat DSMC, cells were challenged with selective P2Y1 receptor agonist (MRS2365), a full agonist of P2Y1 receptors (157). Our result showed [Ca^{2+}]_i signals were evoked by ADP and P2Y1 selective agonist (MRS2365; EC_{50} = 0.40 nM), suggesting that P2Y1 purinergic receptors present in rat DSMC. Previous study showed that this P2Y1 receptor agonist (MRS2365; EC_{50} = 8.56 ± 0.06) has evoked concentration-dependent increase in [Ca^{2+}]_i (156). On the other hand, P2Y1 receptors antagonist (MRS2279) is a selective antagonist with high affinity to the P2Y1 receptor; this antagonist confirmed the ability to block P2Y1 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 P2Y1 purinergic receptor.

Rat DSMC were also challenged in this study with both P2Y4 and P2Y6 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 P2Y4 and P2Y6 receptors. I also used P2Y2 receptor antagonist (AR-C118925XX; 10 µM) to assess its effect on [Ca^{2+}]_i signalling and confirm the presence of P2Y2 receptors in cultured rat DSMCs, and this also blocked the [Ca^{2+}]_i signalling. This suggested that P2Y2 receptor present in rat DSMC that investigated in this study. In a previous study, the P2Y2 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 P2Y2 or P2Y4 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$_{50} = 6.9 \pm 2.45$ μM) of rats with pulmonary arterial hypertension, as compared to rats with no evidence of pulmonary arterial hypertension (EC$_{50} = 19 \pm 5.01$ μM). In the same way, ATP-dependent venous contraction was strongest in rats with pulmonary arterial hypertension (EC$_{50} = 14.3 \pm 1.9$ μM) as compared to healthy rats (EC$_{50} = 28.6,3 \pm 1.1$ 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-stimulated 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$^{2+}$], indicating the presence of purinergic receptors. Further application of selective P2Y and P2X agonist and/or antagonists highlighted that the changes in [Ca$^{2+}$] 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.
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<sub>1</sub> and P2Y<sub>2</sub> 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 Gαq and suppress PLC signalling (90,103,175), we used the double mutated version (i.e. \( \text{D110A,K220R} \) GRK2 or \( \text{D110A,K220R} \) 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 MgCl₂, 1.2 mM KH₂PO₄, 10 mM glucose, 10 mM HEPES, 1.3 mM CaCl₂; 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+}]], changes are displayed as the fluorescence emission relative to basal (F/F₀). Agonists were applied via the perfusion line as indicated. Briefly, to determine P2Y receptor desensitization, DSMC were exposed to an approximate EC₅₀ 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 EC₅₀ 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 P2Y₁ and P2Y₂.

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), D110A, K220R GRK2 (176), K215R GRK5, or 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 Gαq/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\textsuperscript{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\textsubscript{1} and P2Y\textsubscript{2} receptor populations, and should provide an 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 washout 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\textsuperscript{2+}]\textsubscript{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 \(\geq3\) 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 P2Y₁ receptor stimulated [Ca²⁺]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 P2Y₁ 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).
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 P2Y2 receptor-stimulated PLC signalling mediated by UTP signalling in isolated DSMC

Since P2Y1 receptors are not activated by UTP (156) and our evidence suggests that neither P2Y4 nor P2Y6 receptor are present in our cultures we assumed that UTP is stimulating only P2Y2 receptors in DSMCs. Previous work from our group has highlighted that in vascular smooth muscle cells purinergic calcium signals generated by P2Y2 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 EC50 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 P2Y2 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 P2Y2 receptor activity.
Figure 4-4: Desensitization and re-sensitization of P2Y₂ receptor-stimulated Ca²⁺ signalling in DSMC.

Cells loaded with the Ca²⁺-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 [Ca²⁺]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 \([\text{Ca}^{2+}]_i\) 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).

![Western blots showing protein expression for GRK2 and GRK5 in cultured DSMC and whole bladder.](image)

The immunoblot band was detected at 80 kDa and 66kDa for GRK2 and GRK5, respectively.
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 P2Y₁-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 P2Y₁ 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 P2Y₁-receptor-mediated PLC/Ca²⁺ signalling. These findings strongly suggest that GRK3 is a key mediator of ADP-induced P2Y₁ receptor desensitization.
Figure 4-7: GRK3 inhibition decreases P2Y₁-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 [Ca²⁺]ᵢ changes in cells transfected with pcDNA3 (A), D₁₁₀A,K₂₂₀R GRK2 (B), D₁₁₀A,K₂₂₀R GRK3 (C), K₂₁₅R GRK5 (D), or K₂₁₅R GRK6 (E). P₂Y₁ 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 P2Y₂-purinoceptor mediated PLC signalling in DSMC

4.5.4.1. Effects of dominant-negative GRKs expression on P2Y₂ receptor PLC signalling in DSMC

Analysis of P2Y₂ 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 P2Y₂-receptor-mediated PLC/Ca²⁺ signalling. These findings strongly suggest that GRK2 is a key mediator of UTP-induced P2Y₂ 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 H₁) (174) or exclusively regulated by GRK6 (oxytocin) (130). To study histamine H₁ receptor desensitization an identical protocol was applied as that utilised for the P2Y₂ receptor, however, in this case cells were previously transfected with the extensively characterised IP₃ biosensor eGFP-tagged PH domain of PLC₅ (0.5 µg, for 48 h) (37,109,130,174). Receptor/PLC activity was assessed by measuring IP₃ 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 (H₁) 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 H₁ 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, Rₘₐₓ, R₂) protocol (see Methods). Representative traces show the changes in IP₃ 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 IP₃ (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$_3$ (R$_2$ relative to R$_1$) 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\textsubscript{2} but not P2Y\textsubscript{1} receptor signalling, one would expect paroxetine to block P2Y\textsubscript{2} but not P2Y\textsubscript{1} receptor desensitization. To test this theory DSMC were loaded with the Ca\textsuperscript{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\textsubscript{2} receptor desensitization protocol (R1, R2 = 10 μM UTP, for 30 sec; R_{\text{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\textsubscript{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\textsubscript{2} receptor PLC activity in DSMC (Figure 4-12).

To examine the specificity of paroxetine’s actions on P2Y\textsubscript{1} receptor activity we examined whether paroxetine could alter the desensitization of P2Y\textsubscript{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\textsubscript{1} receptor desensitization.
Figure 4-12: Paroxetine but not fluoxetine inhibits the desensitization of P2Y₂ receptor PLC activity in DSMC.

Cells were loaded with the Ca²⁺-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 [Ca²⁺]. D) Cumulative data show the means ± SEM [Ca²⁺], 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 P2Y1 receptor PLC activity in DSMC by paroxetine and fluoxetine.

Cells were loaded with the Ca\(^{2+}\)-sensitive dye Fluo4-AM (4 \(\mu\)M) and incubated with paroxetine or fluoxetine (5 \(\mu\)M) for 1 h, before being subjected to the standard desensitization protocol (R1, R2 = 10 \(\mu\)M 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 P2Y₁, P2Y₂ purinergic receptors promote increased [Ca²⁺], in rat DSMC, thus are likely to induce contraction of the urinary bladder. Consequently, it is likely that P2Y₁ and P2Y₂ 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 P2Y₁ and P2Y₂ 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 P2Y₂-receptor PLC signalling was subject to desensitization in isolated mesenteric smooth muscle cells MSMC (109). Using similar protocols, I have shown that Ca²⁺ measured using (Fluo4) can be used to assess purinergic receptor desensitization in the DSMC. P2Y₂ 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 P2Y₂ receptor activity. Desensitization experiments show that after a 5 minutes washout period [Ca²⁺] signals were markedly suppressed. P2Y₂ receptor responses had recovered gradually over time, and recovering totally after 15 minutes. However, P2Y₁ responses remained suppressed after 10 minutes. Interestingly, these findings suggest an initial desensitization of receptor activity followed by re-sensitization of P2Y₂, but not P2Y₁ receptor responsiveness.

Since GPCR desensitization is usually mediated through the action of GRK proteins we next investigated the expression of GRK isozymes 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\(_\delta\) (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 histamine (H\(_1\)) receptor 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 \( \text{P2Y}_2 \) 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 \( \text{P2Y}_1 \) 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 \( \text{P2Y}_1 \) 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 \textit{in vitro} and \textit{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 \( \text{P2Y} \) 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 resensitization kinetic of endogenously expressed P2Y₁ and P2Y₂ receptor mediated PLC signalling in rat DSMC. Both P2Y₁ and P2Y₂ 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 P2Y₂ receptor desensitization. In addition, the putative GRK2 inhibitor paroxetine was able to prevent the desensitization of P2Y₂ receptor PLC activity in DSMC. Collectively these findings suggest that GRK2 is the key kinase in regulating P2Y₂ receptor driven PLC activity. In contrast, DNM GRK3 attenuated P2Y₁ receptor desensitization, suggesting a selective role for GRK3 in the negative regulation of P2Y₁ 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 P2Y₁ receptor desensitization was attenuated by expression of the GRK3 dominant negative construct. In contrast, P2Y₂ 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 P2Y₁ and GRK2 phosphorylating P2Y₂ receptors. Since GRK mediated GPCR phosphorylation is a prelude to subsequently promote arrestin recruitment it is likely that both P2Y₁ and P2Y₂ 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 P2Y₂ activated PLC signalling in rat arterial smooth muscle cells (108). Further studies utilising exogenous P2Y receptors expressed in model cells systems indicate that both P2Y₁ and P2Y₂ receptors are capable of recruiting arrestin
subtypes (189) collectively, these data suggest that agonist-occupied P2Y₁ and P2Y₂ 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 P2Y₁ and P2Y₂ 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 (IP₃) or calcium to assess the effects of arrestin knockdown on P2Y₁ and P2Y₂ 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/P2Y₁- and UTP/P2Y₂-stimulated IP₃ and Ca²⁺ 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 2x10⁶ 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 37°C and 5% CO₂ 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, 175cm² 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 2x10⁶ 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′-GCCACUGACUCGCUACAAAtt-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% CO₂ 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 PLCδ1 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 P2Y1-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 IP3 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 IP3 and Ca2+ 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 (F0) (F/F0). Selective depletion of arrestin3 markedly reversed ADP-induced decreases in R2 relative to R1 which inhibit P2Y1 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 P2Y₁ 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 IP₃ were measured as the change in cytosolic fluorescence. Representative traces show ADP-stimulated (10 μM; R1 and R2; for 30 sec) IP₃ 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 IP₃ 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\textsubscript{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\textsubscript{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/R\text{max}/R2) desensitization protocol. DSMC were challenged with approximate EC\textsubscript{50} concentration of UTP (10 µM) for 30 s before (R1) and after (R2) addition of a maximal UTP concentration (R\text{max}: 100 µM, for 30 s). The data shown inhibition of arrestin3 expression enhanced UTP-stimulated P2Y\textsubscript{2} receptor signalling (Figure 5-4). While suppression of arrestin2 was less effect on P2Y\textsubscript{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 P2Y2 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/Rmax/R2 desensitization protocol. AR3 depletion significantly enhanced UTP-stimulated IP3 signals when compared with negative control, and arrestin 2- transfected cells significantly enhanced UTP-stimulated IP3 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 P2Y₁ and P2Y₂ receptor PLC function was determined by assessing changes in IP₃ production in DSMC. However, as IP₃ 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 P2Y₁ and P2Y₂ 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).
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 P2Y2 receptor desensitization, whilst depletion of GRK3 prevented P2Y1 receptor desensitization. Interestingly, our study demonstrated the first report that P2Y1 and P2Y2 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 P2Y2 receptor PLC signalling in rat VSMC (37,109), suggesting a common role for this protein in the regulation of P2Y2 receptor contractile signalling in smooth muscle cells. However, although arrestin regulation of P2Y2/PLC activity in VSMC is exclusive (109), in DSMC arrestin3 appears equally adept at inducing P2Y2 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 Goq/11 but not to GoS, Gai or Gα12/13 to inhibit PLC-β activation (38). GPCR activation drives the initiation of multiple intracellular signalling routes, including second messenger pathways controlled by adenyllyl 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 P2Y₁ and P2Y₂ PLC signalling in DSMC. These data suggest despite that fact that P2Y₁ receptors are substrates for GRK3 and P2Y₂ 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).
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 M₃ 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 37°C 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.](image)

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 ± 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 M₃ 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 potentially 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 P2Y₂ receptor activity it is likely that obstructive bladder leads to increased desensitization of P2Y₂ receptor activity and thus reduced contractile force through P2Y₂/PLC signalling. Contrastingly, GRK3 expression was reduced, which suggests that during bladder obstruction P2Y₁ 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 M₃ receptor PLC signalling (185) may reflect a physiological to enhance DSMC contraction to acetylcholine during periods of bladder obstruction (185). In constrast 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), D$_{110}$$A$,K$_{220}$$R$GRK3, K$_{215}$$R$GRK5, or K$_{215}$$R$GRK6 showed that the changes in R2 responses compared with R1 were comparable to those observed in non-transfected cells show the presence of D$_{110}$$A$,K$_{220}$$R$ 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 P2Y2 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 P2Y1 receptor desensitization, as a result of that arrestin3 depletion enhanced ADP-stimulated P2Y1 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 P2Y2 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\textsubscript{1} and P2Y\textsubscript{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.
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