TRPV4 receptors in bladder physiology and age-related pathology – a potential novel target for treatment

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ABSTRACT

Bladder dysfunctions associated with overactive bladder are highly prevalent in the aging population and impose a huge financial cost, however the underlying mechanisms are poorly understood. The newly recognised sensory role of the urothelium provides a new direction of research into the pathogenesis of overactive bladder. As the activity of this sensory structure is highly important for correct bladder function, it is imperative to identify new regulators of this tissue. A role for transient receptor potential vanilloid 4 (TRPV4) channels in bladder function has been recently evidenced. This work aimed to explore the role of TRPV4 in urothelial ATP release, the key step in urothelial sensory transduction, elucidate the underlying mechanisms and assess changes to TRPV4 expression and function in aging and overactive bladders.

Immunohistochemistry and western blotting assessed the expression and localization of TRPV4 throughout the bladder. Bladder strips from animals and humans were challenged with the selective TRPV4 agonist GSK1016790A and ATP release and tissue contractility measured. Various antagonists were employed to uncover the mechanisms. The role of Ca^{2+} in TRPV4-mediated responses was explored using live-cell Ca^{2+} imaging. The effect of TRPV4 activation on reactive oxygen species production was also measured. The interaction between TRPV4 and the principal urothelial receptor P2Y was assessed, supplemented with P2Y_{2} knockout mice. The responses between young and aging guinea pigs and normal and overactive human bladders were compared.

This is the first study to establish a role for TRPV4 in mucosal ATP release and the underlying mechanisms. This study demonstrates increased TRPV4 expression with age and a functional link between TRPV4 and P2Y receptors as a result of aging. This work also provides preliminary evidence for an increased TRPV4-mediated ATP release in overactive human bladders. These novel findings identify a fundamental role for TRPV4 in bladder physiology and pathophysiology and present experimental evidence that pharmacological manipulation of this receptor may provide a novel method for treatment of overactive bladder.
AUTHORS DECLARATION

I confirm that the submitted work is my own work and that I have clearly identified and fully acknowledged all material that is entitled to be attributed to others (whether published or unpublished) using the referencing system set out in the programme handbook. I agree that the University may submit my work to means of checking this, such as the plagiarism detection service Turnitin® UK. I confirm that I understand that assessed work that has been shown to have been plagiarised will be penalised.

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Date 20.09.16
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<th>Definition</th>
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<tr>
<td>$[\text{Ca}^{2+}]$</td>
<td>Calcium concentration</td>
</tr>
<tr>
<td>$[\text{Ca}^{2+}]_i$</td>
<td>Intracellular calcium concentration</td>
</tr>
<tr>
<td>2AG</td>
<td>2-Arachidonoylglycerol</td>
</tr>
<tr>
<td>4α-PDD</td>
<td>4alpha-phorbol 12,13 didecanoate</td>
</tr>
<tr>
<td>A$_{1-3}$</td>
<td>Adenosine receptor subtypes</td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ABMA</td>
<td>alpha-beta methylene-ATP</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AEA</td>
<td>Anandamide</td>
</tr>
<tr>
<td>AF-1</td>
<td>Antifadent-1 compound</td>
</tr>
<tr>
<td>AIP$_4$</td>
<td>Atrophin-interacting protein 4</td>
</tr>
<tr>
<td>ARs</td>
<td>Adrenoreceptors</td>
</tr>
<tr>
<td>ASIC</td>
<td>Acid-sensing ion channel</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>B1-2</td>
<td>Bradykinin receptor subtypes</td>
</tr>
<tr>
<td>Benz</td>
<td>Benzamil</td>
</tr>
<tr>
<td>BM</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>BOA</td>
<td>Bladder overactivity</td>
</tr>
<tr>
<td>Bref A</td>
<td>Brefeldin A</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CaCC</td>
<td>$\text{Ca}^{2+}$-activated chloride channel</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic-adenosine monophosphate</td>
</tr>
<tr>
<td>Carb</td>
<td>Carbenoxolone</td>
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<tr>
<td>CCD</td>
<td>Charge-coupled device</td>
</tr>
<tr>
<td>CCh</td>
<td>Carbachol</td>
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<tr>
<td>Cx</td>
<td>Connexin</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DDS</td>
<td>Drug delivery system</td>
</tr>
<tr>
<td>Deg</td>
<td>Degenerin</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DO</td>
<td>Detrusor overactivity</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal root ganglion</td>
</tr>
<tr>
<td>DSM</td>
<td>Detrusor smooth muscle</td>
</tr>
<tr>
<td>EC$_{50}$</td>
<td>half maximal effective concentration</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EET</td>
<td>epoxyeicosatrienoic acid</td>
</tr>
<tr>
<td>EFS</td>
<td>Electrical field stimulation</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>Em</td>
<td>Emission</td>
</tr>
<tr>
<td>ENaC</td>
<td>Epithelial sodium channel</td>
</tr>
<tr>
<td>FPH</td>
<td>Frimley park hospital</td>
</tr>
<tr>
<td>Gen</td>
<td>Genistein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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</tr>
<tr>
<td>Pi</td>
<td>Protease inhibitor</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol 4,5-</td>
</tr>
<tr>
<td></td>
<td>bisphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLA₂</td>
<td>Phospholipase A2</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PMC</td>
<td>Pontine micturition centre</td>
</tr>
<tr>
<td>PTK</td>
<td>Protein tyrosine kinase</td>
</tr>
<tr>
<td>RBL</td>
<td>Rat brain lysate</td>
</tr>
<tr>
<td>RO</td>
<td>Reverse osmosis</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RSCH</td>
<td>Royal surrey county hospital</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>S2-S4</td>
<td>Somatic spinal regions</td>
</tr>
<tr>
<td>SFKs</td>
<td>Src-family tyrosine kinase</td>
</tr>
<tr>
<td>SM</td>
<td>Smooth muscle</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>T10-L2</td>
<td>Thoracolumbar spinal regions</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline W/ Tween-20</td>
</tr>
<tr>
<td>TCC</td>
<td>Transitional cell carcinoma</td>
</tr>
<tr>
<td>TG</td>
<td>Trigeminal</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
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<tr>
<td>TCC</td>
<td>Transitional cell carcinoma</td>
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CHAPTER 1. INTRODUCTION
1.1 THE URINARY BLADDER

The urinary bladder is a hollow, smooth muscle organ that functions to periodically store and expel urine. Control of the bladder requires complex coordinated signalling between the neurones and various components of the bladder wall to achieve the necessary relaxed phase for storage and contractile phase for voiding. Importantly therefore, the bladder must be capable of recognising the volume of urine present and sensing a threshold volume, upon which a contraction is elicited.

Disruptions in the control mechanisms for urine storage may often lead to lower urinary tract symptoms (LUTS), including urge incontinence, urgency and frequency, all of which comprise the overactive bladder syndrome (OAB) (Abrams P et al. 2002). This is a very common chronic disorder in both sexes that increases in prevalence with age, posing a major problem to our aging society (Stewart et al., 2003). Currently, a poor understanding of the causative mechanisms of OAB means specific treatments are lacking, thus presenting the immediate need for further research. Accordingly, recent advances in our understanding of bladder physiology have revealed the importance of the urothelium in mediating bladder sensation (Birder et al., 2001). Therefore, progressing our knowledge of the mechanisms underlying the sensory functions of the urothelium may provide novel drug targets for control of micturition, and indeed treatment of OAB.

1.1.1 ANATOMY OF THE LOWER URINARY TRACT

The lower urinary tract (LUT) consists of the urethra (outflow region) and the urinary bladder, the latter of which can be divided into two further components: the main bladder body and the base. The bladder base refers to the urethrovesical junction, the trigone and anterior bladder wall. The bladder body, or dome, is composed of several muscular layers forming the detrusor smooth muscle (DSM), and is internally lined by a thin mucosal layer consisting of the urothelium, basement membrane and lamina propria (LP), or suburothelium. The urothelial layer directly separates the urine from the underlying tissues and the LP lies between the basement membrane and the DSM. Schematics demonstrating the structure of the bladder are shown in figure 1–1.
Figure 1 – 1. The lower urinary tract. A: Schematic diagram depicting the structure of the LUT, including the bladder body and outflow region. B: H&E stain of a transverse section of human bladder wall, highlighting in detail the detrusor muscle, urothelial and suburothelial layers (adapted from [Chan, 2005]).

Figure 1 – 2. Organisation of the bladder wall. The urinary bladder wall consists of three highly organised cell layers; the urothelium, the lamina propria and the detrusor smooth muscle, which work together as a functional syncytium to achieve correct bladder function. The urothelium prevents urine leakage into underlying tissues and plays an important role in bladder sensory pathways, the lamina propria provides nutritional and communicative support to the adjacent tissues and the detrusor smooth muscle generates force for successful voiding.
1.1.2 BLADDER WALL MORPHOLOGY

1.1.2.1 DETRUSOR SMOOTH MUSCLE (DSM)

The DSM is formed of many cell bundles arranged into three distinct layers, where the cells of the outer and inner layers are orientated longitudinally, compared to the circularly orientated middle layer (Andersson and Arner, 2004)(figure 1-2). The specific orientation of the bladder smooth muscle cells and their interactions between one another are important, as these factors will determine what effect activity in these cells has on bladder shape, intraluminal pressure and ultimately how the bladder wall will behave. Bladder smooth muscle cells exhibit the typical smooth muscle cellular morphology – long, spindle shaped cells containing a central nucleus and a cytoplasm packed with normal myofilaments and scattered dense bodies. When fully relaxed, these cells may reach lengths of up to several hundred microns. In human detrusor, the smooth muscle cells are arranged into large bundles, commonly a few mm in diameter, surrounded by collagen-rich connective tissue. These bundles, formed from smaller sub-bundles, have no clear arrangement and run in all directions throughout the detrusor. The smooth muscle cells found within these bundles commonly exist in groups of small functional units, termed fascicles or functional modules (DeLancey et al., 2002). These modules are defined by their discrete supplies of individual intramural bladder ganglia and contraction of these modules is synchronised via various neural and hormonal control systems for correct and full bladder voiding (Drake et al., 2001).

1.1.2.2 BASEMENT MEMBRANE AND LAMINA PROPRIA

The basement membrane (BM) separates the urothelium from the lamina propria and has a composition typical of all basement membranes (including collagen IV, fibronectin, laminin), which function to maintain the spatial relationships between epithelia and the adjacent interstitial connective tissues (Deen and Ball, 1994). The lamina propria (LP) is situated between the BM and detrusor muscle and is composed of an extracellular matrix (ECM) with a rich vascular supply, lymphatic channels, elastic fibers and smooth muscle fascicles (Wein et al., 2014). The cells found directly in the LP include interstitial cells (ICs), fibroblasts, adipocytes and sensory nerve endings. The ECM functions to provide support for the surrounding cells and mediates transduction of signals between the tissue layers, importantly those from nerve endings. It is mainly composed of collagen I and III, which orientate from a loose mesh to organised bundles during filling, providing tensile strength and structure to the bladder wall. After micturition, the elastic fibres pull the bladder into its original resting shape (Andersson and McCloskey, 2014).
1.1.2.3 INTERSTITIAL CELLS

The role of interstitial cells (ICs) in the bladder is yet to be fully established. There are two distinctly different populations of ICs in the bladder, those which reside in the LP and those of the detrusor, where each population may serve different functions. ICs in the LP are vimentin-containing stellate-shaped cells which are located close to both the urothelium and nerve endings in both animals and humans (McCloskey 2013; Wiseman et al. 2003). immunofluorescent staining shows that these IC cells form connections with other nearby IC cells, creating an interconnecting cell network, mediated by connexin 43 (Cx43) gap junctions. Bladder ICs are also found in the detrusor, which comprise a further two subgroups; stellate-shaped detrusor ICs, which reside in the interstitial space between muscle bundles, and elongated ICs, which are organised in parallel lines on the boundaries of the muscle bundles (McCloskey, 2013). Both form close physical connections with detrusor nerves (Davidson and McCloskey, 2005). Although our understanding of these cells is currently limited, available evidence has indicated a role for LP ICs in coordinating local bladder signalling (Andersson and McCloskey, 2014). Of importance, suburothelial interstitial cells (myofibroblasts) form a cellular network via expression of connexin 43, in close contact with the sensory nerves (Sui et al., 2002; Wiseman et al., 2003) and have been shown to be electrically active and respond to ATP, which is released by the urothelium(Sui et al., 2008, 2004; Wu et al., 2004). This would allow an interaction with urothelial cells and amplification of “ATP signals”, hence serving as a gain of this function.

1.1.2.4 UROTHELIUM

The urothelium is the epithelial lining of the lower urinary tract that functions both as a physical barrier, separating urine from the underlying tissue, as well as actively participating in bladder signalling, as revealed by recent studies (de Groat, 2004). This internal bladder lining is a transitional epithelium composed of three cell layers, each with a morphologically distinct cell type (Figure 1 - 3).

![Figure 1 – 3. Schematic of urinary bladder urothelium. Cross section highlighting the three morphologically distinct cellular layers of this transitional epithelial lining.](image-url)
Basal cells are 5-10μm in diameter and lie across the continuous basal lamina. These cells are germinal in nature and the fusion of these cells forms intermediate cells, allowing for cell replacement and total urothelial turn over (~6 week in mice) (Martin, 1972). These intermediate cells are slightly larger, with diameters of 20μm and again fuse to form the large umbrella cells that line the bladder lumen, forming a superficial barrier layer. The umbrella cells have a diameter between 50-120μm depending on the degree of bladder stretch and function partly to prevent equilibration of substances between the urine and blood. In order to achieve this, the umbrella cells are coated in hexagonal plaques composed of crystalline proteins termed ‘uroplakins’, which increase the impermeability of the urothelial barrier. Additionally, the cell stratum of the umbrella layer contains tight junctions, impeding the exchange of ions between the urine and blood supply (de Groat, 2004).

1.1.3 MECHANICS OF DETRUSOR CONTRACTION

Correct voiding of the bladder contents requires contraction of the bladder in synchrony with relaxation of the urethra. In order to develop effective therapies for OAB and other urological pathologies through manipulation of micturition, we must possess an extensive knowledge of the control mechanisms of bladder wall contraction and relaxation, both under physiological and pathological conditions. Although the urothelium has in fact recently been found to have a vital role in the afferent sensation of bladder fullness and can exert direct effects on detrusor function, it is the detrusor that houses the contractile machinery for bladder contraction.

Detrusor contraction is achieved in a similar manner to other smooth muscle types, where cell shortening occurs via interaction between thin (α- and β-actin-containing) and thick (SM1B and SM2B myosin-containing) (Martin et al., 2007) filaments. The actin filaments are anchored to dense bodies on the cell membrane and provide binding sites for the thick myosin filaments. This system is myosin-activated, where upon activation; the myosin sub-units attach and cyclically interact with the actin filaments via cross-bridges formed by the myosin head groups. Once this interaction is established, the myosin sub-unit rotates in a rowing motion about its attached head group allowing the muscle to contract (Holmes, 1995). This interaction requires the hydrolysis of MgATP to MgADP and Pi, providing the energy for muscle shortening and force generation (Andersson and Arner, 2004).

The contractile system is myosin-activated and this activation is initiated up-stream by an increase in sarcoplasmic Ca\(^{2+}\) concentration, [Ca\(^{2+}\)] (Fry et al., 2010). Contraction is stimulated once [Ca\(^{2+}\)] is raised from basal levels of 50-100 nM, where the half-maximal activation has been determined to be around 1μM (Wu et al., 1995). This rise in [Ca\(^{2+}\)] is achieved either via entry of extracellular Ca\(^{2+}\) into the sarcoplasm through L- and T-type Ca\(^{2+}\) channels.
(Montgomery & Fry 1992; Sui et al. 2003), or by release of intracellular Ca\textsuperscript{2+} stores found in the sarcoplasmic reticulum (SR). The latter can be triggered either by Ca\textsuperscript{2+} (Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+}-release) via ryanodine receptors, or by inositol trisphosphate (IP\textsubscript{3}) via IP\textsubscript{3} receptors (Harriss et al., 1995). The resulting increase in [Ca\textsuperscript{2+}], causes activation of various Ca\textsuperscript{2+}-dependent proteins such as calmodulin (CaM). Binding of Ca\textsuperscript{2+} to CaM generates the active Ca\textsuperscript{2+}-CaM complex, which in turn binds and activates myosin light chain kinase (MLCK).

Activated MLCK is then able to phosphorylate myosin regulatory light chains, activating the contractile machinery and permitting bladder smooth muscle contraction (Andersson and Arner, 2004)(figure 1-4).

Figure 1 – 4. Diagram summarising the important nerve-activated signalling pathways in detrusor smooth muscle cells. Here, the M3 and P2X\textsubscript{1} receptor pathways are shown, both of which result in elevated [Ca\textsuperscript{2+}], levels leading to subsequent detrusor contraction. M3 receptor activation initiates cleavage of membrane phospholipids (PIP\textsubscript{2}) to inositol trisphosphate (IP\textsubscript{3}) and diacylglycerol (DAG) via phospholipase-C (PLC) activity. IP\textsubscript{3} triggers intracellular Ca\textsuperscript{2+} release from SR stores, which binds to calmodulin (CaM), in turn activating myosin light chain kinase (MLCK), facilitating the binding of myosin to actin for contraction. Active M3 receptors may also activate the rho-kinase pathway, which inhibits MLC phosphatase, also promoting contraction. P2X\textsubscript{1} receptors activated by ATP provide an additional influx pathway for cations including Ca\textsuperscript{2+}. Ca\textsuperscript{2+} influx through L-type Ca\textsuperscript{2+} channels is also shown.(Fry et al., 2010)
Relaxation of the smooth muscle requires subsequent dephosphorylation of the myosin light chain, catalysed by a myosin light chain phosphatase (MLCP). Hence, smooth muscle contraction is controlled by phosphorylation of the myosin light chain by MLCK and dephosphorylation catalyzed by MLCP. Agonist stimulation of smooth muscle not only activates MLCK, but also causes inhibition of MLCP activity resulting in a net increase in MLC phosphorylation, culminating in contraction.

Modulation of MLCK or MLCP activity can therefore attenuate the sensitivity of the contractile system. Various intracellular messengers possess the ability to modulate the activity of these enzymes and can therefore also alter contractile activity. Phosphorylation of MLCK results in decreased activity and a number of responsible kinases have been identified, including CAM kinase II, mitogen-activated protein (MAP) kinase, p21-activated kinase and cAMP-dependent kinase (PKA) (Yamaguchi, 2004). MLCP activity is also reduced upon phosphorylation, which increases the sensitivity of the system to Ca^{2+}. Importantly, Inhibition of MLCP is achieved through phosphorylation by rho-associated kinases (ROCK), which in the detrusor are activated by the rho-family G proteins ROCK I/II (Kimura et al., 1996)(figure 1-4). One study demonstrated attenuated agonist-induced detrusor contractions with several ROCK inhibitors (Y-27632, HA-1077), where these had no effect on depolarisation-mediated contractions (Durlu-Kandilci and Brading, 2006). This suggests an important role for the rho-kinase pathway in regulating bladder contraction.

### 1.1.4 SPONTANEOUS DETRUSOR ACTIVITY

Detrusor smooth muscle in the bladder exhibits involuntary spontaneous contractile activity during the filling stage of the micturition cycle (figure 1-5). This activity seems to occur in localised areas of the bladder wall within individual muscle bundles and results in small areas of the detrusor contracting in isolation, which is unusual for general smooth muscle tissue. This spontaneous activity in the bladder allows individual muscle bundles to adjust their length in response to filling and maintain muscle tone. To achieve this, spontaneous contractions remain low during the filling phase, however they seem to enhance once the bladder becomes increasingly full, resulting in a rise in intravesical pressure promoting bladder contraction (Brading, 2006). Of pathological significance, upregulation of spontaneous activity may lead to uncontrolled whole bladder contractions and contribute to bladder overactivity.
There are several hypotheses for this spontaneous activity and whether it is of neurogenic, urotheliogenic or myogenic origin remains unclear, with evidence to support each, however it is likely a combination. Spontaneous contractions are insensitive to neurotoxins, but are attenuated by Mg²⁺ and Ca²⁺ extracellular antagonists, suggesting a partly myogenic origin (Fry et al., 2004). Of particular interest, spontaneous activity is significantly attenuated in isolated detrusor preparations that have been denuded, compared to intact (Gevaert et al., 2007; Kanai et al., 2007). This evidence could suggest that the spontaneous activity is generated within the interstitial cells of the suburothelium, or from the urothelium itself, and is propagated to the detrusor, triggering contractions. Importantly, it has been observed that this activity is increased in bladders of patients with detrusor overactivity suggesting that this phenomenon may contribute to the overactive bladder syndrome (Mills et al., 2000).

1.1.5 RECEPTORS IN THE BLADDER

1.1.5.1 MUSCARINIC RECEPTORS

Muscarinic receptors are G protein-coupled acetylcholine receptors widely expressed throughout the body that mediate a range of vital physiological functions depending on location and receptor subtype. Five muscarinic receptor subtypes have been characterised (M₁, M₂, M₃, M₄, M₅) and these act through two distinct signal transduction pathways; M₁, M₃, and M₅ are coupled to the Gαq/11 second messenger, activating phospholipase C (PLC) and resulting in mobilisation of intracellular calcium; in contrast, M₂ and M₄ signal predominantly through Gᵢₒ proteins reducing intracellular cAMP concentration via inactivation of adenylate cyclase (Langmead et al., 2008). Both M₂ and M₅ muscarinic receptors are expressed in the detrusor, with M₂ expression ranging from three to nine times greater than that of M₃ depending on the
species (Wang et al., 1995). Despite this, it is in fact the M₃ receptor subtype that is responsible for the muscarinic contribution to contraction in normal bladder. In M₂ KO mice, no significant difference in the normal bladder response to carbachol (CCh), a muscarinic agonist, was observed in vivo and in vitro compared to WT (Igawa et al., 2003). Additionally, pharmacological studies using muscarinic receptor subtype specific antagonists revealed M₃ as predominantly responsible for contraction in detrusor samples from stable human bladders (Hegde, 2006).

Within the bladder, the M₃ receptor is found both on the smooth muscle and in the sympathetic and parasympathetic nerve endings (Somogyi and de Groat, 1999). Upon stimulation, either by ligand (ACh) or agonist (CCh) binding, the coupled Gₐₙ/11 protein is able to activate the enzyme phospholipase C (PLC), which initiates hydrolysis of membrane phosphoinositides (PIP₂) into the second messengers inositol-1,4,5-triphosphate (IP₃) and 1,2-diacylglycerol (DAG)(figure 1-4). IP₃ then proceeds to activate SR bound IP₃-Ca²⁺ release channels, initiating release of Ca²⁺ from cellular Ca-stores to the cytosol for contraction. Various studies provide evidence to support this contractile signalling mechanism; Harris et al. (1995) demonstrated an increase in both IP₃ production and force upon stimulation of M₃ receptors using subtype specific agonists in detrusor strips (Harris et al., 1995); Wu et al. (2002) showed that blocking IP₃ receptors, using for example heparin, prevents Ca²⁺ release, confirming the importance of IP₃ signalling here. DAG, the other substrate of PIP₂ hydrolysis, may also play a supplementary role in the contractile signalling mechanism, causing an increased Ca²⁺ sensitivity of the contractile system in a similar manner to the ROCK pathway discussed previously. It has been reported that DAG may activate PKC, which in turn phosphorylates MLCP causing a decrease in the enzymes activity, thus increasing the Ca²⁺ sensitivity of the contractile system (Wang et al., 2009).

M₂ receptors signal through G₁-proteins that reduce cAMP production via inhibition of adenylate cyclase activity. The more abundant M₂ receptor population present in the bladder appears to have a key functional role in modulating bladder contractions, through indirect enhancement of the contractile response to M₃ receptor activation. It has been suggested that this is achieved by M₂ receptor stimulation opposing sympathetically mediated smooth muscle relaxation, mediated by β-adrenoreceptors (Yamanishi et al., 2000). Additionally, muscarinic receptors present on parasympathetic nerve terminals at the neuromuscular junction can suppress (via M₄) or enhance (via M₁) transmitter release. Which of these occurs is dependent on the intensity of neural firing (Somogyi et al., 1998).

M₂ and M₃ receptors have also been identified in the urothelium, again with M₂ being the predominantly expressed subtype (Chess-Williams, 2002). Several studies have demonstrated
release of an unknown urothelium-derived factor capable of inhibiting detrusor contractions, upon stimulation of urothelial muscarinic receptors. Additionally, blockade of these urothelial muscarinic receptors in bladder mucosa preparations reduces stretch-induced Adenosine triphosphate (ATP) release, strongly suggesting a role for these receptors in overall bladder function.

1.1.5.2 ADRENERGIC RECEPTORS

Direct sympathetic innervation of the detrusor is low and thus has a much lower contribution to contraction than parasympathetic activation. It is possible to evoke detrusor contractions using drugs stimulating α-adrenoreceptors (ARs) in most species, where those stimulating α₁-ARs have the greatest effect, although high (possibly not physiologically relevant) concentrations are required (Anderson, 1993). In normal human detrusor, small and variable contractions are observed when stimulating isolated tissue strips using α-AR selective drugs (Anderson, 1993). Various studies have examined the expression of α₁-ARs in the human bladder, where the conclusive finding was that α₁D and α₁A-ARs are expressed at relatively low levels (6.3±1.0 mol/mg total protein), with no α₁B expression (Malloy et al., 1998). However two studies attempting to determine the relative expression of these two subtypes found contrasting data, where one found a predominance of α₁A (Walden et al., 1997) and the other an expression of 66% α₁D and 34% α₁A (Malloy et al., 1998).

Although the importance of α-ARs in normal bladder function is questionable, it is quite possible that these receptors are involved in the progression of LUT disorders, possibly due to altered subtype expression. For example, in rats displaying detrusor overactivity associated with outflow obstruction, α₁A-AR density did not change, however upregulation of α₁D-ARs was observed (Hampel et al., 2002). This could be of potential importance as α₁D-ARs have a 10-100 fold higher affinity for noradrenaline than the other two subtypes expressed, and α₁D-ARs are the predominantly expressed subtype in human bladders (Malloy et al., 1998). Recent studies have identified all α₁-receptor subtypes in the urothelium, with evidence in rat to suggest α₁A-ARs are able to modulate bladder afferent activity during normal and pathological bladder states (Yazaki et al. 2011; Kurizaki et al. 2011).

Interestingly, it is possible for noradrenaline to exert an inhibitory effect on detrusor contraction (i.e. relaxation) through activation of adrenergic nerves innervating parasympathetic ganglia, resulting in prejunctional inhibition of the parasympathetic nerves responsible for contraction. This is imperative for the filling phase of the micturition cycle, allowing urine to be accumulated without leakage. Noradrenaline is released upon electrical stimulation of detrusor adrenergic nerves. All three β-receptor subtypes are expressed in DSM, where β₃ is the most abundant
These receptors are G-protein coupled and their activation results in elevated smooth muscle cAMP, activating cAMP-dependent PKA, which subsequently phosphorylates and inactivates MLC kinase, resulting in detrusor relaxation (Yamaguchi, 2002). Isolated human detrusor strips where effectively relaxed by β₃-AR selective agonists, suggesting this subtype may be the most important β-subtype involved in the relaxation of human bladder. As the normal detrusor exhibits a greater expression of β-ARs compared to α-ARs, the physiological response to noradrenaline is relaxation (Anderson, 1993). Evidence from many animal studies supports the theory that the sympathetic nervous system contributes to bladder storage function through inhibition of the reflex detrusor contraction during bladder filling. Therefore there is current research aimed at developing β-AR specific agonists for clinical use as a therapy for bladder overactivity (Leon et al., 2008). All β-AR subtypes have been identified in the human urothelium and it has been suggested that these receptors are able to modulate detrusor activity by inducing the release of a urothelial factor that inhibits the β-AR relaxation of human detrusor (Otsuka et al., 2008).

1.1.5.3 PURINOCEPTORS

Further to its function as the key cellular energy source, ATP can also act as a potent extracellular messenger, eliciting its response via activation of the distinct P2 cell surface receptor family (Ralevic and Burnstock, 1998). ATP acting in this manner can be released into the extracellular space by nerve terminals or non-neuronal cells (including epithelia and glia), where its availability for receptor activation is regulated by ectonucleotidases, which hydrolyse ATP into ADP, AMP and adenosine (Zimmermann, 1999).

The first indications that ATP played a role in transduction of sensory information were provided by Holton & Holton (1954), who demonstrated that ATP released from sensory nerves in the rabbit ear triggered artery vasodilation, and later showed this vasodilation was accompanied by ATP release (Holton & Holton 1954; Holton 1959). Further study indicated that ATP, as well as the pyrimidine nucleotide uridine triphosphate (UTP) and dinucleotides adenosine diphosphate (ADP) and uridine diphosphate (UDP) act as ligands for this P2 receptor superfamily (table 1), which together play key roles in tissue functions such as platelet homeostasis, pulmonary, auditory and ocular function, neurotransmission and nociception (Burnstock and Williams, 2000).

The involvement of purinoceptor signalling in correct bladder function was first demonstrated by Kasakov and Burnstock, who observed a noncholinergic, nonadrenergic, atropine (muscarinic antagonist) resistant fraction of the parasympathetic contractile response in the detrusor of GPs (Kasakov and Burnstock, 1982). This atropine-resistant component varies
among species, with 75% of the contraction being atropine-resistant in GPs, 70% in mice, 60% in rabbits and 25% in pigs (Wüst et al. 2002; Brading & Inoue 1991). This, along with numerous additional studies has established a role for purinoceptors in bladder signalling, which exist as two distinct families; the ligand-gated ionotropic P2X receptors, and the G protein-coupled metabotropic P2Y receptors. P2X receptors respond preferentially to ATP, whereas P2Y receptors are activated by UTP, UDP, ATP and ADP in a subtype dependent manner (table 1).

1.1.5.3.1 IONOTROPIC P2X RECEPTORS

To date, seven functionally active mammalian P2X receptor subtypes (P2X1-7) have been identified. These are non-selective cation channels reported to play roles in the transduction of sensory signals such as hearing and nociception. P2X receptors have been further subdivided into 3 groups based on agonist efficacy and desensitization dynamics (Dubyak et al., 1996). P2X1 and P2X3 make up group 1, which have high affinity for ATP (EC$_{50}$ - 1μM) and are rapidly activated/desensitized. Group 2 consists of P2X2, P2X4, P2X5, P2X6 which all have a lower affinity for ATP (EC$_{50}$ - 10μM) with slow desensitization current, and P2X7 makes up group 3, with very low ATP affinity (EC$_{50}$ - 300-400μM) and little desensitization, which can also act as a nonselective ion pore. Functional purinoceptors are composed of either three identical P2X subunits (homomeric) or three different subunits (heteromeric e.g. P2X2/3) resulting in variation in receptor pharmacology and kinetics.

purinoceptors are expressed in the bladders of a number of species including human (Tempest et al., 2004), rat (Lee et al., 2000), guinea pig (Sui et al., 2014) and mouse (Vial and Evans, 2000), where P2X1 is found on the DSM and P2X2 and P2X3 on the urothelium and suburothelial nerve plexi (Elneil et al. 2001; Tempest et al. 2004). With regards to function, P2X2 & P2X3 play key roles in mediating the nociceptive action of ATP, where multiple studies have blocked pain-related behaviours in animals with chronic neuropathic/inflammatory pain using either P2X3 or P2X2/3 specific antagonists or knockout models (McGaraughty et al. 2003; Honore et al. 2002; Cockayne et al. 2000).

P2X receptors also play a key role in mechanosensation and transduction of sensory information in the bladder. Distension of the urinary bladder stimulates the release of ATP from the urothelium (Ferguson et al. 1997; Mochizuki et al. 2009), and intravesically administrated ATP or α, β-methylene ATP stimulates bladder hyperactivity. Non selective purinoceptor antagonists abolish this hyperactivity induced by administered ATP (Namasivayam S1, Eardley I, 2001), and interestingly reverse the bladder overactivity resulting from bladder obstruction (BO) in rats (Velasco et al., 2003). As P2X receptors have a much higher affinity for ATP than the P2Y receptors identified in the bladder, it is likely these findings result from P2X activation.
However, knockout mice have served as an important model to confirm the comparative contributions of P2X and P2Y receptors in functional bladder outputs.

P2X3 deficient mice display normal stretch-evoked urothelial ATP release, however show increased bladder capacity and decreased voiding frequency i.e. bladder hyporeflexia. These findings suggest P2X receptors play a key role in bladder afferent function by receiving information from urothelially released ATP, allowing communication between tissues (Vlaskovska et al. 2001; Cockayne et al. 2000). A more recent paper revealed expression of P2X2 receptors in human bladder urothelium and demonstrated increased expression of both this receptor and P2X3 in interstitial cystitis (IC) bladders (Tempest et al., 2004). Although under physiological conditions human detrusor contraction is mediated entirely through M3 receptor activation, it has been shown that a role for P2X receptors in detrusor contraction emerges in pathologically overactive bladders (the hypothesis for which is discussed in section 1.3.2) and indeed, receptor expression is altered. Together, these findings highlight the importance of purinoceptor signalling in the normal and pathogenic bladder, suggesting that with further understanding, these may serve as useful drug targets.

1.1.5.3.2 METABOTROPIC P2Y RECEPTORS

Thus far, eight molecularly distinct and functionally active P2Y receptors have been reported (P2Y1, 2, 4, 6, 11, 12, 13, 14), where P2Y1, P2Y2, P2Y4, P2Y6 and P2Y12 have been identified in the bladder. In contrast to the P2X family, these receptors are heptahelical G protein-coupled receptors which respond to UDP, UTP, ATP and ADP (Ralevic and Burnstock, 1998). P2Y2, P2Y4 and P2Y6 are sensitive to pyrimidine (uracil) nucleotides (Burnstock and Williams, 2000). P2Y1, P2Y2, P2Y4, P2Y6 and P2Y11 are coupled to the Gq signalling pathway and therefore once activated, induce phospholipase C (PLC) activity liberating intracellular calcium stores, resulting in activation of the protein kinase C (PKC) pathway. Interestingly, it is these receptors only that have been identified in human tissues (von Kügelgen and Wetter, 2000). P2Y12, P2Y13 and P2Y14 are coupled with Gi inhibitory proteins, which prevent conversion of ATP to cAMP by inhibition of adenylate cyclase.

P2Y receptor function has been less extensively studied than P2X, however P2Y1, P2Y2, P2Y4 and P2Y6 have been identified in rat dorsal root ganglia (DRG) by (RT)-PCR, suggesting a role for these receptors in peripheral sensory transduction. Studies have demonstrated an increase in intracellular calcium (an output of Gq coupled P2Y receptors) with UTP in rat DRG cells (Ruan and Burnstock, 2003). ATP liberated calcium from intracellular stores via the IP3 pathway in mouse DRG cells (confirmed by thapsigargin, an inhibitor of sarco/endoplasmic reticulum calcium stores) and this was attributed to
the P2Y<sub>6</sub> receptor (Svichar et al., 1997). Calcium release from intracellular stores of nociceptive rat DRG cells was also triggered by ADP-β-S and was inhibited by the specific P2Y<sub>1</sub> antagonist MRS 2179 (Borvendeg et al., 2003). These results imply a role for P2Y receptors in sensory nerve signalling and nociception.

P2Y<sub>6</sub> has been identified in the GP urothelium (Sui et al., 2006), and P2Y<sub>1</sub>, P2Y<sub>2</sub> and P2Y<sub>4</sub> have been reported in the feline urothelium, where P2Y<sub>2</sub> expression is reduced in cats with naturally occurring IC (Birder et al., 2003). This suggests P2Y<sub>2</sub> may play an important role in normal bladder function, and this decrease may coincide with the increase in P2X2 receptors observed in the human IC study. This highlights the possible importance of relative purinoceptor expression in pathogenic bladders, where further evaluation is needed and may prove interesting. An important role for P2Y receptors in the release of ATP from the urothelium has been demonstrated in rodents, where both UTP and UDP can evoke ATP release from this structure (Carneiro et al., 2014; Sui et al., 2014; Timóteo et al., 2014). These findings suggest that P2Y<sub>2</sub>, P2Y<sub>4</sub> and P2Y<sub>6</sub> are the subtypes responsible for purinoceptor-mediated ATP release from the urothelium.

Expression of P2Y receptors in the DSM has not been definitively shown. However their presence, particularly that of P2Y<sub>6</sub>, has been strongly inferred by functional experiments using nonspecific purinoceptor agonists (Yu et al. 2013; Bolego et al. 1995). Interestingly, several studies have demonstrated a relaxatory effect of ATP and its analogues on rodent bladder preparations, suggesting a role for P2Y receptors in bladder relaxation (McMurray et al., 1998; Tong et al., 1997). One group suggested the weak contractile potency of ATP observed in muscle preparations is due to the opposing effect of ATP in the bladder (relaxatory on P2Y, excitatory on P2X) (Boland et al., 1993). P2Y receptors may also modulate other receptors and channels, indirectly influencing nociception and mechanosensation. Multiple studies have demonstrated co-localization and co-expression of P2Y<sub>1</sub> with both P2X3 and TRPV1, a channel that mediates nociception and mechanosensation (Ruan & Burnstock 2003; Gerevich et al. 2004). A recent study also identified a functional interaction between P2Y<sub>2</sub> and TRPV1 receptors in cultured neuroblast cells (neuro2a), whereby active P2Y<sub>2</sub> receptors can activate TRPV1 in the absence of any other stimuli, however the underlying mechanisms are unclear (Lakshmi and Joshi, 2005). A more recent myographical study using mouse detrusor muscle revealed that activation of P2Y<sub>6</sub> by UDP selectively maximises P2X1–mediated contractile force through the PLC/IP<sub>3</sub> pathway. This culminates in raised intracellular calcium, however the mechanism by which calcium enhances P2X1 activity remains unknown (Yu et al., 2013). These findings infer that P2Y receptors are able to significantly influence bladder function and have the potential to modulate other receptors present in the bladder tissues.
1.1.6 NEURAL CONTROL OF BLADDER FUNCTION

The bladder operates in only two modes: storage and elimination, where it is believed suprapontine influences switch the bladders neural control circuits between the two. These circuits are located in the brain, spinal cord and peripheral ganglia and together coordinate the muscles in the bladder body and bladder outlet to achieve periodic urine storage and voiding (figure 1-6). Unlike many other visceral organs, micturition is under voluntary control that relies on learned behaviour that develops during infancy. Prior to this, switching between storage and elimination phases occurs in a reflex manner, such that voiding occurs involuntarily. Therefore, if the CNS becomes damaged due to spinal cord injury or disease, this reflex-type voiding may re-emerge in adulthood, and changes in the peripheral innervation of the LUT muscles may also result in bladder dysfunction.
Afferent signals from the LUT stimulate micturition in normally functioning bladders (Fowler et al., 2008). However, it is the coordinated pattern of both afferent and efferent signalling in autonomic (parasympathetic [pelvic] & sympathetic [hypogastric]), and somatic (pudendal) nervous pathways that allows controlled bladder filling and voiding. These complex pathways either hold the bladder in a relaxed state, granting low intravesical pressure for urine collection and filling, or initiate voiding by relaxing the bladder outlet and contracting the detrusor.

The parasympathetic pathways originating from the sacral region (S2-S4 level) of the spinal cord mediate both detrusor contraction and outlet relaxation. Parasympathetic nerves from the pelvic nerve innervating the urethral smooth muscle in the outflow region provide inhibitory input by the release of nitric oxide (NO) (Anderson, 1993; Andersson and Persson, 1995; Mumtaz et al., 2000). Postganglionic parasympathetic neurones also from the pelvic nerve highly innervate the detrusor and release both cholinergic (ACh) and non-adrenergic, non-
cholinergic (NANC) neurotransmitters. In most animal species, it is the co-release of ACh and ATP from these parasympathetic nerves that stimulates contraction and this has been demonstrated in various studies (Ambache and Zar, 1970; Burnstock et al., 1972; Carpenter, 1977). However, cholinergic transmission alone is the major excitatory mechanism in normal human detrusor (Andersson and Arner, 2004), although some degree of NANC transmission can be demonstrated in functionally altered or pathological human bladders (Bayliss et al. 1999; Andersson & Wein 2004).

The sympathetic pathways originating from the thoracolumbar region (T10-L2) innervate both the bladder body and urethra, travelling through either the pelvic nerve or the hypogastric nerve. The main role of the sympathetic drive is to contract the urethra and bladder base, however sympathetic innervation also inhibits parasympathetic nerves at the spinal and ganglionic level. Additionally, in many animal species, noradrenaline released from sympathetic nerves relaxes the bladder detrusor during filling, however this has not been fully established in humans (Anderson 1993; Wein et al. 2014).

Motor drive to the external urethral sphincter is supplied by somatic cholinergic nerves originating from the sacral spinal cord (S2-S4) in a region termed Onuf’s nucleus and these reach the periphery via the pudendal nerves. The pelvic floor musculature is also innervated by nerves supplied by a motor nucleus arising from the same spinal level.

The afferents most crucial for micturition are myelinated Aδ-fibres and unmyelinated C-fibers which convey information from receptors in the bladder wall to the spinal cord via the pelvic nerve. The Aδ-fibres convey information about bladder filling, as they respond to passive distension and active contraction (Jänig and Morrison, 1986) with an activation threshold of 5-15mmHg. This is the intravesical pressure at which humans first report a sensation of bladder fullness, which is followed by urge to void once pressures of 20-25 mmHg are reached. Discomfort occurs at pressures above 25mmHg and if bladder pressure exceeds 30 mmHg (requires prevention of micturition) pain is perceived (Rong et al. 2002; Chancellor MB & Yoshimura N 2006). The C-fibers are termed ‘silent’ as they have a high mechanical threshold and respond primarily to chemical irritation (Häbler et al., 1990) or cooling (Fall et al., 1990). Information regarding volume of urine present in the bladder is continuously conveyed by these afferents through spinal-tract neurons that project to the pontine micturition centre (PMC) for voluntary bladder control.

More recently, sensory nerves projecting into the suburothelial layer surrounding the bladder neck, and less so throughout the dome, have been identified in both humans and animals (Wiseman et al., 2002). These are in close contact with the cells of the urothelium, and growing evidence suggests the urothelial cells engage in reciprocal communication with these
neighbouring nerves in order to convey sensory information about the physical and chemical environment in the bladder (Birder et al., 2001).

### 1.1.7 UROTHELIAL FUNCTION

Communication between the various tissues of the bladder is integral in maintaining proper bladder function. As well as acting as a primary barrier, growing evidence supports the idea that through its own specialised sensory and signalling properties, the urothelium is able to respond to chemical and mechanical stimuli in its surrounding environment and engage in reciprocal chemical communication with the underlying tissues. To support this, numerous studies demonstrate the expression of an extensive collection of ‘sensor molecules’ (receptors/ion channels) in the urothelium, similar to those observed in nociceptors and mechanoreceptors. These include receptors for purines (P2X1-7 and P2Y₁,₂,₄), Ach (M₁-M₅), norepinephrine (α and β), adenosine (A₁,₂a,₂b,₃), bradykinin (B₁ and B₂), various neurotrophins and oestrogens, and expression of various TRP channels (TRPV₁, TRPV₂, TRPV₄, TRPV₈ and TRPA₁) and mechanosensitive epithelial sodium channels (ENaC) (Beckel et al., 2006; Birder, 2006; Birder et al., 2003; Carattino et al., 2008; Chopra et al., 2008, 2005; Du et al., 2007; Kullmann et al., 2009; Taylor and Al-Azzawi, 2000; Yu et al., 2006). These enable the urothelium to respond to a range of sensory inputs such as increased stretch during filling (mechanical), soluble factors found in the urine (e.g. ATP and growth factors) and chemical mediators (transmitters, peptides) released from neighbouring nerves, inflammatory cells and blood vessels (Birder, 2010).

The urothelium is then able to elicit an according response to such stimuli by secreting a variety of chemical mediators, further lending to its role in sensory function. These include ATP, ACh, NO, cytokines and prostaglandins which can act in a paracrine or autocrine manner, either activating, inhibiting or modulating the surrounding tissues (Birder and de Groat, 2007) (figure 1–7). Of importance, ATP can activate underlying sensory nerves by acting on P2X₃ receptors and thus contribute to the micturition reflex (Cockayne et al., 2000; Vlaskovska et al., 2001). These findings, together with the observation that bladder distension induces ATP release from the urothelium (Ferguson et al., 1997), provide experimental evidence that ATP is a novel sensory mediator for bladder sensation. In addition, ACh released from the urothelium can also stimulate the muscles and nerves in underlying tissues, or stimulate the urothelium itself via urothelium-associated muscarinic receptors. As previously mentioned, activation of these receptors causes an increase in [Ca²⁺], triggering the release of ATP (Kullmann et al., 2008), which can modulate the activity of bladder afferent nerves (Birder and de Groat, 2007). Additionally, evidence suggests that urothelium-derived ATP acting in an autocrine manner can induce membrane turnover and enhance stretch induced exo- and endocytosis, regulating
Figure 1 – 7. Sensory input & output pathways of the urothelium and communication pathways between the various tissues of the bladder. The urothelium receives sensory input in the form of: stretch (as bladder fills); signalling molecules released from the urothelium itself [1]; signalling molecules released from adjacent afferent/efferent nerves [2]. Activation of receptors expressed by the urothelium (on the apical [3] or serosal [4] surface) triggers the release of mediators such as ATP, ACh, NO, adenosine and prostaglandins which serve as sensory outputs[5]. Additionally, autocrine acting ATP may stimulate umbrella cell membrane turnover at the apical surface (e.g. during filling)[6]. ATP is released from the urothelial cells by exocytosis, through conductive pathways and via ATP carrier proteins [7]. The ATP and other soluble mediators released by the urothelium act as sensory outputs, which can either alter urothelial function [1] or modulate activity of afferent nerves [8] and smooth muscle cells [9][Adapted from(Khandelwal et al., 2009)]. Abbreviations: Ad, adenosine; AdR, adenosine receptor; αβAR, α,β-adrenergic receptor; ENaC, epithelial sodium channel; McR, muscarinic receptor; NcR, nicotinic receptor; NE, norepinephrine; NO, nitric oxide; PGs, prostaglandins; TRP, transient receptor potential channel family member. Created using public access images from(Servier Medical, 2015).

apical surface area (important during filling) and receptor number and function (Wang et al., 2005). Interestingly, removal of the urothelium from detrusor bladder strips significantly increases in vitro muscle contractions to Ach, suggesting the release of an inhibitory factor from the urothelium (Hawthorn et al., 2000). Although this urothelium-derived inhibitory factor is not yet known, studies have ruled out the possible participation of GABA, NO, adenosine, prostaglandins and Ca²⁺/K⁺ sensitive ion channels (Birder and Andersson, 2013).
1.1.7.1 UROTHELIAL STRETCH AND ATP RELEASE

The phenomenon of stretch-evoked ATP release from the urothelium has been demonstrated in many studies using both intact tissue and cultured cells (Birder et al., 2003; Ferguson et al., 1997; Mochizuki et al., 2009; Sun and Chai, 2006). As previously mentioned urothelial cells possess ion channels, some of which resemble the stretch-activated (mechanosensitive) channels found on nerves and therefore these channels may be involved in mechanotransduction in the bladder. It is likely that these channels are activated by increased tension at the apical surface of umbrella cells imposed during bladder filing. Amiloride (epithelial Na channel [ENaC] blocker) and Gd\(^{3+}\) (non-specific mechanosensitive channel blocker) are capable of suppressing stretch-evoked ATP release in both cultured urothelial cells and mucosal tissue strips in rat (Du et al. 2007; Sadananda et al. 2009). The TRP receptor family have also been implicated in bladder mechanosensation and the subsequent release of urothelial ATP, with both TRPV1 and TRPV4 antagonists and null mice inhibiting release of ATP in response to stretch and specific agonists (Gevaert et al., 2007; Mochizuki et al., 2009; Sadananda et al., 2009). These findings strongly imply the involvement of some mechanosensitive channels in the stretch-evoked release of ATP from the urothelium.

This ATP is released from both the serosal and apical surfaces of the urothelium (Ferguson et al., 1997) (figure 1-7) via several mechanisms including vesicular exocytosis, conductive pathways (connexin and pannexin channels) and various transporters (Wang et al. 2005; Birder & Ruggieri 2012). The process is therefore multifactorial, where different receptor-mediated or stretch-related mechanisms may utilize one, or a combination of these possible pathways in varying proportions. Using various inhibitors, Wu et al. previously demonstrated that basal ATP release from the mucosa is facilitated by both vesicular transport and conductive pathways, with an approximate 70% / 30% proportional contribution respectively. However, UTP-mediated (purinoceptor-mediated) ATP release from the mucosa was dependent on intracellular Ca\(^{2+}\) release, rather than extracellular Ca\(^{2+}\) influx, and did not involve vesicular transport, highlighting the variety of pathways available to be utilized for ATP release induced by different stimuli (Sui et al., 2014). Interestingly, studies in astrocytes and various cell lines have demonstrated that cell-swelling induced ATP release is tightly dependent on intracellular calcium elevations (Boudreault & Grygorczyk 2004; Coco et al. 2003), and indeed in primary urothelial cell cultures, stretch-evoked ATP release is closely correlated with calcium influx (Mochizuki et al., 2009). Therefore it is likely that the mechanism by which ATP is released from the urothelium in response to stretch is dependent on intracellular calcium elevations.
Serosally released ATP acts on both P2X3 and P2X2/3 heterometric receptors found in the suburothelial afferent nerve plexus (Rong et al., 2002). It is possible that the degree of afferent stimulation may signal the degree of bladder fullness to the central nervous system, eventually inducing contraction. Supporting this, P2X3 and P2X2/3 KO mice, still capable of releasing ATP, exhibit significantly diminished bladder afferent activation with reduced micturition frequencies and increased bladder capacities (Vlaskovska et al., 2001).

The purpose of apically released ATP is unclear, however it has been shown that both detrusor activity and umbrella cell membrane turnover is increased upon exposure of the apical surface of the urothelium to exogenous ATP (Brown et al. 1979; Wang et al. 2005). In theory, this ATP could bind to purinoceptors expressed on the apical surface, inducing serosal ATP release which could in turn act on the P2X receptors mentioned above, increasing afferent nerve stimulation and detrusor activity. Therefore apical ATP release could function to amplify the original signal through ATP-induced ATP-release (Sun and Chai, 2006). In summary, both apically and serosally released ATP can influence and modulate the activity of adjacent urothelial cells, interstitial cells, nerves and smooth muscle cells both directly and indirectly, and as such, has an important influence on bladder function.

1.2 TRP CHANNELS

The superfamily of mammalian transient receptor potential (TRP) channels contains 28 members, divided into 6 distinct subfamilies based on sequence homology; canonical (TRPC1-7), vanilloid (TRPV1-6), melastatin (TRPM1-8), muculipin (TRPML1-3), polycystin (TRPP1-3) and ankyrin (TRPA1) (Clapham et al., 2005). Bioinformatic searches through genome sequencing data banks has suggested that no further mammalian TRP channel subtypes remain to be discovered (Ramsey et al., 2006). TRP channels are cation-permeable pores formed from four, six-transmembrane (6TM) polypeptide subunits assembled into a tetramer and are sensitive to a wide range of stimuli depending on their homo- or heteromeric formation (Clapham et al., 2005). In general, they are ubiquitously expressed throughout the body with most cell types expressing several TRP channel proteins. These channels are most well-recognised for their functional roles in mechanosensation and nociception, with an interesting function in humans being to differentiate between sweet, bitter and umami tastes and hot and cold temperatures (Zhang et al. 2003; Clapham 2003).

The main function of TRP channels is to mediate the flux of cations across the cell membrane, resulting in elevated intracellular Ca\(^{2+}\) and Na\(^{+}\) concentrations ([Ca\(^{2+}\)]\(_i\) and [Na\(^{+}\)]\(_i\)) which in turn, depolarise the cell. This is a crucial event underlying many cell processes such as propagation
of neuronal action potential and muscle contraction (Hille, 2001). Cellular Ca\(^{2+}\) influx is also an important signalling event where the raised [Ca\(^{2+}\)]i activates Ca\(^{2+}\)-sensitive effector proteins such as calmodulin, regulating a vast collection of cellular processes such as migration and proliferation (Berridge et al., 2003). Most TRP channels can be activated by a number of stimuli i.e. polymodal activation, such that the physiologically relevant stimulus for a specific TRP channel may be governed by its cellular environment, such as cellular lipid content, available interacting proteins, concentrations of ligands and importantly, phosphorylation state (Ramsey et al., 2006). Acute post translational modifications, including phosphorylation, are capable of activating/deactivating TRP channels, with an additional level of TRP channel modulation implemented by Ca\(^{2+}\)/calmodulin (Strotmann et al., 2003). The number of functional TRP channels in a cell is regulated by insertion of TRP-carrying vesicles into the membrane. Therefore, it is important to recognise that agents or conditions that alter the number of spare channels may effectively desensitize, or indeed sensitize TRP channels to agonist stimulation (Ramsey et al., 2006). TRP channels can be activated by three general mechanisms: receptor activation (through GPCR and receptor tyrosine kinase [RTK] modulation); Ligand activation (e.g. endogenous lipids) and direct activation (changes in ambient temperature, mechanical stimuli, channel phosphorylation, cell swelling) and these will be discussed later in further detail specifically for TRPV4.

1.2.1 TRP CHANNELS IN THE LUT

Thus far, TRPV1, TRPV2, TRPV4, TRPM8 and TRPA1 have been identified in the bladder, with TRPV1 being most extensively investigated. TRPV1 channel expression has been demonstrated in both bladder neurons and urothelial cells (Birder et al., 2002), however there is conflicting data as to whether urothelial TRPV1 receptors are functional. TRPV1 is a non-selective cation channel activated by heat, protons, pH<5.9, noxious stimuli, capsaicin and endogenous ligands such as anandamide (Tominaga and Tominaga, 2005). These receptors are expressed in sensory nerves throughout the body and are generally involved in nociception and temperature sensing (Caterina et al., 1997). Current evidence surrounding the function of TRPV1 in the human bladder is limited, however intravesical administration of capsaicin results in a dose-dependent reduction in pressure threshold for micturition, first desire to void and bladder capacity, suggesting the presence of functional TRPV1 receptors that may modulate the micturition reflex (Maggi et al., 1989). Interestingly, it seems TRPV1 plays a more prominent role in pathological bladders, contributing to overactive bladder and pain, with increased mucosal expression in neurogenic detrusor overactive (NDO) bladders (Avelino and Cruz, 2006). Agonist activation of TRPA1 and TRPM8 induce similar phenomena such as increased micturition frequency and reduced voiding volume, and it is likely these channels
again play a more significant role in bladder pathologies, rather than in normal bladder function (Streng et al. 2008; Mukerji et al. 2006). The case for TRPV2 is similar, where the roles of these receptors in normally functioning human bladders, if any, remain to be fully established.

### 1.2.2 TRPV4 CHANNELS

The TRPV4 receptor is a non-selective cation channel preferentially permeable to Ca$^{2+}$, permitting influx of this major secondary signalling ion upon activation by a variety of physical and chemical stimuli. This channel plays important roles in many different cellular functions including osmo- and thermo-regulation, mechanosensation and nociception. Interestingly, the gating properties of this channel depend on the surrounding cellular environment (temperature, osmolarity, [Ca$^{2+}$], etc), and as such TRPV4 can be defined as a physiological integrator.

#### 1.2.2.1 STRUCTURE AND TRAFFICKING

The molecule is composed of 871 amino acids with 6 membrane spanning segments, including an intracellular N- and C- terminus, a pore forming loop between S5 and S6 and 6 ankyrin domains in the N terminus thought to have involvement in the mechanosensitive ability of the receptor (figure 1-8). There are also several putative calmodulin binding sites contained within the C terminus, one of which is found between H812-H831 and is involved in Ca$^{2+}$ dependent activation of TRPV4 (Strotmann et al., 2003). The other sites may also enable modulation of the receptor resulting in alternate changes to its function. Coexpression studies have thus far concluded that TRPV4 preferentially forms homomers, with no evidence for heteromeric assembly with other TRPV molecules (Hellwig et al., 2005).

Correct maturation of TRPV4 is characterized by complex glycosylation which takes place in the Golgi apparatus before the receptor is targeted and inserted into the plasma membrane (Lei et al., 2013). Deletion of the full C terminus or specific C terminal sections impedes translocation of TRPV4 from the ER to the plasma membrane (Becker et al., 2008). C residues are not only required for glycosylation, but are also involved in oligomerization, a process that is commonly necessary before translocation of proteins to the plasma membrane can take place. As such, the C terminus plays an important role in maturation and correct targeting of TRPV4 to the cell membrane. Insertion of TRPV4 into the cell membrane is regulated by ubiquitination. Atrophin-interacting protein 4 (AIP4) facilitates this process (Wegierski et al., 2006), where the degree of ubiquitination influences the fate of the protein. Once ubiquitinated, TRPV4 is available for endocytosis and once internalized, is transported to multivesicular bodies, where TRPV4 channels are sorted and or degraded in lysosomes. Mono- and multi-ubiquitinated TRPV4 channels occupy vesicles that sit below the plasma
membrane forming a reservoir of proteins, ready for incorporation. Poly-ubiquitinated channels are degraded and removed from the vesicular traffic (Katzmann et al., 2002). Interestingly, overexpression of AIP4 results in increased retention of TRPV4 within these vesicles, preventing their incorporation into the cell membrane and therefore reducing basal function (Wegierski et al., 2006). This may have implications in disorders involving TRPV4. Protein-protein interactions are also important for the correct trafficking and function of TRPV4 e.g. interaction with pacsin 1 or 2 shift the ratio of membrane bound TRPV4 compared to cytosolic in favour of membrane-associated TRPV4. Additionally, pacsin 3 binding modulates endocytosis of the channel, affecting its subcellular localization (Cuajungco et al., 2006).

![Figure 1 – 8. Schematic model of TRPV4. TRPV4 is composed of an intracellular C terminus with an incorporated CaM binding site (orange), six transmembrane domains (blue), a pore forming loop (green) and a cytosolic N terminal head containing the ankyrin repeat domain (ARD) which consists of six ankyrin (ANK) repeats.](image)

1.2.2.2 EXPRESSION & GENERAL FUNCTION

The TRPV4 receptor is widely expressed and has been detected by northern hybridization in the heart, brain, lungs, liver, trachea, pancreas, prostate and endothelium among other tissues (Delany et al., 2001). Strong expression has been confirmed in the kidneys within the cortical collecting duct cells, where it functions to recognise changes in osmolarity and shear stress. Evidence suggests that once activated by luminal flow, TRPV4 triggers K⁺ secretion and Na⁺ reabsorption in these cells (Wu et al., 2007). TRPV4 is also highly expressed in the airway epithelia of the lungs and trachea, with greatest expression observed in the bronchial cilia
where it is thought to play a role in ciliary beating and mucociliary clearance (Lorenzo et al., 2008). TRPV4 has also been identified in other ciliated epithelia such as that lining the fallopian tube, suggesting a role in oocyte transport (Teilmann et al., 2005). TRPV4 can be found throughout the cardiovascular system in high abundance in the vascular endothelium, and to a lesser extent in the smooth muscle cells of the pulmonary artery, aorta and cerebral arteries (Hartmannsgruber et al. 2007; Yang 2006). Many studies have demonstrated the role of TRPV4 in both vasodilation in the vascular endothelium and neovascularisation in capillaries through its mechanosensitive properties (Hartmannsgruber et al. 2007; Loot et al. 2008). Data suggests that TRPV4 detects shear stress in low-resistance arterioles, converting this to a Ca$^{2+}$ signal, resulting in release of endothelial relaxing factors and flow mediated dilation of these vessels (Mendoza et al., 2010). Activation of TRPV4 through shear stress can also stimulate proliferation of endothelial cells, in some cases leading to arterial occlusion, implicating TRPV4 as a promising therapeutic target for prevention of this disorder (Troidl et al., 2009).

In the brain, expression has been demonstrated in neurons of the circumventricular, vascular and subfornical organs, ependymal cells of the choroid plexus and in scattered neurons throughout the brain (Benfenati et al. 2007; Liedtke et al. 2000). TRPV4 is also functionally expressed in parasympathetic and sympathetic nerves and sensory trigeminal (TG) and dorsal root ganglia (DRG) neurons (Delany et al., 2001), and has been suggested to play a role in communication between neurons and astrocytes (Dunn et al., 2013). An important function of TRPV4 is its role in detecting nociceptive osmotic and mechanical stimuli at the peripheral nerve ending in these TG and DRG cells, confirmed by use of TRPV4$^{-/-}$ mice, which demonstrated lower sensitivity to these stimuli (Alessandri-Haber et al., 2003). TRPV4 is present throughout the inner ear in epithelial cells of the stria vascularis, inner and outer hair cells of the organ of corti, and transitional and sensory cells (Takumida et al., 2005), suggesting a role for TRPV4 in mechanosensation in the cochlea for response to sound and pressure. TRPV4 is also expressed in osteoblasts where it regulates bone remodelling (Masuyama et al., 2008) and in epidermal keratinocytes where it plays a role in thermo-regulation and –sensation (Chung et al., 2004). Finally, TRPV4 has been demonstrated in abundance throughout the urothelium of the bladder, where it is functionally expressed on the plasma membrane of these urothelial cells (Everaerts, Vriens, et al. 2010; Birder et al. 2007). One Immunoprecipitation assay revealed that TRPV4 expressed in the cell membrane has a physical connection to adherence junctions via the actin-microfilament network (Janssen et al., 2011). In this way, TRPV4 is mechanically connected to structural cellular networks, which may facilitate its mechanosensitive abilities, in turn enabling TRPV4 to detect bladder stretch.
1.2.2.1 TRPV4 AND STRETCH-INDUCED ATP RELEASE

One important functional output of TRPV4 activation is stretch-induced cellular ATP release, which has been demonstrated in a number of tissues (Shahidullah et al., 2012) and cell types (Egbuniwe et al., 2014; Mihara et al., 2011; Silva and Garvin, 2008), including those of the bladder (Gevaert et al., 2007; Mochizuki et al., 2009). The full mechanism by which TRPV4 facilitates ATP release, either induced by mechanical stretch or agonist-induced, remains to be identified. This is a challenging undertaking, as the general mechanisms by which mechanical stimuli trigger ATP release from cells in other systems, which are likely similar for TRPV4, are still poorly defined. Studies using various hypotonicity, cell-swelling and cell-stretch systems to mimic physiological stretch have however identified vesicular transport, exocytosis (Bodin and Burnstock, 2001; Boudreault and Grygorczyk, 2002; Maroto and Hamill, 2001) and conductive ATP release (Espelt et al. 2013; Cotrina et al. 1998) as the main pathways for this, coinciding with those identified for ATP release in the urothelium (Wang et al., 2005). One study also demonstrated that cell-swelling induced ATP release is dependent on intracellular Ca\textsuperscript{2+} elevations (Boudreault and Grygorczyk, 2004) and another that purinoceptor-mediated ATP release through conductive pathways requires both intracellular Ca\textsuperscript{2+} mobilization and active Cl\textsuperscript{-} channels (Cotrina et al., 1998). With regards to the bladder, stretch-induced ATP release from isolated and cultured feline urothelial cells requires vesicular transport, mobilization of intracellular Ca\textsuperscript{2+} and to a lesser extent extracellular Ca\textsuperscript{2+} entry (Birder et al., 2003). Currently, the mechanisms underlying TRPV4-induced ATP release remain unclear. However, a study in porcine lens tissue revealed that TRPV4-induced ATP release (by the TRPV4 agonist GSK1016790A) was hemichannel-mediated and dependent on extracellular Ca\textsuperscript{2+} (Shahidullah et al., 2012). A more recent study showed that cell swelling in human bronchial epithelial cells caused TRPV4-mediated ATP release, which was facilitated entirely through pannexin-1 channels (Seminario-Vidal et al., 2011).

1.2.2.3 ACTIVATION AND REGULATION

1.2.2.3.1 HEAT

TRPV4 is expressed in the thermosensitive regions of the hypothalamus indicating a role for thermosensation in this tissue. As with TRPV1, 2 and 3, TRPV4 can be activated by heat. However, unlike the 3 others, which are activated by temperatures exceeding 40°C up to those that are noxious, TRPV4 responds to temperatures which lie within the physiological range (30-35°C)(Watanabe et al., 2002b). Thus far, the mechanism by which this occurs is unclear, however evidence suggests activation occurs indirectly, through production or enhancement of a heat-dependent ligand, rather than direct activation by, for example, a conformational change
An important finding is that TRPV4 activation by temperature is modulated by osmolarity and vice versa, meaning changes to one may modulate the activation parameters for other stimuli, or indeed change the activation mechanism itself (Guler et al. 2002; Liedtke et al. 2000). It seems temperature is a critical modulator for channel gating of TRPV4, as at room temperature, activation by shear stress and hypotonic swelling is slow, but once raised to body temperature (37°C), activation by these is rapid.

1.2.2.3.2 OSMOLARIITY

TRPV4 expression in mammalian tissues involved in osmoregulation (circumventricular organs) and exposed to changes in osmolarity (kidney collecting ducts) provided the first indication that TRPV4 may be an osmotically sensitive channel. Importantly, changes in cellular osmolarity result in both osmotic stimuli due to cell swelling and mechanical stimuli due to membrane stretching/shear stress.

TRPV4 can be activated by changes in extracellular osmolarity or hypotonicity, where a reduction in osmolarity stimulates increased \([\text{Ca}^{2+}]\) in endothelial cells (Strotmann et al., 2000). Consistent with this, TRPV4\(^{-/-}\) mice exhibit defects in osmoregulation (Liedtke and Friedman, 2003) as well as a loss of the endothelial cell response to hypotonicity (Vriens et al., 2005). The underlying mechanisms are again unclear, however as the response to osmo-stimuli are relatively slow, it is thought that this type of TRPV4 activation is through an indirect mechanism. Some reports suggested that tyrosine kinase-dependent phosphorylation of TRPV4 mediates the channels response to hypotonic cell swelling (Xu et al., 2003), however further replicative studies failed to confirm this (Vriens et al. 2003; Plant & Strotmann 2007). One study revealed that TRPV4 is activated by arachidonic acid breakdown (Watanabe et al., 2003b). Through various further investigations, Watanabe et al. suggested that changes in osmolarity activate a phospholipase A2 (PLA\(_2\)) dependent endogenous signalling pathway, which results in the breakdown of arachidonic acid (AA) to epoxyeicosatrienoic acid (EET), a mechno- and osmotransducing messenger which can activate TRPV4. Despite this, it is still unclear whether EETs directly activate TRPV4, or further mediators are involved, however there is evidence to support a direct activation (Fernandes et al., 2008). These findings suggest that the mechno- and osmo-sensing properties of TRPV4 are mediated through either EET binding or PTK-dependent phosphorylation, or indeed both.
1.2.2.3.3 MECHANICAL STIMULI

Mechanosensitivity i.e. membrane stretch/shear stress may be a result of osmotic changes or simply the occurrence of the physical stretch of cells. For example, during bladder filling the bladder wall stretches to accommodate increasing volumes of urine, or when artery walls stretch during the periodic increases of pressure stimulated by each ventricular contraction. Both of these physiological events will cause a degree of shear stress, which can be recognised by stretch receptors. With regards to TRPV4, one mechanism by which the channel can detect shear stress is thought to be similar to that described for osmotic changes. It is likely mechanical stimuli, for example those triggered by osmotic pressure, are detected by PLA$_2$, triggering downstream signalling to indirectly activate TRPV4, as again, activation of this receptor by shear stress is relatively slow. However, evidence regarding the receptors physical incorporation into the cell membrane also provides possible means by which the receptor could recognise shear stress. TRPV4 mechanosensitivity is significantly impaired upon deletion of the N terminal ARD region, partly responsible for correct trafficking (Vriens and Nilius, unpublished observations (refer to Everaerts, Nilius, et al. 2010; Liedtke et al. 2000). It is postulated that this domain anchors the receptor into the membrane, forming a mechanical link for stretch activation and gating. Additionally, immunoprecipitation studies in human and mouse urothelial cells observed a molecular connection between TRPV4 and α-catenin, a component of adherence junctions, which ultimately forms another physical link between TRPV4 and the cells cytoskeleton (Janssen et al., 2011). However, cell-swelling activation models have revealed that this process relies on protein-protein interactions e.g. activation of TRPV4 by hypotonicity in salivary gland epithelial cells requires binding of aquaporin 5 (Liu et al., 2006), and pacsin 3 has also been implicated in this activation mechanism (D’hondt, unpublished data, refer to [Sharif-Naeini et al., 2008]). Thus, although there are several lines of evidence for direct mechanical activation of TRPV4, the majority would suggest its ability to recognise mechanical stimuli is through activation by endogenous openers generated by stretch.

1.2.2.3.4 LIGAND ACTIVATORS

Thus far, the identified endogenous activators of TRPV4 come from the endocannabinoid class of lipids, including anandamide (AEA) and its related endocannabinoid 2-arachydonyl glycerol (2-AG), which are both able to trigger whole-cell currents in TRPV4 expressing cells (Watanabe et al., 2003b). These lipids are transported into the cell where they are degraded by lipoxygenase, prior to being hydrolysed to arachidonic acid by various hydrolases and lipases. Through various pharmacological studies, it seems that EETs (the breakdown product of AA) are the final product of this pathway able to activate the plasma membrane-bound portion of
TRPV4 receptors, however it is still unclear whether EETs can directly bind to TRPV4 (Vriens et al., 2005). As previously described, this is the proposed mechanism by which TRPV4 can recognise osmo- and mechno- stimuli.

Various well-characterized synthetic agonists for TRPV4 have been utilized to examine its function, including 4αPDD, 4α-PDH and GSK1016790A. 4αPDD (EC\textsubscript{50} 200-400nM) is a phorbol ester, a group of compounds which bind directly to TRPV4 at a binding pocket formed by TM3 and TM4 residues (Vriens et al., 2003). The structurally similar 4α-PDH agonist (EC\textsubscript{50} 70nM) exhibits 5-fold potency of 4αPDD. GSK1016790A was produced through screening of small molecule libraries and shows very high efficacy for TRPV4 activation (EC\textsubscript{50} 1-10nM) with a 300-fold higher potency than 4αPDD (Thorneloe et al., 2008).

### 1.2.2.3.5 MODULATION BY CALCIUM AND PHOSPHORYLATION

Feedback regulation by Ca\textsuperscript{2+} on TRPV4 is an important process, which either potentiates or inhibits channel activity depending on the [Ca\textsuperscript{2+}]i and in doing so, can also alter the time course and dynamics of channel activity. Ca\textsuperscript{2+} is involved in both the activation phase and the decay phase of TRPV4 responses to osmo-, mechano- and ligand stimuli. The decay phase is delayed in the absence of extracellular Ca\textsuperscript{2+}, however the negative feedback mechanism for this remains unknown. In contrast, onset of the decay phase is accelerated during conditions of raised [Ca\textsuperscript{2+}]e. This involves residues in TM6, where a specific mutation in this region causes slower inactivation of TRPV4 by this high [Ca\textsuperscript{2+}]e, with unchanged sensitivity to [Ca\textsuperscript{2+}]i (Watanabe et al., 2003a). Increases in [Ca\textsuperscript{2+}]i first potentiate TRPV4 activity and then result in channel inhibition, where the IC\textsubscript{50} for [Ca\textsuperscript{2+}]-dependent inactivation of TRPV4 is 400-600nM (Watanabe, Vriens, Janssens, et al. 2003; Watanabe, Davis, et al. 2002). A calmodulin (CaM) binding site, found to be functional in other TRP channels, has been identified in the intracellular C terminus of TRPV4 (Strotmann et al., 2003). Various studies have suggested a Ca\textsuperscript{2+}-dependent component of TRPV4 activation; Ca\textsuperscript{2+} entering through TRPV4 binds CaM forming Ca\textsuperscript{2+}-CaM complex, this binds to the C terminal domain of TRPV4 channels, resulting in potentiation of TRPV4 activity, increasing both speed and amplitude of the response (Strotmann et al., 2003). Interestingly, IP\textsubscript{3} has also been shown to sensitize TRPV4 receptors to osmotic, mechanical and direct EET stimulation through protein-protein interaction at the previously described CaM binding site, coupling membrane TRPV4 receptors to intracellular Ca\textsuperscript{2+} stores (Garcia-Elias et al., 2008). Therefore, elevated [Ca\textsuperscript{2+}], resulting from other physiological processes may be partly responsible for basal/spontaneous activation of TRPV4 in the absence of stimulatory events, by intrinsic activators such as EETs and those yet to be identified.
TRPV4 contains two major phosphorylation sites for Src-family tyrosine kinases (SFKs) in the N- and C- terminus at Y110 and Y805. Y110 plays an important role in activation of the channel by all stimuli, however this site is phosphorylated before activation of TRPV4 and as such phosphorylation of this site probably sensitizes the receptor rather than causing direct activation (Wegierski et al., 2009).

As previously stated, phorbol esters such as PMA and 4αPDD are able to activate TRPV4 via direct interaction with the channel. However, concentrations of these ligands that only stimulate negligible effects at room temperature are much more potent when the temperature is raised to that of the body (37°C). This has been found to be dependent on Protein kinase A (PKA), and as such phorbol esters can activate TRPV4 via both direct and indirect mechanisms, depending on modulation by temperature, highlighting the diversity in activation parameters of this channel.

In summary, TRPV4 is not only capable of recognising multiple stimuli and integrating these signals, but its activity can be highly influenced by the cellular environment (e.g. heat, osmolarity) and modulated by protein-protein interactions, post-translational modifications and indeed mutations. These traits, taken with the fact TRPV4 responds to noxious stimuli, such as those encountered in inflammation and tissue obstruction and hypertrophy, mean the activity of this receptor can be highly influenced by pathologies, and therefore likely has important pathological implications in the bladder.

1.2.3 TRPV4 IN THE BLADDER

High TRPV4 expression has been demonstrated in mouse urothelium, localized mainly to the basal and intermediate urothelial cell layers, with a uniquely abundant mRNA expression here compared to other TRP channels (Gevaert et al., 2007; Janssen et al., 2011; Mochizuki et al., 2009) and in rat urothelium (Kullmann et al., 2009). TRPV4 positive staining was also reported in human urothelial umbrella cells, albeit from limited samples (Yamada et al., 2009). TRPV4 expression has been observed in both the peripheral neurons that innervate the bladder and in the bladder smooth muscle (Birder et al. 2007; Thorneloe et al. 2008). Its expression in the urothelial lining can be observed throughout the urinary tract (Yamada et al., 2009).

TRPV4 was first shown to function in cultured bladder urothelial cells as a Ca\(^{2+}\) influx pathway activated by hypotonic cell swelling and the synthetic TRPV4 agonist 4α-phorbol 12,13-didecanoate (4α-PDD)(Gevaert et al., 2007). In the same study, cystometry in TRPV4 null mice revealed lower frequency of overall voiding with an increase bladder capacity, but with a higher frequency of non-voiding contractions. This is consistent with the notion that TRPV4 may partly facilitate voiding. TRPV4 deficient mice also exhibited a decreased spontaneous activity in
isolated bladder strips, indicating that TRPV4 may promote spontaneous bladder contraction, which could underlie bladder overactivity (Gevaert et al., 2007). This suggests an important role for TRPV4 in regulating correct voiding in vivo, likely through mediating transduction of intravesical pressure, owing to the channels previously known characteristics. In the same paper, a decreased stretch-induced ATP release in isolated whole bladders of these TRPV4 deficient mice was reported (Gevaert et al., 2007), although direct measurement of ATP release from the urothelium was not made. A more recent in vitro mechanical stretch stimulation performed on cultured urothelial cells confirmed that stretch activates TRPV4, resulting in increased $[\text{Ca}^{2+}]$ and ATP release (Mochizuki et al., 2009). This finding was also shown by Birder et al. who demonstrated functional expression of TRPV4 in cultured rat urothelial cells, where again both hypo-osmolarity and the agonist 4α-PDD induced calcium influx and ATP release. From this evidence it has been proposed that TRPV4 senses bladder distension and communicates the extent of this to primary afferent nerves via an ATP signal for correct bladder function during the urine storage phase and voiding initiation. Finally, whole animal studies revealed that conscious WT mice exhibit bladder overactivity upon intravesical administration of GSK1016790A, with no effect in KO mice. As such, TRPV4 also has pathological implications and can contribute to bladder disorders associated with overactivity. Additionally, this study provided indirect evidence that TRPV4 receptors present in the DSM could contract the muscle, revealing separate roles for TRPV4 in both the urothelium and DSM.

A major step forward in our understanding of TRPV4 physiology awaits clarification of the mode of action for TRPV4 in native urothelium and smooth muscle.

### 1.2.4 TRPV4 AND DISEASE

The most well-characterized group of TRPV4-related pathologies are autosomal dominant skeletal dysplasias, such as brachyloma, which are caused by various point and missense mutations within TRPV4 resulting in mutant channels. Missense mutations within the TM5 domain have been associated with kozlowski-type spondylometaphyseal dysplasia, where two particular mutations result in increased basal channel activity in vitro (Krakow et al., 2009). The involvement of TRPV4 in pathologies of other systems is less well studied, however current knowledge suggests it does play a role in the development of neuropathic pain and hypo-osmotic hyperalgesia (Alessandri-Haber et al., 2006). With regards to bladder disorders, as a definitive role for TRPV4 has yet to be fully established, its role in bladder pathologies remains unclear. However, Everaerts et al. showed that development of cystitis-induced bladder dysfunction is greatly reduced in TRPV4 deficient mice. Additionally, using a potent TRPV4 antagonist HC-067047 the group demonstrated increased bladder capacity and reduced micturition frequency in WT mice and rats (Everaerts et al., 2010c) with cystitis. This not only...
implicates the involvement of TRPV4 in this bladder disorder, but also presents the channel as a promising target for novel drug therapies. Despite the fact little else is known about the role TRPV4 may play in bladder pathologies such as overactivity, the evidence supporting an important role for this channel in normal bladder function would strongly suggest the involvement of this channel in the progression of such disorders. It is imperative to examine the role of TRPV4 in human conditions, hence determining the value of targeting this receptor for the treatment of overactivity.

1.3 BLADDER PATHOLOGIES & THE OVERACTIVE BLADDER SYNDROME

Bladder dysfunctions are extremely common, with urinary urge incontinence (UUI) affecting up to 13.3% of males and 30.3% of females of age 40 and above in western countries, with an estimated annual cost of €295 million in the UK (Milsom et al., 2014). Disorders affecting control of the bladder can be classed as either disorders of filling/storage or voiding (Wein and Rovner, 2000). Failure in the storage phase of the micturition cycle, leading to incontinence, can result from anatomical defects or weakness in the bladder outlet, or from bladder overactivity i.e. overactive bladder syndrome (OAB). OAB is defined as urgency, with or without urge incontinence, usually with frequency and nocturia and is typically caused by detrusor overactivity. Epidemiological studies have revealed an overall prevalence of 17% in Western Europe and 16.5% in the US, increasing further still with advancing age (Milsom et al. 2002; Stewart et al. 2003). Only 1/3 of patients with OAB exhibit urge incontinence, suggesting that the source of the dysfunction may not always be from the bladder itself.

![Figure 1 – 9. Contributing mechanisms underlying overactive bladder. Factors influencing disruption of the normal voiding reflex resulting in involuntary detrusor contractions.](image-url)
Studies using isolated bladder strips have shown a significant association between OAB and increased spontaneous detrusor activity (Fry et al., 2010). Therefore, as with the theories for generation of spontaneous contractions, there are three principle factors that may influence the generation of overactive bladder: urothelial changes, myogenic changes and/or neurogenic changes (figure 1-9).

Neurogenic changes that may contribute to an overactive state include: an increase in the afferent input from the bladder, physical damage to the neurones in the spinal cord innervating the bladder, and reduced activity of inhibitory pathways due to disrupted central processing of afferent input. It is thought that myogenic OAB arises from superior muscarinic sensitivity and increased coupling between DSM cells leading to increased detrusor excitability (Fry et al., 2004).

1.3.1 ANTIMUSCARINICS

Antimuscarinics are the first-line pharmacological treatment for overactive bladder. In a study assessing over 7 million patients with diagnosed OAB through a 45 year period, 24.4% were treated with antimuscarinics, with the remaining 75.6% staying untreated (Helfand et al., 2010).

However, these drugs exhibit increased side-effects and low efficacy in many patients meaning drug compliance and successful treatment is very low. Antimuscarinic agents selectively block muscarinic receptors. During treatment of OAB these agents elicit their therapeutic effect by blocking the muscarinic receptors on the DSM, which normally respond to Ach released from neighbouring parasympathetic nerves, thereby reducing bladder contractions. However, these receptors are widely expressed throughout the body and therefore blockade causes a range of side effects including constipation, dry mouth and various cognitive impairments which limits their clinical use (Klausner & Steers 2008; Abrams et al. 2006). It is highly likely the commonly low efficacy is due to the multimodal nature of this disorder and the emergence of other signalling pathways governing contraction that emerge during pathology (discussed below). Therefore there is an immediate need to develop both uroselective antimuscarinics and drugs aimed at other targets capable of treating OAB.

1.3.2 ATP AND OVERACTIVE BLADDER – EMERGENCE OF A NANC COMPONENT

In the normal human detrusor, contraction is mediated entirely through binding of acetylcholine (ACh) to muscarinic M₃ receptors located on the cell membrane (Hegde 2006). This has been demonstrated by a complete loss of contraction observed with atropine, implicating ACh as the sole activating neurotransmitter. Interestingly however, varying levels of atropine-resistant
emerge in detrusor samples from pathologically overactive human bladders, where the remaining contractions can be suppressed with alpha beta-methylene-ATP (ABMA), which desensitizes P2X receptors (Bayliss et al., 1999). ATP is co-released from parasympathetic nerves alongside ACh and it has been hypothesised that under physiological conditions the ATP released into the neuromuscular junction is completely broken down by extracellular ectoATPases, such that none is able to activate the smooth muscle. During human bladder pathologies however, it is possible that either the released ATP is less effectively broken down in the junction, or an excess of ATP is released. Previously it has been shown that ectoATPase activity is reduced in detrusor samples from human detrusor overactive (DO) bladders, supporting the former hypothesis; a lower ectoATPase activity will result in concentrations of ATP high enough to activate purinoreceptors, thus contributing to contraction and causing the observed atropine resistance (Harvey et al., 2002). However, conclusive studies must be performed using ectoATPase-inhibitors in normal human bladders to fully evaluate this; if ATP is in fact released as a co-transmitter under physiological conditions and the purinoreceptors present on the nerve terminals are always functional, you would expect inhibition of ectoATPase activity to again contribute to contractions if the mechanism stands.

Studies have also demonstrated an augmented release of ATP from the urothelium during bladder pathologies. Using a feline interstitial cystitis model, Birder et al. observed an increase in stretch-evoked urothelially derived ATP release and altered purinoceptor profiles in urothelial cells (Birder et al., 2003). Chronic spinal cord injury and irritated bladders in rats also causes enhanced release of ATP into the bladder lumen (Salas et al. 2007; Girard et al. 2008). Thus far this augmented release of ATP has only been linked to enhanced sensation of pain via activation of purinergic receptors on nearby sensory fibres. Further investigation is needed to evaluate whether this increased ATP release is responsible for or indeed capable of contributing to the atropine resistant contractile fraction observed in overactive human bladders. However, as it is known that purinoceptor contribution to contraction does emerge in the pathologically overactive human bladder, it provides an important potential target for reducing the contractions observed with bladder overactivity (BOA). These findings also provide an indication as to why the muscarinic antagonists currently used for treatment of LUTS only exhibit a moderate efficacy.
1.3.3 INFLUENCE OF THE UROTHELium IN OVERACTIVE BLADDER

As the urothelium possesses the ability to alter the excitability of bladder neurons, changes in the sensory mechanisms responsible may increase signals of bladder sensation and contribute to OAB. One explanation is that there is an increase in $M_2$ and $M_3$ receptors in the pathologic state, meaning ACh released from the urothelium will have a more profound effect (Kim et al., 2008). Another theory is that there is simply increased ACh release from the urothelium during the filling phase, again leading to increased sensation. Importantly, advances in our understanding of the urothelium have revealed many more receptors and signalling pathways able to influence bladder function, which may in turn also malfunction (e.g. in aging) and contribute to OAB. Indeed, studies have demonstrated changes in urothelial function during pathologies (i.e. augmented ATP release) and importantly changes in purinoceptor profiles in urothelial cells, suggesting that urothelial sensor molecules exhibit plasticity to response to pathologic conditions (Birder et al. 2003; Sun et al. 2001). Therefore, increasing our understanding of the urothelium’s sensory function and how it achieves this (for example, through TRPV4 receptors) may expose novel candidates for therapeutic intervention.

1.3.4 THE AGING UROTHELium

Various studies have revealed the sensitivity of the urothelium to, for example inflammation, injury, pathological insult and aging, which can damage and alter urothelial properties. Studies have shown the urothelium to be sensitive to ischemia and hypoxia (associated with aging and overactive bladders)(Christiaanssen et al., 2011), urothelial cell degeneration and mucosal denudation in the bladders of aged animals (Akram Al-Motabagani, 2005). Additionally, various bladder disorders can impact on urothelial integrity and function, such as outlet obstruction, painful bladder syndrome and overactive bladder itself. There are therefore many triggers that may alter urothelial properties and processes, particularly associated with aging, that may result in impaired urothelial function and potentially lead to overactive bladder and other disorders. Regulation of urothelial function in aging bladders is an important but understudied area. Identification of pathological regulators from the urothelium that predispose the aging bladder to overactivity may provide new therapeutic targets for management of bladder dysfunction in the elderly.
1.4 CURRENT RESEARCH QUESTION

Previous studies have focused only on cultured urothelial cell preparations, which are subject to phenotypic changes (Cross et al., 2005), and stretch as a method of investigating TRPV4, which activates many stretch-sensitive processes other than TRPV4 channels; however the mode of action of TRPV4 in native tissue, in particular by specific activation of this receptor, has not yet been examined. Given the recognition of the urothelium and associated structure as a whole functional syncytium, it is crucial to understand the function of TRPV4 in intact multicellular preparations. More importantly, the role of TRPV4 in human conditions has not been explored, prompting urgent need for this translational investigation. The primary aim of this thesis is therefore to characterise the properties of TRPV4 and its contribution to urothelial activation, through specific activation of the receptor in physiologically relevant tissue strips from both animals and humans. Importantly, the effect of aging on the channels activity will also be investigated. These studies will provide crucial evidence and translational information about TRPV4 that may reveal the therapeutic potential of this receptor in treatment of overactive bladder and other age-related sensory disorders.
1.5 HYPOTHESES

1. TRPV4 channels significantly regulate urothelial function through release of urothelium-derived ATP.

2. Changes in the expression and function of TRPV4 channels occur in aging and overactive bladders.

1.5.1 AIMS AND OBJECTIVES

The overall aim of this project is to understand the physiological role of TRPV4 receptors in urothelial and bladder function, demonstrate its pathological significance and explore the therapeutic potential of targeting this receptor.

The project will test the former hypotheses by achieving the following objectives:

(1)

- Demonstrate the presence of TRPV4 in the urothelium of human and animal bladders.
- Determine the relative tissue expression of TRPV4 throughout the bladder.
- Determine whether TRPV4 receptors are functional regarding urothelial ATP release.
- Explore the mechanisms underlying TRPV4-mediated urothelial ATP release.
- Explore the effect of channel antagonists on TRPV4 function to evaluate the therapeutic potential of the channel.

(2)

- Identify any age-related changes in the levels of expression and tissue localization of TRPV4.
- Identify any age-related changes in the function of TRPV4.
- Explore the relationship of TRPV4 receptors with urothelial purinoceptors associated with ATP release, and whether this is affected by aging.
- Identify any changes in TRPV4 channel activity that occur in overactive aging human bladders.

The outcomes of this study will identify a novel therapeutic target for management of bladder over-activity and other sensory disorders and reveal the role of sensory TRPV4 channels in contributing to abnormal bladder activity in older people.
CHAPTER 2. MATERIALS & METHODS
2.1 CHEMICALS & SOLUTIONS

All final solutions and buffers were prepared fresh on the day of experiment unless otherwise stated.

2.1.1 FUNCTIONAL ORGAN BATH EXPERIMENTS

2.1.1.1 HEPES – BUFFERED TYRODE’S SOLUTION

Tissue samples were incubated and superfused with a HEPES-buffered Tyrode’s solution prepared weekly using dry powders and pre-made stock solutions dissolved in ‘ultra-pure’ distilled water (Milli-Q system, Millipore, UK)(Table 2). The pH was adjusted to 7.4±0.05 with 2M NaOH using a pH meter (Oaktron, acorn series) and stored at +4°C until required. This solution is commonly used as a tissue superfusate for physiological experiments, emulating extracellular physiological conditions.

Table 2 – HEPES-buffered Tyrode’s solution

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final concentration (mM)</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stock powders</strong></td>
<td>NaCl</td>
<td>132 Sigma Aldrich, UK</td>
</tr>
<tr>
<td></td>
<td>HEPES</td>
<td>10 Merck Millipore, UK</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
<td>6.1 BDH, UK</td>
</tr>
<tr>
<td></td>
<td>Na Pyruvate</td>
<td>5 BDH, UK</td>
</tr>
<tr>
<td><strong>Stock solutions (1M)</strong></td>
<td>KCl</td>
<td>4 -</td>
</tr>
<tr>
<td></td>
<td>MgCl2</td>
<td>1 -</td>
</tr>
<tr>
<td></td>
<td>NaH₂PO₄</td>
<td>0.4 -</td>
</tr>
<tr>
<td></td>
<td>CaCl₂</td>
<td>1.8 BDH, UK</td>
</tr>
</tbody>
</table>

A Ca²⁺-free modified Tyrode’s solution was prepared by substituting the CaCl₂ stock with the same concentration of MgCl₂ (total MgCl₂: 2.8mM) to achieve the same concentration of divalent cations and thus avoid any changes to cell membrane potential. The chelating agent EGTA (0.1mM, [Sigma, UK]) was added to buffer any residual calcium to pCa of 8 (Sui et al., 2009).

2.1.1.2 SODIUM BICARBONATE TYRODE’S SOLUTION

For the purpose of live-cell calcium imaging, a more commonly used sodium bicarbonate version of Tyrode’s was prepared. This was prepared on the experimental day as above (table 1), where HEPES was omitted and replaced with sodium bicarbonate (NaHCO₃) at a final concentration of 24mM. The final solution was bubbled with a gas mix of 95% O₂ : 5% CO₂ resulting in a final pH of 7.4 ± 0.1 at 37.0°C ± 0.5°C.
2.1.2 CALIBRATION SOLUTIONS FOR LIVE-CELL CALCIUM IMAGING

Both Ca²⁺-free and high-Ca²⁺ variations of HEPES-buffered high K⁺-based intracellular solutions were required to mimic the intracellular environment for accurate calibration of the Ca²⁺ imaging setup. The Ca²⁺-free intracellular solutions contained 120mM KCl, 10mM NaCl, 1mM MgCl₂ with no added CaCl₂ and 5mM EGTA. High-Ca²⁺ Tyrode’s was prepared to a final [Ca²⁺] of 2.0mM by addition of further CaCl₂ stock. The pH of both solutions was adjusted to pH7 using 2M NaOH.

2.1.3 DRUGS & COMPOUNDS

The drugs and compounds used for interventions during physiological experiments were dissolved in either deionised water or DMSO (BDH, UK) and stored at -20°C as stock solutions for further dilution on the day of each experiment (Table 3). The experimental concentration of agonists was chosen based on preliminary work that demonstrated a reproducible effect without causing significant desensitization, being above the EC₅₀ but below the maximal effect. Experimental concentrations of antagonists were calculated based on their affinity for receptors for predominant blockade, but not too high to generate a non-specific effect; and where appropriate, to oppose the concentration of same-receptor agonist used, such that >95% of receptors were inhibited.

2.1.3.1 TRPV4 AGONIST / ANTAGONIST INTERACTION

The TRPV4 antagonist HC-067047 (HC) was used at a concentration of 1μM for predominant blockade (>95%) of TRPV4 receptors (based on previous literature and calculations using the equilibration dissociation constant of HC-067047 [Kᵯ = 7.8] [IUPHAR/BPS 2010; Everaerts, Zhen, et al. 2010]). During challenge of tissue with both GSK and HC, it is important to consider the activity and interactions between the two that may influence the concentrations required to achieve desired blockade of TRPV4. In the presence of agonist, the Kᵯ of competitive antagonists is altered (as is that of the agonist), such that a higher concentration of antagonist is required to achieve desired blockade. The Schild-slope factor equations are used for this purpose and in this case, the Kᵯ of HC and GSK (7.7 [IUPHAR/BPS 2007; Thorneloe et al. 2008]) would be used (Harvey Motulsky, 2004) to determine the required concentrations. However, as previous experiments(Everaerts et al., 2010c) suggest that HC is a non-competitive antagonist, any TRPV4 agonist should not compete/interfere with the activity (and Kᵯ) of HC. As such, HC at 1μM should still achieve the same percentage blockade with or without GSK. To be sure of this, during simultaneous challenge of tissue with both GSK and
HC, the concentration of GSK was lowered to 0.5μM, which has been shown to still elicit physiological responses by the literature and experiments in this study.

2.1.4 LUCIFERIN – LUCIFERASE ASSAY SOLUTIONS

2.1.4.1 ADENOSINE 5′-TRIPHOSPHATE (ATP) ASSAY MIX (ATP MIX)

This was purchased as a lyophilised powder (Sigma, UK) and reconstituted with 5mL sterile water to minimise the background ATP from bacteria. The solution was stored at -20°C until use for ATP measurement (in conjunction with ATP buffer below).

2.1.4.2 ATP ASSAY MIX DILUTION BUFFER (ATP BUFFER)

This compound (Sigma, UK) was reconstituted with 50mL sterile water and aliquoted into several vials which were then stored at -20°C until use for ATP measurement.

2.1.5 IMMUNOHISTOCHEMISTRY & WESTERN BLOTTING

2.1.5.1 PHOSPHATE BUFFERED SALINE SOLUTION (PBS) AND PBS WITH 1% BOVINE SERUM ALBUMIN (1% BSA PBS)

PBS was prepared by the addition of 1 PBS tablet per 100ml dH2O (Oxoid, UK), where 1% BSA PBS also required the addition of 1g BSA powder per 100ml. These solutions were prepared in advance and stored at 4°C until use.

2.1.5.2 RIPA LYSIS BUFFER

RIPA buffer was chosen to lyse cells for total protein extraction, as this is the most applicable buffer for investigating membrane-bound proteins, such as TRPV4. This buffer has the capacity to denature samples further than others (e.g. NP-40), liberating any membrane-bound proteins of interest for further treatment. This was prepared in advance and stored at room temperature, with final concentrations (mM) of 50 Tris-HCl, 150 NaCl, 2 ethylene diamine-tetraacetic acid (EDTA), 50 NaF, 1% NP-40, 1% Sodium deoxycholate dissolved in ‘ultra-pure’ distilled water. The pH was adjusted to 7.5±0.05 with HCl.

Protease inhibitors (Pi) were added (‘cOmplete, Mini, EDTA free PI cocktail’ [Roche, UK]) to inhibit the endogenous proteolytic enzymes released from subcellular compartments during cell lysis that would otherwise degrade proteins of interest, and the final solution was then aliquoted (1ml eppendorfs) and stored at -20°C until use.
2.1.5.3 SAMPLE LOADING BUFFER

On the day of experiment, samples (stored in lysis buffer at -20°C) were diluted 3:1 in 4x laemmli buffer (277.8 mM Tris-HCl, pH 6.8, 4.4% SDS, 44.4% (w/v) glycerol, 0.02% bromophenol blue 10% 2-mercaptoethanol [Bio-Rad, UK]) for loading. The glycerol increases sample density for improved layering in sample wells. The SDS acts as the denaturing agent and ‘labels’ each protein with a negative charge for electrophoretic size-based separation. The 2-mercaptoethanol reduces the disulphide bonds and bromophenol blue acts as the ‘dye front’.

2.1.5.4 TRIS-BUFFERED SALINE (TBS) AND TBS-TWEEN (TBST)

A 10x TBS solution was prepared weekly and stored at room temperature. This was then diluted 10 fold on the day of experiment to provide a solution with final concentrations of 20mM Tris-base and 150mM NaCl (pH 7.6). For TBST, 0.1% Tween 20, a detergent that helps remove nonspecifically bound material, aiding in clarity of blots, was also added (1ml Tween 20 : 1L 1x TBS). For blocking and antibody solutions, a 5% milk TBS or TBST solution was prepared by adding 5% w/v dry milk powder (Marvel, UK).

2.1.5.5 ELECTROPHORESIS RUNNING BUFFER

Pre-prepared 10x running buffer stock solution was diluted to a 1x working solution with dH₂O for gel electrophoresis. The final 1x solution contained 192mM glycine, 25mM Tris and 0.1% w/v SDS dissolved in dH₂O with a pH of 8.3 (all reagents purchased from Fisher Scientific Chemicals, UK).

2.1.5.6 TRANSFER BUFFER

A standard wet-transfer buffer was freshly prepared in dH₂O, containing 192mM glycine, 25mM Tris and 20% methanol (Sigma, UK).

2.1.5.7 ANTIBODIES (Abs)

The Abs listed in table 4 and 5 were stored in aliquots at -20°C to avoid repeated freeze-thaw cycles. For the purpose of western blotting, these were diluted in TBST 5% milk on the day of experiment and after use were stored in -20°C to be used again. The diluted Abs were used a maximum of 3 times before a fresh preparation was made. For Immunohistochemistry, the Abs were diluted in 1% BSA/PBS on the day of experiment.
2.1.5.8 STRIPPING BUFFER

Stripping buffer was used to remove any Abs from an already probed membrane to allow re-probing for further proteins of interest. This allowed for assessment of multiple proteins in the same target samples without having to re-run gels, minimizing use of precious samples. A low pH (2.5) glycine-HCL (0.1M) buffer was used (Restore™ Stripping buffer, Cat. #21059, Thermo Fisher, UK), where the low pH removes Abs by altering their structure such that the binding site is no longer active. This is a mild stripping method, which does not remove any protein but in some cases may leave traces of Ab. A harsher variation uses heated SDS buffer, however this can remove significant amounts of protein. The mild version was successful for the Abs employed here.
Table 3 – List of drugs and compounds employed during physiological organ bath experiments.

<table>
<thead>
<tr>
<th>Drug/Compound</th>
<th>Stock Solvent</th>
<th>Experimental concentration</th>
<th>Effect</th>
<th>Company</th>
<th>Product reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSK1016790A</td>
<td>DMSO</td>
<td>0.2-1μM</td>
<td>Potent and selective TRPV4 agonist; enhances Ca(^{2+}) influx in human and mouse TRPV4-expressing HEK cells (EC(_{50}) = 2.1 nM and 18 nM respectively). Has 300-fold higher potency that 4-α-PDD in activating TRPV4 channels (Thorneloe et al. 2008; Jin et al. 2011). Does not affect TRPM8 and TRPA1 channels up to concentrations of 20 μM. Suggested by one study to act by recruiting previously inactive channels, rather than through increasing elevation of basal activity (Sullivan et al., 2012). Others suggest the agonist is a true ligand (IUPHAR/BPS 2007; Thorneloe et al. 2008).</td>
<td>Sigma-Aldrich</td>
<td>G0798</td>
</tr>
<tr>
<td>HC-067047</td>
<td>DMSO</td>
<td>1μM</td>
<td>Selective non-competitive TRPV4 antagonist. Reversibly inhibits mouse, human and rat TRPV4 (IC(<em>{50}) = 17, 48, and 133 nM respectively in whole cell voltage-gated current measurement). Does not inhibit other TRP receptors up to concentrations of 5μM (TRPM8 IC(</em>{50}) = 780 nM, little or no activity toward hK(_i)1.3, hTRPA1, hTRPC5, hTRPV1/2/3/6, mTRPM7, mTRPV2, or rK(_v)1.2) (Everaerts et al., 2010c).</td>
<td>Sigma-Aldrich</td>
<td>SML0143</td>
</tr>
<tr>
<td>Uridine 5’-trisphosphate trisodium salt dihydrate (UTP)</td>
<td>H(_2)O</td>
<td>100μM</td>
<td>Endogenous P2Y receptor agonist. Preferentially activates P2Y(_2) and P2Y(_4) but may agonise P2Y(_6) to small degree. (Ralevic and Burnstock, 1998)</td>
<td>Sigma-Aldrich</td>
<td>94370</td>
</tr>
<tr>
<td>Uridine 5’-diphosphate disodium salt hydrate (UDP)</td>
<td>H(_2)O</td>
<td>20μM</td>
<td>Endogenous P2Y receptor agonist, which preferentially activates P2Y(_6) (Ralevic and Burnstock, 1998)</td>
<td>Sigma-Aldrich</td>
<td>94330</td>
</tr>
<tr>
<td>MRS2578</td>
<td>DMSO</td>
<td>10-20μM</td>
<td>Selective antagonist of P2Y(<em>6) nucleotide receptors (IC(</em>{50}) = 37 and 98 nM in human and rat respectively. Displays no activity at P2Y(_1), P2Y(_2), P2Y(_4) and P2Y(<em>11) receptors (IC(</em>{50}) &gt; 10 μM). (Mamedova et al., 2004)</td>
<td>TOCRIS Biosciences</td>
<td>2146</td>
</tr>
<tr>
<td>Brefeldin A</td>
<td>DMSO</td>
<td>10μM</td>
<td>Fungal metabolite which disrupts the structure and function of the Golgi apparatus. Inhibits vesicular transport-mediated ATP release. (Dinter and Berger, 1998)</td>
<td>Sigma-Aldrich</td>
<td>B7651</td>
</tr>
<tr>
<td>Carbenoxolone</td>
<td>H(_2)O</td>
<td>100μM</td>
<td>Glucocorticoid that inhibits pannexin and connexin channels. Blocks conductive ATP release. (Espelt et al., 2013)</td>
<td>Sigma-Aldrich</td>
<td>C4790</td>
</tr>
<tr>
<td>Genistein</td>
<td>DMSO</td>
<td>100μM</td>
<td>Inhibitor of tyrosine protein kinase. (Akiyama et al., 1987)</td>
<td>Sigma-Aldrich</td>
<td>G103</td>
</tr>
<tr>
<td>Autocamtide 2-related inhibitory peptide</td>
<td>H(_2)O</td>
<td>0.5μM</td>
<td>Potent inhibitor of calmodulin-dependent protein kinase II. Selective over PKC, PKA and CaM kinase IV (IC(_{50}) &gt; 10 μM). (Ishida et al., 1995)</td>
<td>Sigma-Aldrich</td>
<td>A4308</td>
</tr>
<tr>
<td>Substance Description</td>
<td>Solution</td>
<td>Concentration</td>
<td>Effect</td>
<td>Source/Notes</td>
<td></td>
</tr>
<tr>
<td>-----------------------</td>
<td>----------</td>
<td>---------------</td>
<td>--------</td>
<td>--------------</td>
<td></td>
</tr>
<tr>
<td><strong>5-Nitro-2-(3-phenylpropylamino)benzoic acid (NPPB)</strong></td>
<td>DMSO</td>
<td>100μM</td>
<td>Inhibits calcium-sensitive chloride currents. (Keeling et al., 1991)</td>
<td>TOCRIS Biosciences 0593</td>
<td></td>
</tr>
<tr>
<td><strong>Carbamoylcholine chloride (Carbachol)</strong></td>
<td>H₂O</td>
<td>50μM</td>
<td>Non-selective cholinergic agonist. (Yan et al., 1995)</td>
<td>Sigma-Aldrich C4382</td>
<td></td>
</tr>
<tr>
<td><strong>Angiotensin II</strong></td>
<td>DMSO</td>
<td>0.2-1μM</td>
<td>Endogenous AT-1 and AT-2 receptor agonist. (Inagami et al., 1988)</td>
<td>Sigma-Aldrich A9525</td>
<td></td>
</tr>
<tr>
<td><strong>Amiloride Hydrochloride</strong></td>
<td>Made fresh on day in Tyrode’s</td>
<td>1mM</td>
<td>Na⁺ channel blocker. Blocks transient receptor potential polycystic 3 (TRPP3) (Dai et al., 2007), TRPA1 (Nagata et al., 2005), acid sensing- (ASIC) (Jetti et al., 2010) and mechanogated membrane-ion channels (epithelial sodium channels (ENaC))(Ferguson et al., 1997), urokinase-type plasminogen activator(Vassalli and Belin, 1987), as well as the Na⁺/H⁺ exchanger (Kleyman &amp; Cragoe 1988; Rievaj et al. 2013).</td>
<td>Sigma-Aldrich 1019701</td>
<td></td>
</tr>
<tr>
<td><strong>Benzamil</strong></td>
<td>H₂O</td>
<td>100μM</td>
<td>Selective and potent blocker of Na+/Ca²⁺ exchanger (NCX) (IC₅₀ ~ 100 nM)(Fischer et al., 2002); TRPP3 channels (IC₅₀ = 1.1 μM for inhibition of TRPP3 channel activity)(Dai et al., 2007), non-selective ENaC blocker; reduces colonic afferent mechanosensitivity. More potent derivative of amiloride.</td>
<td>TOCRIS Biosciences 0890</td>
<td></td>
</tr>
</tbody>
</table>
Table 4 - List of primary Abs employed during immunohistochemistry and western blotting

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Optimised Concentration/ dilution</th>
<th>MW / kDa</th>
<th>Host</th>
<th>Clonality</th>
<th>Supplier</th>
<th>Product reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-TRPV4 (Alomone)</td>
<td>2.0μg/ml</td>
<td>98</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>Alomone</td>
<td>ACC-034</td>
</tr>
<tr>
<td>Anti-TRPV4 (ABCAM)</td>
<td>1.0μg/ml</td>
<td>98</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>ABCAM</td>
<td>Ab94868</td>
</tr>
<tr>
<td>β-actin</td>
<td>0.5μg/ml</td>
<td>45</td>
<td>Mouse</td>
<td>Monoclonal</td>
<td>New England Biolabs</td>
<td>8H10D10</td>
</tr>
</tbody>
</table>

Table 5 - List of secondary Abs employed during immunohistochemistry and western blotting

<table>
<thead>
<tr>
<th>Secondary Antibody</th>
<th>Optimised Concentration/ dilution</th>
<th>Host</th>
<th>Conjugate</th>
<th>Excitation/Emission</th>
<th>supplier</th>
<th>Product reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Rabbit IgG</td>
<td>0.1μg/ml</td>
<td>Goat</td>
<td>IRDye® 800CW</td>
<td>-</td>
<td>Li-cor</td>
<td>926-32211</td>
</tr>
<tr>
<td>Anti-Mouse IgG</td>
<td>0.1μg/ml</td>
<td>Goat</td>
<td>IRDye® 800CW</td>
<td>-</td>
<td>Li-cor</td>
<td>926-32210</td>
</tr>
<tr>
<td>Anti-Mouse IgG</td>
<td>0.1μg/ml</td>
<td>Donkey</td>
<td>IRDye® 680RD</td>
<td>-</td>
<td>Li-cor</td>
<td>926-68072</td>
</tr>
<tr>
<td>Anti-Goat IgG</td>
<td>0.1μg/ml</td>
<td>Rabbit</td>
<td>IRDye® 800CW</td>
<td>-</td>
<td>Li-cor</td>
<td>926-32214</td>
</tr>
<tr>
<td>Anti-Rabbit IgG Alexa 568</td>
<td>1μg/ml</td>
<td>Goat</td>
<td>Alexa Fluor® 568</td>
<td>578/603</td>
<td>Life Technologies</td>
<td>A-11011</td>
</tr>
<tr>
<td>To-PRO3</td>
<td>1:5000 / 0.2μg/ml</td>
<td>-</td>
<td>Cy®5</td>
<td>642/661</td>
<td>Invitrogen</td>
<td>T3605</td>
</tr>
</tbody>
</table>
2.2 EXPERIMENTAL MODELS

2.2.1 GUINEA-PIGS

Guinea-pigs (GP) have been used extensively as an experimental model in research for the study of bladder function under both normal and pathological states, as the physiological properties of guinea-pig bladders are similar to those of human bladders. Importantly, guinea-pig urothelial and smooth muscle tissues share similarities to human. Many studies have identified the fundamental receptor groups in both species (refer to introduction), however, important similarities in bladder activity have also been determined. Spontaneous and carbachol induced mucosal contractions are observed in both species with a similar magnitude, both accompanied by ATP release. In both species, this ATP release increases in aging models, and other phenomenon, such as purinoceptor and muscarinic agonist-induced ATP release have been consistently demonstrated in both species, highlighting important similarities in fundamental signalling properties (Sui et al., 2014). Other phenomenon conserved between the two species include expression and activity of fundamental receptors (Creed et al., 2010; Inoue and Brading, 1991; Shabir et al., 2013), contractility parameters (Groen et al., 1994) and similarities in IC populations (Monaghan et al., 2012) to name a few.

In addition, guinea-pig bladders are readily available and provide a homogeneous tissue source for detailed investigation of the mechanisms. The size of the animal allows for precise surgery under anaesthetic, which is well tolerated, permitting excellent urodynamic studies. Precise dissection of the bladder tissue allows clean separation of tissue layers, providing tissue uniquely suited to many organ bath and electrophysiological techniques (Mostwin et al., 1994). Many pathological bladder models have also been developed in the GP, such as partial and progressive bladder outlet obstruction (Mostwin et al., 1991).

Laboratory GPs exhibit a median life span of three years until death by natural causes, and therefore at this age represent a 70 year old man in western society (Yao et al., 1988). GPs between 2 and 5 months old have not yet reached full maturity but are capable of breeding. From the age of 18 months GPs begin to exhibit age-related structural and functional changes in both general and bladder-specific physiology and therefore represent early old age (Fini et al., 2008; Gomez-Pinilla et al., 2007; Karim et al., 1992). Qualitative age-related changes observed in humans, such as an increase in prostatic stromal volume, begin to
emerge in GPs from 2 years and thereafter, indicating GPs of above this age as a good representative model for translational age-related studies (Horsfall et al., 1994; Jones et al., 2004). In addition, aging GP bladders commonly exhibit detrusor overactivity and as such, are a useful model for specific study of pathological bladders associated with aging (Gomez-Pinilla et al., 2007).

Generally speaking, larger animals exhibit a closer bladder physiology to humans, however research using larger animals is impractical and expensive, especially if it is necessary to maintain animals for long periods of time to reach ‘aging’ criteria. The main physiological disadvantage of using rodents for bladder research is that they exhibit a certain level of atropine resistance, i.e. there is purinoceptor contribution to contraction, whereas in humans there is not. This has been shown to be around 50% in GPs (Sibley, 1984). Although under physiological conditions human bladder contraction is mediated entirely through muscarinic receptor activation, it has been shown that purinoceptor contribution to contraction emerges in pathologically overactive bladders. Moreover, the atropine resistance observed in GPs stems from the presynaptic release of neurotransmitters, whereas the postsynaptic properties of the smooth muscle and the urothelial tissue are similar between GP and humans (Inoue and Brading, 1991), which is the focus of the current project. Therefore, the GP bladder model is suited for physiological investigation of the current project and also for translational pathological studies, despite the atropine resistance, which is not the subject of the current project. The advantages listed above make the GP both a useful and practical tool in bladder research, where their size also permits cost-effective aging studies.

Based on this information, young (2-5 months) and aging (24-36 months) male Dunkin-Hartley guinea-pigs (GPs) were used as the main animal model for this study.

2.2.2 MICE

Mice are widely used animal models in biomedical research, with the best-characterised genetics and readily available genetically modified models for investigation of molecular mechanisms. Of relevance to this project, mice have been extensively used to study the mechanisms and treatments of OAB, bladder cancer and the effect of aging on bladder function (Chan et al., 2009; Parsons and Drake, n.d.). Despite these small rodents having small differences in physiology, the fact that these animals can be bred easily and genetically manipulated presents a great advantage and tool for bladder research.

For the purpose of this project, both wild type (WT) C57BL/6 and P2Y₂ knockout (KO) (JAX® laboratories, USA) male mice were used. Young and aged mice were used during this
2.3 HUMAN SAMPLES

2.3.1 NORMAL CONTROL (OAB NEGATIVE)

Mucosal tissue biopsies were obtained by cystoscopy from the bladders of patients with no symptoms of OAB during routine surgery to obtain biopsies for diagnostics (table 6). Samples were obtained from well informed, consenting patients with the approval of the National Research Ethics Committee (South East Coast – Surrey) in accordance with the Helsinki declaration. In patients with suspected tumours or cancer, bladder samples were taken from macroscopically normal areas. These samples were collected from either Frimley Park Hospital (FPH) or The Royal Surrey County Hospital (RSCH) immediately after surgery and transported to the laboratory on ice in Tyrode’s solution. These samples were then subjected to physiological experimentation (section 2.5.1) using a variety of drug and compound interventions, after which they would be snap frozen in liquid nitrogen and stored at -80°C for future western blot analysis and immunohistochemistry (IHC).

Table 6 - List of human tissue biopsies collected for experimentation to date (Muc - mucosa, SM - smooth muscle)

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Date</th>
<th>Hospital</th>
<th>Gender</th>
<th>Age</th>
<th>Diagnosis</th>
<th>Tissue</th>
<th>Additional information</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16.11.12</td>
<td>FPH</td>
<td>Male</td>
<td>77</td>
<td>Haematuria</td>
<td>Muc alone</td>
<td>Post-Radical Prostatectomy</td>
</tr>
<tr>
<td>2</td>
<td>20.11.12</td>
<td>FPH</td>
<td>Male</td>
<td>69</td>
<td>Transitional cell carcinoma (TCC)</td>
<td>Muc w/ SM attached</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>24.7.13</td>
<td>RSCH</td>
<td>Male</td>
<td>83</td>
<td>Suspected Tumour</td>
<td>Muc - very little SM</td>
<td>Unknown histology</td>
</tr>
<tr>
<td>4</td>
<td>1.8.13</td>
<td>RSCH</td>
<td>Female</td>
<td>25</td>
<td>Suspected Cystitis</td>
<td>Muc w/ little SM attached</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1.8.13</td>
<td>RSCH</td>
<td>Male</td>
<td>69</td>
<td>Suspected Tumour</td>
<td>Muc w/ little SM attached</td>
<td>Frozen in OCT for IHC</td>
</tr>
<tr>
<td>6</td>
<td>3.9.13</td>
<td>RSCH</td>
<td>Male</td>
<td>80</td>
<td>TCC</td>
<td>Muc w/ little SM attached</td>
<td>Prior Brachytherapy</td>
</tr>
<tr>
<td>7</td>
<td>3.9.13</td>
<td>RSCH</td>
<td>Male</td>
<td>64</td>
<td>-</td>
<td>Muc - very little SM</td>
<td>Transurethral resection of prostate</td>
</tr>
<tr>
<td>8</td>
<td>18.11.14</td>
<td>FPH</td>
<td>Male</td>
<td>70</td>
<td>TCC</td>
<td>Muc - No SM</td>
<td>Obstructed</td>
</tr>
<tr>
<td>9</td>
<td>16.12.14</td>
<td>FPH</td>
<td>Male</td>
<td>83</td>
<td>TCC</td>
<td>Muc w/ little SM attached</td>
<td>Highly obstructed w/ chronic retention</td>
</tr>
<tr>
<td>10</td>
<td>19.12.14</td>
<td>FPH</td>
<td>Male</td>
<td>87</td>
<td>TCC</td>
<td>Muc w/ little SM attached</td>
<td>Obstructed</td>
</tr>
<tr>
<td>11</td>
<td>13.1.15</td>
<td>FPH</td>
<td>Male</td>
<td>77</td>
<td>Suspected tumour</td>
<td>Muc alone</td>
<td>Radiotherapy</td>
</tr>
</tbody>
</table>
Bladder biopsies were again obtained by cystoscopy from well informed, consenting patients under the ethics approval of a collaborative research group. These samples were collected from Medway Maritime Hospital (MMH) during operations and immediately transported to the laboratory. Samples from this cohort of patients were taken from female bladders diagnosed with idiopathic detrusor overactivity (IDO) (table 7), for comparison with the biopsies from ‘normal’ bladders (no IDO). These samples were then subjected to the same physiological experiments as the ‘normal’ biopsies’ and were also then snap frozen in liquid nitrogen.

Table 7 - List of human tissue biopsies collected for experimentation to date (Muc - mucosa, SM - smooth muscle)

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Date</th>
<th>Hospital</th>
<th>Gender</th>
<th>Age</th>
<th>Diagnosis</th>
<th>Tissue</th>
<th>Additional information</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.9.15</td>
<td>MMH</td>
<td>Female</td>
<td>&gt;45</td>
<td>IDO</td>
<td>Muc alone</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>3.9.15</td>
<td>MMH</td>
<td>Female</td>
<td>&gt;45</td>
<td>IDO</td>
<td>Muc w/ little SM attached</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>17.9.15</td>
<td>MMH</td>
<td>Female</td>
<td>&gt;45</td>
<td>IDO</td>
<td>Large mucosal sheet</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>25.9.15</td>
<td>MMH</td>
<td>Female</td>
<td>&gt;45</td>
<td>IDO</td>
<td>Muc, highly vascular, no SM</td>
<td>-</td>
</tr>
</tbody>
</table>

2.4 ANIMAL TISSUE DISSECTION

Animals were humanely culled by schedule 1 procedure in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986. All experiments were approved by the local ethics committee and followed UK home office regulations.

The urinary bladders were immediately removed and transported to the laboratory in Tyrode’s solution. These were then transferred to a sylgard dissection dish and submerged in fresh Tyrode’s. Under a dissection microscope, a longitudinal incision was made from the bladder neck to the dome and the full bladder was pinned out as a sheet with the urothelium uppermost. Longitudinal strips were then dissected from the bladder. For the purpose of
thorough investigation, ‘full thickness’, ‘smooth muscle only’ and ‘mucosal’ strips were required for experimentation. The mucosa (urothelium and suburothelium) was dissected away from the underlying tissue by gentle peeling for mouse tissue and by careful microdissection along the natural cleavage plane for GP tissue, using microdissection scissors. The various bladder tissue strips were then subjected to further preparation, appropriate for either physiological experimentation, immunohistochemistry, or ‘snap-frozen’ in liquid nitrogen and stored at -80°C for future investigation.

2.5 TECHNIQUES

2.5.1 FUNCTIONAL ORGAN BATH EXPERIMENTS

Longitudinal bladder strips (full-thickness, smooth muscle only, mucosal) dissected from animal bladders, or human bladder biopsies, were mounted within an organ bath/perfusion trough (Figure 2-2. B) for experiments measuring ATP release and contractile activity. Suture thread was used to tie one end of the tissue to a fixed hook and the other to an isometric force transducer with micromanipulator, allowing precise orientation of the tissue. The full experimental set-up, including trough dimensions, is displayed in figure 2-1. Mounting of strips was performed under a dissection microscope to improve accuracy and avoid accidental stretch and damage, which can stimulate ATP release and invalidate results. A water-jacket system and circulator (Techne C-400, GoIndustry DoveBid, USA) was used to encase the solution tubing and perfusion trough to maintain experimental solutions and tissue at 37°C. The tissue preparations were superfused with Tyrode’s solution at a resting rate of 2ml min⁻¹ for at least 45mins prior to experimentation to allow full tissue stabilization.

In order to monitor and record changes in tissue contractility during experiments, tissue was tied at one end to a force-displacement transducer (Model FT03, GRASS, USA) (Figure 2-2). This instrument enabled detection of changes in isometric force exerted by the tissue in response to the specific drug interventions performed, providing a force output in the form of volts (V). The force transducer was calibrated to the system using weights of known mass (g) to produce a voltage reflection, where the range of weights covered the range of isometric force generated by tissue during experiments. This yielded a standard curve of force (V) against weight (g), where the voltage output of the bridge amplifier could be used to determine force, which was expressed in Newtons from the following relationship, where ‘F’ is force, ‘m’ is mass and ‘g’ is the acceleration of gravity directed downwards:
\[ F = mg \ (g = 9.8 \text{ m.s}^{-2}) \]

There is a linear relationship between the force and voltage output where the line passes through the origin. The signal was amplified using a bridge-amplifier (TBM4M amplifier, WPI, USA) and recorded and stored as a digital real-time tracing using AxoScope version 9.0.2.03 (Axon instruments, USA). To minimise trace interference due to vibration, the set up was built on an ‘Active-air vibration control platform’ (Benchmate 2212 series, Kinetic Systems, USA).

For measurement of ATP, samples of solution were taken from the sampling point at specified time-points (refer to experimental protocol – figure 2-3) using a micropipette. The sampling point was situated downstream of the tissue, such that all ATP released by the tissue would be collected and none lost due to solution outflow (Fig. 2.2 C). The samples were later measured for ATP content using the luciferin-luciferase assay (section 2.5.1.2).
Figure 2 – 2. Perfusion trough set up. A: Image of two organ baths mounted on vibration control platform. B: Close-up image of single organ bath. Dimension: 4mm (width) x 5mm (depth) x 30mm (length) 1. Isometric force transducer 2. Organ bath #1 3. Organ bath #2 4. Solution tubing 5. Fixation hook attached to force transducer 6. Tissue strip 7. Fixation hook 8. Solution inlet C: Schematic diagram of perfusion trough. Tyrode’s solution flows from solution bottles through insulated tubing and solution inlet over tissue at the desired flow rate. Changes in force exerted by tissue are detected by the force transducer, amplified, digitized and recorded as a digital real time tracing, observed on a computer monitor. Samples of solution are taken from the sampling point at specified time-points using a pipette and later measured for ATP content.
2.5.1.1 EXPERIMENTAL PROTOCOLS

2.5.1.1.1 STANDARD PROTOCOL

Tissue strips were mounted and incrementally stretched until exerting approximately 1 gram of tension. Strips were then left to stabilize in Tyrode’s solution at a constant flow rate of 2 ml min\(^{-1}\) for between 30-60 mins until a stable baseline tension was achieved, after which the protocol in figure 2-3 was initiated. The flow rate was then increased to 5 ml min\(^{-1}\) and the tissue was continuously perfused with Tyrode’s for a specified control period, after which superfusate was collected from the sampling point using a micropipette tip and snap frozen for later evaluation of ATP content. A sampling volume of 45 μl was instated as this allowed for duplicate ATP measurement, minimized dilution of the superfusate, and did not mechanically disturb the tissue.

Drug compounds were then introduced via the superfusate and samples taken at 1, 3 and 5 minutes for short observations, followed by sampling at 10 min intervals thereafter for longer experiments. This was then followed by a post control period using the appropriate control solutions (DMSO-containing/normal Tyrode’s) during which further superfusate samples were taken. The mean ATP content of pre and post control samples was used as the baseline ATP release, and any changes in ATP release stimulated by the drug intervention was expressed as a percentage difference from this. Additional samples were taken directly from the bottles of prepared solutions (Tyrode’s control, DMSO-containing Tyrode’s control, Drug-containing Tyrode’s) for ATP measurement to ensure all observed ATP was from tissue release and not from solutions and apparatus.
The protocol was then extended for the purpose of interaction and antagonist studies. Antagonist studies involved the use of the TRPV4 agonist (GSK1016790A) to trigger ATP release, whilst simultaneously inhibiting various signalling molecules and mechanisms to observe any effect these may have on this ATP release. The full procedure is shown in figure 2–4. The tissue strips were first subjected to the single drug intervention protocol (figure 2-3), using GSK. Tissue was then superfused with Tyrode’s at 2ml min\(^{-1}\) for a ‘recovery period’ of 1 hour to allow the tissue to return to a stable state and for ATP pool regeneration. This was followed by a similar protocol, but with the addition of a 10minute antagonist step before the GSK, which was maintained throughout the 5minute superfusion with GSK and for 10minutes after. The mean ATP release during the 10minute antagonist periods were used as control and taken as 100%. This was followed by a further recovery period and a repeat intervention of GSK. The GSK intervention was repeated to show that activation of TRPV4 is recoverable/reproducible, and allowed any drift in tissue function over the period of the full experiment (=6 hours) to be observed.
An interaction study was used to determine whether activation of TRPV4 (1μM GSK) with simultaneous activation of specific purinoceptors (100μM UTP) triggers a higher or more sustained ATP release/contraction than their combined individual effects, possibly indicating cross-talk of receptor mechanisms. This involved 5 interventions with 1 hour recovery periods between each. First, tissue was superfused with UTP alone, followed by GSK alone (pre-control interventions), and then UTP and GSK in combination to determine whether they may amplify the ATP signal when activated in simultaneously. This was followed by further post-control interventions of GSK alone and UTP alone. Tissue was superfused with Tyrode’s solution for 1 hour between each intervention as a recovery period.

Figure 2 – 4. Antagonist studies intervention protocol. Schematic of work flow carried out for antagonist studies. Red arrows indicate sampling points, black arrows indicate direction of work flow. Tissue was continuously monitored via real-time tracing throughout the protocol to obtain a record of all tissue activity and ensure tissue stability.
2.5.1.1.3 ASSESSMENT OF MECHANICAL STRETCH-INDUCED ATP RELEASE

A variation of the antagonist study protocol was designed to investigate the effects of mechanical stretch-induced ATP release from GP tissue strips. Instead of the 5minute GSK intervention, tissue strips were stretched to 150% of their original length for 30seconds as the main intervention. First, tissue was repeatedly stretched in small increments until a stable tension was achieved, exerting approximately 9.8mN force. Strips were then stretched using the micromanipulator by 150% of their resting length and ATP content of the surrounding solution was measured at 30seconds, 1, 2 and 3minutes. These time points were chosen from information provided by preliminary optimisation experiments, which revealed the stretch-induced ATP release is greatest at 30-60seconds and lost after 2minutes (refer to figure 3-19). A 30second stretch was chosen as this could be repeated 3 times (with a 1hour recovery period between each repeat) and still elicit a reproducible ATP release with each stretch. A 2minute stretch was also assessed but this was not reproducible over 3 repeats. During the first stretch, tissue strips were constantly superfused with Tyrode’s. During the second stretch (after a 1hour recovery period) tissue was superfused with 1μM HC-067047 (TRPV4 antagonist) for 10minutes prior, during, and 10minutes after the stretch intervention. This allowed the role of TRPV4 in stretch-induced ATP release to be assessed. After stretch, tissue was returned to its original resting tension.

2.5.1.2 MEASUREMENT OF ATP – LUCIFERIN-LUCIFERASE ASSAY

2.5.1.2.1 EXPERIMENTAL PROTOCOL

Samples collected during the aforementioned physiological experiments were measured for ATP content using the ATP-dependent bioluminescent ‘Luciferin-Luciferase’ assay (Sigma, UK). The active working solution was prepared by diluting ‘Sigma ATP assay mix’ with the kit buffer according to manufacturer’s guidelines and stabilised at +4°C for 1hour to allow any contaminated ATP to breakdown. Samples were then combined in a 1:1 ratio with working solution and the luminescence intensity immediately read using an LRB Wallac 1250 luminometer. Appropriate control readings of reagents and chemicals were also taken into account for any background luminescence. The luminometer was calibrated on the day of each experiment using an ATP standard of known concentration, as this is an enzyme based assay where the enzyme activity will vary slightly between experiments. ATP content of samples was determined using a calibration log-log plot of luminescence as a linear function of ATP concentration. An example ATP standard curve is provided in figure 2-5, with an ‘n=5’ demonstrating the consistency of the assay.
DATA ANALYSIS

The background luminescence of the working solution was subtracted from the final ATP content value to provide a true value of ATP content per sample. The ATP content of the control solution sampled directly before and after the drug intervention was averaged, providing a mean value for basal ATP release which was used as the control and taken as 100%. Any ATP released in response to the drug intervention was expressed as a percentage change from this. For each individual experiment, ATP release was normalized to tissue mass.

The nonstatic and nonrecirculating experimental setup used for the purpose of ATP measurement provides a constant flow, which removes any accumulating metabolites and allows any dynamic changes in ATP release to be measured. This also allows controlled delivery of intervention chemicals to the tissue through the perfusion system for reliable quantification of its effect against basal ATP release. However, it is important to recognise that the quantity of ATP release determined is likely an underestimate, as the actual quantities released into the tissue microenvironment will be affected by factors such as tissue ecto-ATPase activity, the diffusion barrier and the flow imposed by the system. Despite this, the setup permits accurate and reproducible determination of the effect of chemical interventions on bladder tissue outputs.

Figure 2 - 5. ATP standard curve. Luminescence emitted by different known concentrations of ATP, providing a linear standard curve for determination of sample ATP concentration. Each concentration was repeated 5 times, with very little standard deviation, demonstrating the consistency of the assay. (n=5).
2.5.1.3 MEASUREMENT OF ISOMETRIC FORCE

For appropriate interventions, the magnitude of contraction elicited by the agonist was determined. Recorded traces were analysed using ClampFit (version 9.0.2, Axon Instruments, USA) and graphical examples of traces were produced using KaleidoGraph software (version 3.5, Synergy Software, USA). Traces were imported into Clampfit and the baseline tensions at the end of the pre and post control solution periods (Figure 2-6) were found. These were averaged and subtracted from the maximal force reached during the drug intervention, providing a value of net increase in force of the tissue (contraction) evoked by the intervention expressed as force μN/mg tissue.

![Graph showing measurement of isometric force with 50 μM Carbachol](image)

2.5.2 MEASUREMENT OF INTRACELLULAR CALCIUM ([Ca²⁺])

The focus of this thesis is on the function of TRPV4 within the bladder mucosa and the mechanisms underlying its physiological outputs. As this receptor is a calcium permeable, non-selective cation channel, it is highly likely that the activity of this channel relies significantly on entry of extracellular Ca²⁺ resulting in downstream intracellular Ca²⁺ signalling. Although functional organ bath experiments can and have been designed to assess this, measurement of changes in the intracellular Ca²⁺ concentration in single isolated cells using the Ca²⁺ fluorophore Fura-2 through epifluorescence microscopy grants a more direct and conclusive method for this purpose.
2.5.2.1 CELL ISOLATION

Bladders were immediately harvested from culled GPs and placed in a Hepes-buffered Tyrode’s solution (described in section 2.1.1.1) to maintain tissue. The mucosa was then gently removed using microdissection tools and cells where then dissociated using a collagenase type -1 (1.0 mg/ml Worthington Biochemical Corp, Lakewood, NJ, USA) -based enzyme mix dissolved in Hepes-buffered Tyrode’s solution with no added CaCl₂. The collagenase hydrolyses native extracellular collagen fibres releasing the various cell types of the mucosa into a single-cell suspension. During the dissociation process, the tissue suspension was carefully triturated using a fire-polished glass pipette to further dissociate the cells and produce the final cell suspension for loading. Variations of enzyme exposure time and incubation temperature were used on the day of each experiment depending on tissue size and observations of the cell suspension when triturating and viewing under the inverted microscope (Nikon eclipse Ti, UK). Commonly, cells were either incubated in the enzyme mix overnight at 4°C, or for 1-1.5 hours at 4°C followed by 2-3 repeats of a 2 minute incubation at 37°C with a subsequent 5min incubation at room temperature. These optimized methods provide a well-dissociated cell suspension, without damaging the cells such that they remain responsive and of high quality.

2.5.2.2 LOADING OF CELLS WITH THE RATIO METRIC CALCIUM FLUOROPHORE FURA-2

Fura-2-acetoxymethyl-ester (Fura-2 AM, Molecular Probes®, Thermo fisher Scientific, UK Cat# F1201) is a ratiometric fluorescent Ca²⁺ indicator dye that allows measurement of changes in intracellular calcium concentrations. A ratiometric dye is a duel excitation or emission indicator that shows a shift in its excitation (or emission) spectra once bound to calcium, which can be detected. Fura-2 is a duel excitation indicator with two excitation wavelengths of 340 and 380nm, with an emission wavelength ranging from 410-510nm.

In the presence of low or 0-Ca²⁺ the excitation max is 362nm with emission max at 512nm. Binding of Ca²⁺ shifts the excitation max to 335nm and emission to 505nm(Grynkiewicz et al., 1985). Therefore, measuring the Ca²⁺-induced emitted fluorescence (510nm) at both 340 and 380nm excitation wavelengths allows the Ca²⁺ concentration to be estimated based on the ratio between the 340/380 values. The ratio between the fluorescence excitation spectra of Fura-2 is a direct function of the intracellular [Ca²⁺], as demonstrated in figure 2-7.
This ratio method minimizes limitations from dye leakage, uneven loading, photobleaching, changes in focus, as well as problems associated with varying cell thicknesses in mixed populations providing more reliable results. In addition, using the ratio to estimate the [Ca$^{2+}$] means the overall dye concentration in the cell will not need to be accounted for. A further advantage of Fura-2 is that it is lipophilic and therefore highly cell-permeable. Once in the cell the acetoxymethyl group is cleaved off by intracellular esterases, thus containing Fura-2 within the cell.

Cells were incubated with 5μM Fura-2 AM at 37°C for 5-10 minutes with an equal volume and concentration of pluronic F127 to aid with uptake. The cell suspension was then stored in this solution at 4°C under dark conditions for 1-2 hours before loading to the experimental set up. Once ready, cells were slightly triturred once more and then pipetted into a chamber (10x20x5mm) within a custom-made Perspex dish contained in a water jacket heated to 37°C. Cells were then left to settle and attach to the dish in 37°C Tyrode’s solution for 20 minutes prior to the experiment.

Figure 2 – 7. Fluorescence excitation spectra of Fura-2. This duel excitation dye can be excited at both 340 and 380nm wavelengths, emitting at 510nm in both cases. The excitation wavelengths at various free Ca$^{2+}$ concentrations are shown (0-39.8μM in buffer at pH7.1). The ratio of light emitted between excitation at 340 and 380nm (dotted lines) is used to estimate the concentration of Ca$^{2+}$ within a cell. Image adapted from (Inc., 2015).
2.5.2.3 CALCIUM IMAGING SET-UP

The set-up for detection of calcium in live cells includes; an inverted Nikon Eclipse Ti microscope equipped with a xenon arc lamp (Cairn research, UK); an excitation filter wheel to supply the required 340nm and 380nm wavelength light; various dichroic mirrors acting as beam splitters to filter and direct the desired wavelength light through the necessary light path; a cooled charge-coupled device (CCD) camera (LucaEM S, Andor™ Technology, UK)(shown in full in figure 2-8) for data acquisition; a Cairn A-D interface to convert the analogue signals to digital signals, which are then passed to a PC. The set-up is controlled using a PC running WinFluor software for data acquisition. The microscope uses a 10x eyepiece with 40x quartz objective lens for imaging, with a numerical aperture exceeding 1.2. Refer to figure 2-8 for full set up and light path.

Figure 2 – 8. Schematic of experimental setup for live-cell calcium imaging by epifluorescence microscopy. The xenon arc lamp emits broad spectrum white light which is passed through a spun filter wheel, filtering the light to 340nm and 380nm wavelengths. This light is then directed to the cell chamber via a 410nm dichroic mirror. Any emitted light is collected by the objective and wavelength light of >410nm passes back through the dichroic mirror and is focused and directed by a light mirror through a light tube. The light then hits a final 510nm dichroic mirror filtering out any >510 wavelength light and delivering the remaining 410-510nm wavelength light to the CCD camera. The CCD camera output is processed via a digitizer and is then displayed on a computer monitor as a real time trace for 340 and 380nm excitation wavelengths, as well as a trace representing the ratio of the two. The data is captured and stored for later analysis.
2.5.2.4 EXPERIMENTAL PROTOCOL

Once cells had settled they were superfused with a constant flow of oxygenated sodium bicarbonate Tyrode’s solution (described in section 2.1.1.2) at approximately 2ml/min$^{-1}$ through a water-jacketed tubing system heated to 37°C. This rate of flow ensured cells were not dislodged and washed away from the cell chamber, but were still supplied with sufficient superfusate to be adequately maintained. Cells were then visualized using the microscope white light source and a cell of interest chosen depending on the size, morphology and general appearance of the cell (ideally, healthy intermediate urothelial cells). Superfusate solutions (sodium bicarbonate Tyrode’s alone, or containing either 100μM UTP or 1μM GSK1016790A [same concentrations used for functional organ bath work for comparison]) were stored in small gas-bubbled flasks connected to a gravity-fed delivery tube above the set-up. Entry of each solution to the delivery tube was controlled by a channel flow switch (three-way tap) (figure 2-9). Superfusate solutions flowed through the gravity-fed delivery tube and into the cell chamber and waste superfusate removed at the same rate via a suction pump (Minipuls 3, Gilson Inc, USA).

Figure 2 – 9. Storage and flow control of superfusate solutions for calcium imaging. 1. Gas delivery tubes supplying superfusate solutions with 95% O$_2$ : 5% CO$_2$. 2. Solution flasks containing superfusate solution and agonist solutions. 3. Channel flow switches controlling entry of solutions into final delivery tube 4. Gas control switch 5. Delivery tube flow switch 6. Final solution delivery tube.
An area of interest was selected to surround the chosen cell and recording initiated. Initially, a control period of Tyrode’s superfusate was recorded providing a basal intracellular Ca\(^{2+}\) level. Single cells were then challenged with the stated agonists for 5 minutes followed by a second control period of Tyrode’s. Throughout the recording period, cells were alternately illuminated at 32Hz with 340nm and 380nm excitation wavelength light. Emitted light between 410nm and 510nm previously focused and split by dichroic mirrors in the light path was then collected by a CCD camera, set at a speed 500 frames per second. The ratio between the emitted light intensities of Fura-2 under the two excitation wavelengths was converted into intracellular [Ca\(^{2+}\)] values and any relative changes to intracellular [Ca\(^{2+}\)] were recorded and displayed as a real time tracing on a video monitor. The relationship between the excitation wavelength ratio \((R [340nm/380nm])\) and intracellular [Ca\(^{2+}\)] is expressed by the equation (Gryniewicz et al., 1985):

\[
[Ca^{2+}]_i = K_d \beta \left( \frac{R - R_{\text{min}}}{R_{\text{max}} - R} \right)
\]

\(K_d\) (224nM) is the specific dissociation constant of Fura-2 for Ca\(^{2+}\) in the cytosolic environment (determined previously [Gryniewicz et al., 1985]). \(\beta\) represents the ratio of the fluorescence intensity at 380nm between saturating and 0 [Ca\(^{2+}\)] conditions and is defined by the specific setup used. \(R\) is the fluorescence ratio between 340/380nm. The \(R_{\text{min}}\) and \(R_{\text{max}}\) refer to the ratio values at saturating and 0 [Ca\(^{2+}\)] conditions and are influenced by the optical system, and are thus determined experimentally. For accurate quantification of [Ca\(^{2+}\)]\(_i\), \(R_{\text{min}}\) and \(R_{\text{max}}\) are determined experimentally using high (1mM) and 0 [Ca\(^{2+}\)] (no added CaCl\(_2\) plus 1mM EGTA) solutions in vivo, i.e. calibrating the system to \(R\) under these conditions in live cells, where the cells under investigation are filled directly with the buffered [Ca\(^{2+}\)] solutions via patch-clamp technique. This provides a more accurate calibration than using an in vitro solution-based method (calibrating to high and 0 Ca\(^{2+}\) solutions only, not in live cells) as Fura-2 will behave differently in live cells due to differences in the surrounding conditions imposed by the cytosolic environment, such as viscosity. However, as the aim during this study is to simply observe relative changes in [Ca\(^{2+}\)], in response to specific agonists, the system was calibrated using the solution-based method (solutions described in section 2.1.2). Here, the calibration solution is a high K\(^+\) based saline to mimic the intracellular environment.
2.5.3 WESTERN BLOTTING

This technique was used to determine the presence, and or quantity of various proteins of interest in the bladders of species used throughout this project. The mucosa and smooth muscle were separated and analysed separately to allow comparison of protein expression between these tissues.

2.5.3.1 PREPARATION OF WHOLE TISSUE LYSATES

Previously snap-frozen mucosal and smooth muscle samples were ground to a fine powder in liquid nitrogen using a pre-cooled pestle and mortar. The powder was lysed in an appropriate volume of RIPA buffer (depending on the sample weight [for full description see section 2.1.5.2]) and sonicated at 100Amp using a VC130PB ultrasonic processor (Sonics & Materials Inc, USA) for 30 seconds to release intracellular components. Samples were stored at -20°C until required.

2.5.3.2 PROTEIN QUANTIFICATION

The protein concentration of whole tissue lysates was determined using a DC™ (detergent compatible) colorimetric protein assay kit (DC™ protein assay, BioRad, UK). This was performed on the day of whole tissue lysate preparation before samples were frozen down to avoid a further freeze-thaw cycle. This particular assay is based on the well-established Lowry assay, which relies on the reaction of protein with an alkaline copper tartrate solution and folin reagent. The initial reaction between protein and copper, and the subsequent reduction of folin results in a blue coloured solution, the intensity of which is proportional to the protein content of the sample.

A serial dilution of known protein concentration ranging from 30.125μg/ml to 1000μg/ml was prepared using a stock solution of 1000μg/ml bovine serum albumin (BSA). Samples were diluted 1:20 and 1:50 in reverse-osmosis (RO) water and the working solution (alkaline copper-tartrate) was prepared as per the manufacturer’s instructions. 50μl of diluted samples and standards were reverse-pipetted (in triplicate) into a NUNC 96-well plate, followed by 25μl working solution per well. A multi-pipette was then used to add 200μl of the folin-containing reagent to each well, initiating the color-producing reaction. After incubation for 5 minutes the colour intensity was read at 695nm using a plate reader (multiskan RC plate reader, Labsystems, Finland) and recorded using Ascent™ multiskan data acquisition software (Thermo-scientific, USA). BSA standards were used to plot a standard curve which was used to determine the protein concentration of samples.
2.5.3.3 PREPARATION OF LOADING-SAMPLES & GEL ELECTROPHORESIS

On the day of experiment, frozen samples were rapidly thawed and combined in a 3:1 ratio with Laemmli buffer. Samples were then incubated at 95°C for 5 minutes on a hot plate to fully resolve the lysate. However, during optimisation of this technique, it was found that this level of heating denatured TRPV4 to an extent where it could no longer be detected. Therefore, when probing for TRPV4, samples were left at RT for 30 minutes, which resolved samples to the optimum degree for TRPV4 detection.

Precast gels (8–16% Mini-PROTEAN® TGX™ 10 well Gel, BioRad, UK) were clamped in a gel cassette and placed in a running tank filled with fresh running buffer. Samples were then vortexed before being loaded (30μg/well) in duplicate alongside a pre-stained protein ladder (PageRuler Prestained NIR Protein Ladder, Thermo-scientific, USA) which produces coloured bands at specified known molecular weights. This allows both visualization of the progress of gel electrophoresis and clarification of the size of the protein bands probed from samples. The tank was then connected to a powerpack (PowerPac™, BioRad, UK) and run at a constant voltage of 120V. A constant voltage was chosen as this insures the current does not rise during the run, which can result in band distortion. Using gels that have a progressively higher percentage of acrylamide from start to finish means resistance increases during the run and thus under a constant voltage, the current slowly drops, insuring no damage or distortion occurs. Gel electrophoresis ended once the ‘dye front’ had run out from the end of the gel or once the proteins had been adequately separated.

2.5.3.4 ELECTROPHORETIC TRANSFER

A nitrocellulose membrane, two sheets of filter paper and two fibre pads were pre-soaked in cold transfer buffer. The gel was carefully placed on top of the membrane, which was placed centrally between the two sections of filter paper and then the two fibre pads (figure 2-10). A roller was used against the filter paper to gently remove any air bubbles from between the gel and membrane. The construct was then clamped within a transfer cassette and placed within the transfer tank filled with pre-cooled transfer buffer, taking care to orientate the cassette correctly such that the proteins would transfer towards the membrane. The transfer was run at 300mA for 90 minutes, which ensured full transfer of proteins to the membrane. The tank was surrounded by ice and contained a cold pack to maintain low temperatures necessary for efficient transfer. An electronic stirrer was also used within the tank to ensure a consistent temperature of buffer throughout the tank, as heating may occur at focal points close to the electrodes which can cause an uneven transfer.
2.5.3.5 IMMUNOBLOTTING

Once the transfer had completed, the membrane was transferred to a small opaque box and rinsed twice in TBS for 5 minutes to remove any residual transfer buffer. All rinsing and incubation steps were carried out on a shaker to ensure even coverage of the membrane with solutions. The membrane was then incubated for 1 hour at RT in a blocking buffer (5% milk TBS) containing a number of proteins, including casein, which bind to the remaining surface of the membrane (not occupied by proteins transferred from samples), which prevents nonspecific binding of the detection Abs to the membrane in later steps. This incubation was followed by the addition of desired primary Abs (diluted in 5% milk TBST) to the membrane. Tween20 is introduced to the incubation and washing solutions from this point onwards as it is a detergent which also aids in the prevention of nonspecific Ab binding. The primary Ab solutions were then decanted into falcon tubes and frozen down at -20°C for re-use. Membranes were then rinsed once and washed 3 x 5minutes in TBST, before addition of the relevant IRDye® – conjugated secondary Ab, also diluted in 5% milk TBST. The membranes were incubated for 1 hour at RT in the secondary Ab before a further rinse and 2 x 5minute washes in TBST and a final wash in TBS (as tween 20 can interfere during signal detection) before visualization. In some cases, once visualized, the membrane was stripped of the original Abs using stripping buffer (refer to chemicals and solutions) to allow re-probing using different Abs. This was done by washing the membrane in 5ml Restore™ stripping buffer for 15minutes, followed by a 5minute wash in TBS, before commencing the immunoblotting protocol from the blocking stage.
2.5.3.6 SIGNAL DETECTION

The Odyssey®CLx infrared imaging system (LI-COR, Germany) was used for signal detection. Membranes were placed protein-down on the scanning surface in RO water. A silicone matt was placed on top and a roller was used to remove any air bubbles. These steps ensure the membrane is flat against the imaging surface for optimal signal detection. LI-COR® image studio software was used for image acquisition and later analysis.

2.5.3.6.1 USE OF NEAR-INFRINGEMENT (IR) SECONDARY ANTIBODIES

Various studies have been performed to characterise the sensitivity and variability of IR Abs used for western blotting. Use of IR Abs commonly yield a 200-400 fold higher sensitivity than classic chemiluminescence, due to a much lower background of membranes in the IR range. The most notable advantage, is that IR Abs are directly labelled with a fluorescent dye and thus do not involve the enzyme/substrate kinetics indicative of chemiluminescent detection. The involvement of enzyme kinetics introduces variation between experiments and the need for development and exposure times. Directly labelled IR Abs avoid these limitations, as the fluorescence intensity observed on the final image is emitted directly from bound Abs, providing data that is more accurate, reproducible and representative of the actual quantity of protein present. To summarise, it has been shown that the IR detection method is quantitative, with a wide, consistent linear detection range, compared to chemiluminescence, which is only semi-quantitative with an unpredictable, lower linear detection range (Schutz-geschwender et al., 2004). Therefore, use of this method provided more accurate and reliable data for quantification.

2.5.3.7 BLOT ANALYSIS

LI-COR® image studio software was used for this purpose. Rectangular areas of interest of identical size were drawn around each band. The software calculates the sum of the individual pixel intensities within the rectangle and then subtracts the mean intensity values of pixels from the background (taken from the area directly surrounding the rectangle, defined by a slightly larger rectangle [figure 2-11]). This provides a final corrected value for the ‘signal’ of each band. Raw images were used during analysis, however adjusting the contrast/brightness did not affect output values as the software always uses the raw image intensity values obtained during acquisition from an ‘image memory’.

β-actin was used as a loading control to indicate the total protein loaded per well. Initially, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was tested as the reference protein,
however this yielded different band strengths between mucosal and smooth muscle samples despite similar loading concentrations, due to the increased energy demand of DSM cells over urothelial. \( \beta \)-actin is a housekeeping gene that is expressed at similar levels in both tissue types and was therefore used as the loading control. The final signal value for the band of the target protein was normalized to its corresponding \( \beta \)-actin, accounting for any variation in total protein levels. Samples were run in duplicate on the same gel, such that duplicate values could be averaged providing a more representative final value of the quantity of target protein in each sample. This, as well as vortexing samples prior to loading, reduced possible errors due to uneven protein concentrations within samples.

![Image](image-url)

**Figure 2 – 11.** Screenshot of example workspace in Image studio®. Inner purple rectangle measures total pixel intensity, outer rectangle calculates mean of background pixels, value under each rectangle and in ‘signal’ column is total intensity of each band minus surrounding background. The upper bands in this example show TRPV4 and the lower bands \( \beta \)-actin. Each TRPV4 signal value would be normalized to its corresponding \( \beta \)-actin, providing final values accounting for variation in total protein loaded between samples.
2.5.4 MEASUREMENT OF REACTIVE OXYGEN SPECIES (ROS) USING LUCIGENIN-ENHANCED CHEMILUMINESCENCE

2.5.4.1 PRE-INCUBATION OF BLADDER TISSUE SECTIONS

Tissue strips were instantly dissected from a previously harvested GP bladder. Ten GP bladders were investigated for the purpose of this experiment. From each bladder two strips were taken, one for incubation in Tyrode’s solution to serve as control, and one which was incubated in 1μM GSK10166790A (TRPV4 agonist) as the intervention. After dissection, the strips were transferred to an Eppendorf containing either the control Tyrode’s solution or TRPV4 agonist and incubated at 37°C for 30 minutes in a pre-heated water bath. The strips were then removed and instantly snap-frozen in liquid Nitrogen before being stored at -80° until further investigation.

2.5.4.2 PREPARATION OF WHOLE-TISSUE LYSATES

This was achieved in a similar manner to that described for preparation of lysates for western blotting. The previously snap-frozen full-thickness bladder wall samples where ground to a fine powder in liquid nitrogen using a pre-cooled pestle and mortar. Each sample was re-suspended in 200μl Hanks Balanced Salt Solution (HBSS) and then sonicated on ice using a VC130PB ultrasonic processor (Sonics & Materials Inc, USA) for 30 seconds at 60 AMPs.

A Bradford assay was then employed to determine protein concentrations of the homogenates. The Bradford reagent contains Coomassie Brilliant Blue G-250 dye, which once bound to protein, changes colour from red to blue. The absorbance at 595nm is proportional to the quantity of blue protein-dye complexes and hence the quantity of protein in the solution (Bradford, 1976). 2μl of tissue homogenate was added to 1ml Bio-rad protein assay dye (Bio-Rad Bradford protein dye, Bio-Rad, UK) and thoroughly vortexed. The dye-homogenate solution was transferred to a 1.5ml plastic cuvette and the optical density was measured at 595nm using a spectrophotometer (UV1101 Biotech photometer, WPA, Cambridge, UK). A standard curve was generated using prepared samples of known BSA concentrations and this was used to determine the protein concentrations (μg/ml) of homogenate samples.
2.5.4.3 MEASUREMENT OF ROS USING LUCIGENIN-ENHANCED CHEMILUMINESCENCE

A lucigenin-enhanced chemiluminescence assay was used to determine the NADPH-dependent superoxide production in bladder wall homogenates. Lucigenin is a chemiluminescent probe that is reduced by ROS to an energy-rich dioxetane molecule. This process causes dioxetane to emit a photon, which can be detected by a luminometer allowing determination of ROS production in samples (Dikalov et al., 2007). The quantity of photons collected is expressed as relative light units, or RLUs.

Following protein determination, 25μl of each sample was reverse-pipetted in duplicate into a NUNC 96-well plate. 25μl sample buffer minus tissue was added to 2 wells to serve as the blank control. Sample buffer (HBSS, 0.8mM MgCl₂, 1.8mM CaCl₂) was then added to provide a final volume of 140μl in each well. The plate was then placed in a pre-heated (37°C) luminometer (BMG Lumistar, Germany) and 40μl lucigenin buffer (25μM lucigenin [Sigma, UK] in sample buffer) was sequentially added to each well using the plate reader’s auto-dispenser function. Basal superoxide production was then recorded over 11 cycles, where the RLU for each well was measured for a 3 second period during each cycle. After this basal reading was taken, 20μl of a 1mM NADPH (Sigma, UK) solution in sample buffer was manually reverse-pipetted into each well. The plate was then immediately returned to the luminometer and RLU recorded over 3 seconds for each well for a further 21 cycles. Finally, 2μl of 1M Tiron (Sigma, UK) was manually added to each well and the RLU measured over a further 21 cycles as before. Tiron is a preferential cell-permeable superoxide radical scavenger (Ledenev et al., 1986; Van Ginkel and Raison, 1980) and thus will remove any superoxide from each well, allowing determination of the proportion of superoxide present among the other ROS being measured by the luminometer. Each well contained a final volume of 200μl providing final working concentrations of 5μM lucigenin, 100μM NADPH and 10mM Tiron. A low lucigenin concentration was used to avoid artifactual superoxide production through redox cycling of the molecule (Li et al., 1998). The light emission was recorded, corrected to the protein concentration of each sample and expressed as median arbitrary light units/min (MLU/min).
2.5.5 IMMUNOHISTOCHEMISTRY

2.5.5.1 SAMPLE PREPARATION & SECTIONING

Following dissection, longitudinal strips of full bladder wall were embedded in optimal cutting temperature compound (OCT [Bright Instrument LTD, UK]), snap-frozen in liquid nitrogen and stored at -80°C. These bladder strips were orientated to enable sectioning through the transverse plane, ensuring that once sectioned, each specimen contained all three distinct tissue layers; the urothelium, suburothelium and detrusor smooth muscle. This enabled analysis of molecular structures across the entirety of the bladder.

Specimens were transported from -80°C in a liquid nitrogen tank to a pre-cooled (-22°C) cryostat (Hyrax C25, Zeiss, Germany). Specimens within OCT were fixed to the cryo-chuck using further OCT and were sectioned at 10μm thickness before being immediately mounted onto poly-L-lysine coated slides (4 sections per slide). Each section was viewed under a light microscope to ensure both structural integrity and presence of all tissue types, before being stored at -80°C prior to immunostaining.

2.5.5.2 IMMUNOSTAINING

On the day of immunostaining, cryosections were fixed in previously cooled methanol for 5mins at -20°C, before undergoing 3x10min washes in PBS. 1%BSA PBS solution was then used to block the sections for 1 hour at room temperate, after which either a diluted primary antibody would be added to the sections, or 1% BSA PBS would be reapplied to one as a secondary control. This secondary control reveals any unspecific secondary antibody binding that could present a false positive stain. Slides were then incubated over night at 4°C in a humidified chamber. On the following day, slides were again washed 3 times in PBS for 10mins, before incubation at room temperature for 1 hour in the appropriate secondary antibody. Following another 3x10min PBS washes, each specimen was incubated for 10mins in a nuclear stain (To-PRO3® [Molecular Probes, Netherlands]) at 1:5000 concentration. A further 3x10min PBS washes were performed, after which sections were mounted using an antifadent-containing fluorescence mounting medium (AF-1 [Citifluor, UK]), sealed using clear nail polish and then stored under dark conditions at +4°C until imaging.

Optimal concentrations of antibodies were experimentally determined previously, and all antibodies were diluted to this respective concentration in 1% BSA PBS. All procedures and incubations involving fluorescent-conjugated antibodies were performed in either complete
darkness or dim-light conditions to reduce photo-bleaching. Additionally, diluted secondary antibodies were centrifuged at 10,000rpm at 4°C for 4mins to separate any artefacts and other particles from the antibody solution to achieve a cleaner final section and thus image.

**2.5.5.3 IMAGING & ANALYSIS**

Immunostained sections were visualized at 40x magnification (oil-emersion lens) using a Bio-Rad Radiance 2000 laser-scanning confocal Nikon TE1000 microscope and images were subsequently analysed using Axovision LSM 510 Meta software. Appropriate filter pathways were employed to omit any autofluorescence and detect only the desired emission wavelength of each specific antibody/stain (alexa-568nm [emission 603], To-PRO3 642nm [emission 661]). For each animal bladder investigated ('n number'), 4 sections would be used; one for the secondary control, and three positively stained sections to confirm expression of the target protein throughout the bladder. For each set (1 secondary control, 3 positively stained) the gain and contrast was calibrated using the secondary control section, such that no fluorescence emitted from the secondary Ab (nonspecific staining) was observed. This ensured all staining observed on positive sections is a true representation of the presence and localization of the desired target protein. Specificity of immunostaining was confirmed in all sets as no or very little fluorescence was displayed in the absence of primary antibody (secondary control) and this was further confirmed using a peptide control.

ImageJ software (NIH, USA) was used to quantify the density of images. Each image was converted to an ‘RGB stack’ to isolate each fluorescent channel. For alexa 568 channel, the image’s threshold was set to highlight the fluorescent tissue from non-fluorescent tissue and background using the ROI manager tool. Highlighted regions of tissues of interest (urothelium, suburothelium or smooth muscle) were selected using the ‘region of interest selection wand’ tool and the ‘density’ measured (refer to figure 2-12 for example workspace). The number of To-PRO3-stained nuclei within this defined area were counted manually (using a ‘marker tool’ to prevent miscounting) and the ‘density’ value was divided by this number, providing an average density/fluorescence per cell (mean cell fluorescence [MCF]). The MCF from the secondary control was subtracted from that of the three positively stained sections, which were then averaged, providing a final MCF for the target protein throughout the bladder with background removed.

This method possesses an advantage over western blotting as it allows more precise differentiation of receptor expression between tissue layers and cell types. For a list of Abs used and their concentrations, refer to tables 3 and 4.
2.6 STATISTICAL ANALYSIS

Data are expressed as medians ± interquartile range (int. range), where n denotes the number of bladders assessed, unless otherwise stated. All statistics were performed using raw data, unless comparing the relative effects of two different interventions, graphs display data normalized to controls (taken as 100%) for quick comparisons. All statistical analysis was performed using Microsoft Excel (2010, version 14.1.6) and GraphPad Prism (2011, version 6.01, GraphPad Software). All data was tested for normality using D’Agostino-Pearson omnibus test and all variables were found to have non-Gaussian distribution. As such, a Mann-Whitney (unpaired, non-parametric student’s t-test) was used to examine two unpaired data sets of unknown distribution. A Wilcoxon matched-pairs signed rank test was used to analyse paired data sets, for example comparing two different interventions.
performed on the same bladder strip. The null hypothesis was rejected at $p<0.05$, with the level of significance indicated by *$p<0.05$, **$p<0.01$ and ***$p<0.001$. When describing results in text, the medians with 25% percentile and 75% percentile will be provided as: median [25%, 75%].
CHAPTER 3: EXPRESSION AND FUNCTION OF TRPV4 RECEPTORS IN THE HUMAN AND GUINEA PIG BLADDER
3.1 INTRODUCTION

Since the identification of TRPV4 receptors in the bladder, the role of these channels in bladder physiology and pathophysiology has become of increasing interest. It is well established that TRPV4 receptors are responsible for many nociceptive and stretch-mediated responses in various tissues throughout the body, such as the vasculature (Alessandri-Haber et al., 2003; Hartmannsgruber et al., 2007; Loot et al., 2008; Mendoza et al., 2010). As such, various studies have investigated the role of TRPV4 in stretch-mediated ATP release in the bladder in cultured cells and knockout models (Gevaert et al., 2007; Mochizuki et al., 2009), however, studies using intact tissue preparations to define the mode of action in different tissue layers, especially those from humans, are lacking. Furthermore, the mechanisms underlying TRPV4-associated ATP release remain to be elucidated. As a purinoceptor-induced component to contraction appears to emerge in various human bladder pathologies, the importance of ATP release and signaling in such pathologies is heightened (Bayliss et al., 1999). Therefore, TRPV4 receptors and their associated signaling may provide novel targets for therapeutic intervention of OAB symptoms.

3.1.1 RELEVANT STUDIES OF TRPV4 FUNCTION IN THE BLADDER

For a detailed review of the current knowledge of TRPV4 function in the bladder, please refer to section 1.2.3 in the general introduction.

The role of TRPV4 in normal bladder function has been previously explored by a collection of studies in animal bladders. Gevaert et al. first identified a crucial role for TRPV4, where TRPV4+/− mice exhibited reduced voiding frequency and reduced stretch-induced ATP release from isolated whole bladders (Gevaert et al., 2007). They suggested that TRPV4 mediates urothelial transduction of intravesical mechanical pressure via release of an ATP signal. In vivo evidence for TRPV4 function was also demonstrated by Everaerts et al. who showed increased functional bladder capacity and reduced voiding frequency in WT mice upon application of the specific TRPV4 inhibitor HC-067047 (Everaerts et al., 2010c). However, it was not definitively confirmed whether these altered voiding patterns were due to lack of TRPV4 function in the urothelium, or lack or TRPV4 in the DSM, or indeed both. Following from this, Thorneloe et al. utilised the selective TRPV4 agonist GSK1016790A (used in the current study) to further investigate TRPV4 function in the bladder. GSK contracted both intact and denuded DSM strips and induced bladder over-activity in TRPV4+/− mice, both of which were not observed in TRPV4+/+ mice. Together with the findings from Gevaert et al (Gevaert et al., 2007) and Birder et al (Birder et al., 2007), they
proposed that ‘GSK1016790A-induced bladder overactivity in vivo (absent in TRPV4−/− mice) is a result of the functional role of TRPV4 in both the urothelium and the UBSM, causing a release of ATP from the urothelium, sensitizing bladder afferents and direct contraction of UBSM, respectively’(Thorneloe et al., 2008). This study not only identified a possible role for TRPV4 in mediating bladder overactivity, but also demonstrated the specificity of GSK with regards to TRPV4-mediated bladder outputs. Mochizuki et al. then demonstrated a reduced agonist and stretch-induced ATP release from cultured primary urothelial cells from mice upon knockout of TRPV4 or with unspecific TRP channel blockers(Mochizuki et al., 2009). This evidenced the role for TRPV4 in stretch-induced urothelial cell ATP release, although native urothelial tissue was not directly examined. Thus specific studies in more physiologically relevant tissue strips, including native urothelial tissue are needed to fully establish the role of TRPV4 in sensation of bladder stretch. Furthermore, as TRPV4 receptors can be activated by inflammatory conditions without stretch, defining the role of TRPV4 receptors in the bladder by selective receptor activation without stretch will provide new insight into the physiological role of these receptors with pathological implications.

The studies in this chapter build upon previous work but take a major step forward. Rather than using stretch as a means of evoking ATP release, which will activate a myriad of other receptors and pathways, the specific TRPV4 agonist GSK1016790A (more potent and specific than 4α-PDD [Thorneloe et al., 2008]) is used to directly activate TRPV4 receptors, to investigate the functional importance of TRPV4 and the underlying mechanisms in physiologically relevant native and intact guinea pig bladder preparations. This will overcome the limitations of phenotypic change in cultured cells and also maintain cell-to-cell interactions.
3.2 SUMMARY

The objectives for characterizing TRPV4 receptors in both human and guinea pig bladder are as follows:

1. **EXPRESSION STUDIES:**
   
   a. Determine the presence and localization of TRPV4 receptors throughout the bladder wall using immunohistochemistry.
   
   b. Confirm and quantify the expression of TRPV4 in the mucosa and smooth muscle using quantitative western blotting.

2. **IN VITRO FUNCTIONAL EXPERIMENTS USING FUNCTIONAL ORGAN BATH TECHNIQUE:**
   
   a. Determine the effect of specific TRPV4 activation on ATP release and contractile function in various bladder tissue preparations.
   
   b. Explore the mechanisms underlying TRPV4-related ATP release using a variety of antagonists and extracellular conditions.

3. **FURTHER IN VITRO STUDIES:**
   
   a. Determine the dependence of TRPV4-related ATP release on extracellular Ca²⁺ entry using live-cell calcium imaging.
   
   b. Investigate the effect of TRPV4 activation on production of reactive oxygen species in bladder tissue preparations using lucigenin-enhanced chemiluminescence.
3.3 METHODS

For a full description of the techniques and protocols used, please refer to the relevant methods section (detailed in text or figure legend where applicable).

3.4 RESULTS

3.4.1 PHYSIOLOGICAL EXPRESSION OF TRPV4 IN THE BLADDER

This section of the study was performed to first establish the expression of TRPV4 in the bladder tissues of the species used during the investigation to corroborate functional findings.

3.4.1.1 IDENTIFICATION AND TISSUE LOCALIZATION OF TRPV4 IN GP AND HUMAN BLADDER TISSUES BY IMMUNOHISTOCHEMISTRY

The distribution of TRPV4 receptors throughout the bladder was assessed in GP and human bladder cryosections and examined using confocal microscopy (figures 3-1 and 3-3). Sections were labeled with a specific Ab for TRPV4 (Alomone, Isreal, ACC-034), which was in turn labeled with an Alexa Fluor® - 568 fluorescent secondary Ab (em=603nm, Cy3 channel) of the relevant species, providing a red signal for TRPV4. Sections were also labeled with a blue nuclear stain (TO-PRO3®, em=661nm, Cy5 channel) to identify cell numbers and provide clearer tissue differentiation for images. Brightfield images were also used to examine tissue morphology and integrity and to identify tissue types more clearly (Cy2 channel). A peptide control was carried out on sections from 4 GP bladders to confirm specificity of the Alomone TRPV4 Ab (figure 3-2). 9 young GP bladders and 4 human bladder mucosa biopsies (taken from position of healthy tissue in male patients >65 years of age [YOA] with suspected or known tumours) were examined. Representative images for each group are shown in figures 3-1 and 3-3. All groups showed clear expression of TRPV4 in the urothelium and suburothelium. The smooth muscle was assessed in 3 GP bladders and 1 human biopsy (which had smooth muscle attached), TRPV4 expression was observed in all. TRPV4 staining seems to be more evident in the urothelium, with weaker expression in the suburothelium and weaker still in the smooth muscle. To assess this, staining was quantified by measuring pixel density of images and calculated per cell, providing final values of TRPV4 expression as ‘mean cell fluorescence’ (MCF) for each tissue type (figure 3-4).
GP urothelium expressed higher levels of TRPV4 compared to the suburothelium in all 9 bladders examined (MCF - uro; 2393 [1928, 3677], suburo; 1207 [605, 2091], SM; 1624 [611, 2098]). GP smooth muscle was assessed for 4 of the bladders providing an indication of expression levels (similar to that of suburothelium), however accurate quantification was not possible. Human TRPV4 expression in the urothelium and suburothelium appears to be similar, however only 4 bladders were examined due to limited biopsies (MCF - uro; 2708 [2263, 3074], suburo; 2019 [1162, 2854]).

However, quantification using confocal images has many limitations (considered in critique of methodology). As such, a more robust western blotting method was also used to examine and quantify TRPV4 expression in these tissues, and results from the two methods compared to see if the results were in agreement.
Figure 3 – 1. Representative images demonstrating expression of TRPV4 in young GP bladder tissues. Cryo-sections (10μm) of young GP bladder tissues at x40 magnification. TRPV4 is labelled with Alexa fluor 568 (red) and nuclei with To-PRO3 (Cy5; blue). Column A: Secondary control; no primary antibody (Ab) added, ensuring no unspecific binding of secondary Ab to sample. Column B: Positively stained sections depicting expression and distribution of TRPV4 in the urothelium (green bar), suburothelium (pink bar), smooth muscle and vasculature. High TRPV4 expression is observed in the urothelium with lower expression in the suburothelium. Images from each row (tissue type) were taken from the same ‘area’ of tissue on sections cut directly after one another, thus providing a reliable comparison between control and positive staining. These images were taken from sections of 1 of 9 young GP bladders examined.
Figure 3 – 2. Representative peptide control for Alomone anti-TRPV4 primary antibody. Cryo-sections (10μm) of young GP bladder tissues at x40 magnification. TRPV4 is labelled with Alexa fluor 568 (red) and nuclei with To-PRO3 (Cy5; blue). A: Brightfield image of peptide control section only, demonstrating general morphology and tissue distribution. B: Secondary control; no primary antibody (Ab) added, ensuring no unspecific binding of secondary Ab to sample. C: Primary TRPV4 antibody was pre-incubated with supplied control peptide for 30min at room temperature before addition to tissue section. Any red staining observed here represents unspecific binding of the primary to proteins other than TRPV4. D: Positively stained sections displaying expression and distribution of TRPV4 in the urothelium and suburothelium. Each image was taken from the same ‘area’ of tissue from serial sections, thus providing a reliable comparison between controls and positive staining. Aging GP tissue was also examined using a peptide control (total 4 bladders)
Figure 3 – 3. Expression of TRPV4 in aging human bladder tissues. 10μm cryo-sections of human bladder wall from a male patient (69yo) with a suspected tumour (Sample #5 - methods table 5), examined by confocal microscopy at x40 magnification. TRPV4 is labelled with Alexa fluor 568 (red) and nuclei with To-PRO3 (Cy5; blue). Column A: Secondary control; no primary antibody (Ab) added, ensuring no unspecific binding of secondary Ab. Column B: Positively stained sections indicating expression and distribution of TRPV4 in the urothelium (green bar), suburothelium (pink bar) and smooth muscle. High TRPV4 expression is observed in the urothelium with some expression in the suburothelium. Images from each row (tissue type) were taken from the same ‘area’ of tissue on sections cut directly after one another, thus providing a reliable comparison between control and positive staining. These images were taken from sections of 1 of 5 aging male patient bladders examined. Unfortunately, due to limited samples, evaluation of vessels was not possible.
3.4.1.2 IDENTIFICATION AND RELATIVE TISSUE EXPRESSION OF TRPV4 IN GP AND HUMAN BLADDER TISSUES BY WESTERN BLOTTING

TRPV4 expression was confirmed in separated mucosal and smooth muscle bladder whole tissue lysates by western blot, run against rat brain lysate (RBL) positive control tissue (recommended by Ab suppliers). Both representative blots in Figure 3-5 A and B show clear bands at the same molecular weight (kDa) as the positive control, thereby confirming presence of TRPV4 in these samples. Two Abs were used as the Alomone Ab was formerly used for immunohistochemistry, however optimising use of this Ab for western blotting was originally unsuccessful. Therefore, the Abcam Ab was purchased for this purpose and yielded positive results. To confirm integrity of the Alomone Ab, western blotting was attempted for a second time and successfully optimised. Expression levels were quantified by densitometry and relative expression between tissues compared (figure 3-5 C).
Quantification by densitometry revealed a much higher expression of TRPV4 in mucosal tissue than smooth muscle when using either primary Ab (Arbitrary units normalized to loading control - Abcam; mucosa – 29.1 [17.8, 74.7], smooth muscle – 4.314 [2.7, 19.1] Alomone; mucosa – 52.3 [31.1, 78.6], smooth muscle – 17.8 [8.6, 21.6]). Mucosal TRPV4 expression was approximately 6-fold higher compared to smooth muscle when probed with the Abcam Ab and 4-fold higher with Alomone. However, the results obtained using one
primary Ab were not significantly different from the other, both confirming the integrity of Abs and providing robust evidence of the presence of TRPV4.

Once the western protocol was optimised, TRPV4 expression was assessed in human mucosal biopsies. Biopsies were very small, therefore it was important the protocol was fully optimised before attempting to evaluate these samples, as failures in the process would waste large proportions of sample. The exact same RBL positive control was again used for these blots, providing reassurance of correct band size. Once again, clear bands can be observed at the same molecular weight (kDa) as the positive control for 3 of the 4 mucosal lysates assessed, thereby confirming presence of TRPV4 in these samples (figure 3-6).

Quantification was not necessary, as the purpose of these blots was to simply confirm presence of TRPV4 in human biopsies and support the previous findings from immunohistochemistry.

Figure 3 – 6. Detection of TRPV4 in human bladder mucosa biopsies. Mucosal bladder biopsies from four aging male patients assessed for presence of TRPV4 by western blot. These biopsies were taken from a healthy position in the bladder from patients undergoing surgery to collect further biopsies from suspected or known tumour sites (sample #11, 12, 13, 14 - methods table 5). The rat brain lysate (RBL) sample used as the control here is the exact sample used for GP western blots previously. The membrane was first probed for TRPV4 (A), which can be observed as bands at 98kDa. The membrane was then stripped and re-probed for β-actin (B) with bands at 42kDa. 3 of the 4 samples assessed have a positive band at 98kDa confirming presence of TRPV4. Sample #11 is negative for TRPV4 however this is likely due to sample degradation due to reuse.
3.4.2 FUNCTIONAL CHARACTERIZATION OF TRPV4 IN THE BLADDER

It was first important to characterise the functional outputs of TRPV4 in bladder tissues and for this purpose, the effect of TRPV4 activation on ATP release and bladder contractions were examined, as ATP release from the urothelium and smooth muscle contraction are two essential physiological responses critical for correct bladder function.

3.4.2.1 GSK1016790A – A POTENT AND SELECTIVE TRPV4 AGONIST

This TRPV4 agonist was used for the majority of experiments in this thesis. As such, it was of high importance to select a concentration that was most physiologically relevant (activate a proportion of receptors similar to that which would occur in response to a stimuli within the body) and provide reproducible results. In current literature, a concentration of 10μM is commonly used for infusion of isolated whole mouse bladders to stimulate contractions (Thorneloe et al., 2008), and concentrations of 100nM-1μM are used for eliciting Ca\textsuperscript{2+} influx (Egbuniwe et al., 2014; Thorneloe et al., 2008) and 30nM-300nM to induce ATP release in various cell types (Mihara et al., 2011; Shahidullah et al., 2012). One study used 300nM GSK to generate contractions in intact bladder strips from mice (Thorneloe et al., 2008). This study, along with another, demonstrated that GSK is inactive against TRPV1 and TRPM8 channels up to concentrations of 20μM, demonstrating no modulation of Ca\textsuperscript{2+} influx in HEK cells expressing these channels(Willette et al., 2008). It is suggested to act by recruiting previously inactive channels, rather than through increasing elevation of basal activity(Sullivan et al., 2012).

Previous preliminary experiments by the research group had identified 1μM GSK as the optimal concentration for eliciting reproducible ATP release and contractions in GP tissue, while remaining physiologically relevant (above EC\textsubscript{50}, below maximal effect). To reinforce this, the effect of 0.3μM, 0.5μM and 1μM GSK on ATP release and contractions in GP bladder strips were assessed to determine the most appropriate concentration for experiments (figure 3-7).

All 3 concentrations significantly enhanced ATP release in both mucosal and full thickness bladder preparations, with no significant difference between any (refer to table 8). No contractions were evoked in mucosal strips by GSK at any concentration. However, 1μM evoked a significantly stronger and more reproducible contraction in full thickness tissue strips than lower concentrations (proportion of successfully evoked contractions; 0.3μM – 20%, 0.5μM – 25%, 1μM – 90%). This concentration is within a physiologically relevant
range and far below that which may have effects on other receptors, but is capable of evoking reproducible ATP release and a contraction in intact tissue strips. Therefore, 1μM was chosen for functional experiments.

Table 8 - Quartiles (median [25%, 75%]) of ATP release and force elicited by various GSK concentrations in bladder tissues (referring to figure 3 – 7 graphs).

<table>
<thead>
<tr>
<th>Tissue strip</th>
<th>0.3μM GSK</th>
<th>0.5μM GSK</th>
<th>1μM GSK</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP release</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Full thickness</td>
<td>248 [171, 327]</td>
<td>221 [173, 281]</td>
</tr>
<tr>
<td>Force generated (μN/mg tissue)</td>
<td>Mucosa</td>
<td>17.8 [12.8, 29.9]</td>
<td>8.5 [4.2, 33.2]</td>
</tr>
<tr>
<td></td>
<td>Full thickness</td>
<td>42.0 [10.1, 138.2]</td>
<td>48.4 [15.6, 216.4]</td>
</tr>
</tbody>
</table>

Figure 3 – 7. Dose dependent effect of GSK1016780A on bladder functional outputs in GP. A: Median force of mucosal and full thickness contractions generated by range of GSK concentrations. No contractions observed in mucosa at any concentration, small contractions evoked at 0.3 and 0.5μM in full thickness (median force: 41.98 and 48.36μN respectively) with significantly greater contractions evoked at 1μM (median force: 358.1) B: Corresponding median ATP release from same bladder strips. All concentrations significantly increase ATP release from basal levels in both mucosa and full thickness. No significant difference in ATP release is observed between mucosa and full thickness or at different concentrations of GSK (data sets compared using Mann-Whitney test). For both graphs: data expressed as % medians [25%, 75%], n=10 (0.3μM), n=8 (0.5μM), n=18 (1μM), Wilcoxon’s, graph B *p<0.05, drug intervention vs. control (basal levels [BL]).
3.4.2.2 TRPV4-INDUCED ATP RELEASE AND CONTRACTIONS IN BLADDER TISSUE STRIPS: GUINEA PIG

The role of TRPV4 in regulation of ATP release and smooth muscle contraction was investigated. Separated mucosal and full thickness GP bladder strips were mounted to a contraction setup (described in figure 2-1 and 2-2). Both changes in ATP release and force were measured as functional outputs in response to specific activation of TRPV4 using 1μM GSK1016790A (GSK-TRPV4 agonist) (refer to section 2.5.1). Superfusate was sampled at 3 time-points across a 5-minute intervention period (example in figure 3-8 A) and measured for ATP content. These values were averaged to provide a median ATP release and expressed as a percentage change from basal ATP levels taken as 100%. All data are normalized to tissue mass. GSK (1μM) significantly enhanced ATP release (figure 3-8 B) in both mucosal (235% [147%, 412%]) and full thickness (256% [217%, 378%]) tissue strips. GSK (1μM) also evoked contractions in full thickness strips (force μN/mg tissue: 246 [175, 669]), which were significantly higher than mucosal sheets (force μN/mg tissue: 0.39 [0, 3.57]) where no contractions were observed. The contractions evoked in full thickness strips were of similar magnitude to those evoked by the gold-standard muscarinic agent carbachol (50μM), however GSK was not capable of evoking contractions in mucosal sheets, whereas carbachol is (Sui et al., 2014) (figure 3-8 D). As can be seen in figure 3-8 C, TRPV4-derived contractions are sustained, usually for between 2-4 hours, unlike transient carbachol contractions. The force of GSK-induced bladder contractions did not coincide with the level of ATP release as demonstrated in figure 3-9, where data shows a Pearson correlation coefficient r value of -0.02963, implying a very weak, non-significant negative correlation. This suggests that ATP release and bladder contractions are independent in this model, where ATP is not required for or does not significantly modulate contraction. This is supported by findings observed with denuded smooth muscle strips (refer to figure 3-10). GSK evoked contractions in 70% of denuded smooth muscle strips (median force μN/mg tissue: 260 [74, 347], however significant ATP release was lost (103% [96%, 123%]) with only 16% of strips releasing ATP beyond basal levels. (>120% of basal ATP release). This suggests that the majority of released ATP is derived from the urothelium, and also that this urothelium-derived ATP is not necessary for smooth muscle contraction.
Figure 3 – 8. Effects of TRPV4 activation on young GP bladder. A: Example of mucosal and full-thickness (FT) ATP release after exposure to 1μM GSK (TRPV4 agonist). B: Combined data – GSK (1μM) increased ATP release. ATP release across the 3 time-points (as seen in A) was averaged for each bladder (n=9 bladders) and data presented as % medians [25%, 75%], p value shown, Wilcoxon’s, drug intervention vs. control. C: Record of contraction induced by 1μM GSK in mucosal and full thickness bladder strips. D: Median values of mucosal and full thickness contractions, with record of single 50μM carbachol (CCh) contraction for reference (note Log2 Y axis). GSK does not trigger a contraction in mucosa, but does in full thickness. The contractions observed in full thickness strips are significantly greater than those observed in mucosa. Data sets are expressed as % medians [25%, 75%], n=8, Mann-Whitney, mucosa vs. full thickness.
Figure 3 – 9. Relationship between GSK1016790A-induced contractions and ATP release. No correlation observed between the force of contraction and level of ATP release in young GP full thickness strips. Pearson correlation r value: -0.02963, n=18.

Figure 3 – 10. Effect of denuding GP bladder strips on GSK1016790A-induced changes in ATP release and force. Shown alongside mucosa and full thickness tissue for comparison. A: Median force of mucosal, full thickness and denuded smooth muscle tissue contractions generated by 1μM GSK. No significant difference observed between force of contraction generated in full thickness or denuded smooth muscle strips. B: Corresponding median ATP release from same bladder strips. Denuded smooth muscle tissue does not release ATP in response to GSK. Data sets expressed as % medians [25%, 75%], n=10, * p ≤0.05, Wilcoxon’s, drug intervention vs. control. Data sets compared using Mann-Whitney, e.g. full thickness vs. denuded smooth muscle. Protocol: figure 2-3 methods.
For this purpose, mucosal, denuded smooth muscle and full thickness strips were challenged with 1μM GSK, allowing differentiation between tissues. To determine the tissue(s) responsible for the ATP release evoked by GSK, the raw data must be analysed (figure 3-11). Observing the ATP release as a relative change from basal levels in the form of a percentage (normalized to controls) will mask the relative quantities of ATP being released from each tissue preparation. Figure 3-12 shows that significant ATP release is evoked by GSK in both mucosa and full thickness tissue, but not denuded (pMoles ATP/min: muc - basal; 55 [43, 172], GSK; 140 [46, 455], denuded - basal; 9 [6, 23], GSK; 16 [6, 37], full thick - basal; 12 [5, 34], GSK; 16 [37, 99]). The quantity of ATP released per gram from full thickness bladder was significantly less than that from separated mucosa. This indicates that the DSM and other underlying tissues in full thickness bladder do not contribute to GSK-induced ATP release, as normalizing to tissue mass in this tissue preparation significantly reduces both basal and agonist-induced ATP release, implying that these tissues are redundant regarding ATP release. This and the fact that denuded smooth muscle is incapable of releasing ATP in response to GSK, implies that all GSK-induced ATP release is from the mucosa.

Additionally, mucosa released significantly more basal ATP per gram of tissue than both denuded smooth muscle and full thickness tissue. This again implies that the majority of basal ATP release originates from the mucosa.
Figure 3 – 11. GSK1016790A-induced ATP release from different bladder tissue preparations: Raw data. Graph of raw data representing pmoles of ATP released per gram of tissue per minute, before normalizing to controls. GSK significantly enhanced ATP release from mucosal and full thickness strips, but not from denuded smooth muscle tissue. The GSK-induced ATP release from mucosa was significantly greater to that from full thickness (not shown). Basal ATP release from mucosa was significantly greater than basal ATP release from both denuded and full thickness tissue (not shown). Basal release from full thickness was not different to denuded. ATP release expressed as pmoles/g tissue/min medians [25%, 75%], mucosa and full thickness n=8, denuded n=10, p values shown, Wilcoxon’s, drug intervention vs. control. Unpaired data sets compared using Mann-Whitney e.g. mucosa vs. full thickness.
3.4.2.3 TRPV4-INDUCED ATP RELEASE AND CONTRACTIONS IN BLADDER TISSUE STRIPS: HUMAN

Human mucosal biopsies taken from macroscopically healthy tissue positions in bladders from male patients >65 YOA with suspected or known tumours were also investigated. GSK (0.3μM and 1μM) significantly enhanced ATP release, however the times after initial exposure for onset and maximal ATP release were different for each concentration. 1μM GSK did not evoke significant ATP release until 3 minutes, with the peak at 10 minutes and return to basal levels at 20 minutes. 0.3μM GSK did not evoke significant ATP release until 5 minutes, with the peak at 20 minutes. The peak ATP release for each concentration were similar (0.3μM; 20min - 235% [155%, 427%], 1μM; 10min – 190% [149%, 467%]). Despite different onset and peak times, ATP release was not significantly different between either concentration at any time-point. However, 1μM was used throughout the study as the dynamics of ATP release were more consistent and this allowed reliable comparison with GP tissue. A noticeable contraction was evoked in 1 of the 11 mucosal sheets (sample #2 in methods table 5)(figure 3-12 B(i)) and it was noted that this mucosal sheet was the only biopsy examined with abundant intact smooth muscle attached, confirmed by confocal microscopy performed on this sample after physiological experimentation (figure 3-12 B ii). All other mucosal sheets remained relaxed (figure 3-12 B2).

![Figure 3 – 12. Effect of TRPV4 activation on aging human bladder biopsies. A: Both 1μM (light grey bars) and 0.3μM (dark grey bars) GSK significantly enhanced ATP release from mucosal bladder biopsies (aging male patients, suspected or known tumours, >65 YOA, biopsy taken from macroscopically normal area) over a 20 minute period. Times of onset and peak ATP release differed between concentrations. Data sets expressed as % medians [25%, 75%], 1μM; n=10, 0.3μM; n=11, * p ≤0.05, ** p ≤0.01, Wilcoxon’s, drug intervention vs. control. Data sets compared using Mann-Whitney, 1μM GSK vs. 0.3μM GSK. B(i): Example trace recordings of tension during challenge with 1μM GSK in human mucosal biopsies. B(i)1: Contractile response of a mucosal biopsy (smooth muscle attached) to GSK (B(ii) confocal image of SM from biopsy used for this tension recording). B(i): 2. Record of common contractile activity of human mucosal biopsies during challenge with GSK.](image-url)
3.4.3 MECHANISMS UNDERLYING TRPV4-INDUCED MUCOSAL ATP RELEASE

Antagonists of various receptors and molecules postulated to be involved in the mechanism for TRPV4-related ATP release were utilized to explore the potential underlying signalling pathways. Refer to methods section, figure 2-4 for detailed description of protocol used for the following antagonist studies.

3.4.3.1 CONTROL EXPERIMENT

The majority of functional experiments in this thesis require 3 repeated exposures of tissue to 1μM GSK, with the second including an antagonist, with ATP release as the focus variable. As such, a control experiment was performed to further assess the reproducibility of ATP release in response to 3 successive exposures to GSK (figure 3-13), in accordance with the protocol used for antagonist studies (refer to figure 2-4), but omitting any antagonist. This was used to confirm that during antagonist studies, any observed reduction in ATP release is due to effects of antagonist, and not a consequence of an inability of GSK to re-evoke ATP release.

All 3 successive exposures to GSK significantly enhanced ATP release in both mucosal and full thickness bladder preparations (refer to table 9), with no significant difference between any. This confirmed that successive use of 1μM GSK in accordance with the 'antagonist protocol' evokes reproducible ATP release. A contraction was evoked with first exposure, however following exposures were incapable of eliciting further contractions and this was observed throughout all the similar experiments performed in this thesis.
Table 9 - Quartiles (median [25%, 75%]) of ATP release and force elicited by 3 successive exposures to 1μM GSK in bladder tissues (referring to figure 3 – 13 graph).

<table>
<thead>
<tr>
<th>Tissue strip</th>
<th>Exposure 1</th>
<th>Exposure 2</th>
<th>Exposure 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP release (% of averaged controls)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full thickness</td>
<td>345 [190, 434]</td>
<td>233 [174, 375]</td>
<td>161 [147, 224]</td>
</tr>
<tr>
<td>Force generated (μN/mg tissue)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucosa</td>
<td>10.7 [2.5, 19.5]</td>
<td>5.6 [2.5, 10.87]</td>
<td>1.1 [0,3.8]</td>
</tr>
<tr>
<td>Full thickness</td>
<td>154.3 [55.9, 187.6]</td>
<td>6.4 [3.5, 8.5]</td>
<td>2.1 [0.9, 13.9]</td>
</tr>
</tbody>
</table>

3.4.3.2 SELECTIVE BLOCKADE OF TRPV4 USING HC-067047

HC-067047 (HC) was used at a concentration of 1μM for predominant blockade (>95%) of TRPV4 receptors. The ATP release triggered by GSK (0.5μM) was significantly reduced by HC-067047 to near basal levels in both tissue types (mucosa; GSK con - 221% [164%, 312%], GSK W/ HC - 105% [94%, 119%], full thick; GSK con - 180% [175%, 182%], GSK W/ HC - 113% [102%, 133%]) (figure 3-14). This confirms that all ATP release observed when using GSK is a result of specific TRPV4 activation and not unspecific action of GSK on other related receptors, such as TRPV1. HC also significantly reduced basal ATP release in mucosa but not full thickness strips (mucosa; 92% [61%, 103%], full thick; 80%
[65%, 106%] of control). Data from human mucosa indicate that antagonism of TRPV4 reduces basal ATP release, although it took a longer time for the effect to occur (relative change in ATP release from basal levels: 10min: 95% [80%, 130%], 15min: 62% [43%, 100%], 20min: 74% [37%, 80%]).

Figure 3 – 14. Effect of TRPV4 antagonist HC-067047 on ATP release in bladder strips. A: HC-067047 (1μM) significantly reduces ATP release triggered by GSK (0.5μM) in GP tissue. Light grey bars: Averaged ATP release from pre and post control interventions of 0.5μM GSK. Dark grey bars: ATP release from middle drug intervention of 1μM GSK with 1μM HC. Data sets expressed as % medians [25%, 75%], n=7, p values shown, Wilcoxon’s, GSK averaged pre/post control vs. GSK W/ HC-067047. Protocol: figure 2-4 methods. B: (i) 1μM HC-067047 significantly reduces basal ATP release in GP mucosal strips, but not in GP full thickness strips. Data sets expressed as % medians [25%, 75%], n=8, p values shown, Wilcoxon’s, drug intervention vs. control. Protocol: figure 2-3 methods. (ii) Raw ATP release data from (i) expressed as median release/g tissue/min. C: (i) 1μM HC-067047 reduces basal ATP release in human mucosal biopsies after 20min. Data sets expressed as % medians [25%, 75%], n=7, p values shown, Wilcoxon’s, drug intervention vs. control. (ii) Raw ATP release data from (i) expressed as median release/g tissue/min.
3.4.3.3 SPECIFIC PATHWAY FOR TRPV4-INDUCED ATP RELEASE

The mechanisms for ATP release from tissue including the urothelium are complex and may involve several processes including vesicular exocytosis, conductive pathways (connexin and pannexin channels) and various transporters (Birder and Ruggieri, 2012). The involvement of these processes in the release of ATP specific to TRPV4 activation was further explored (figure 3-15). The pannexin/connexin channel inhibitor carbenoxolone (100μM) significantly reduced GSK-induced ATP release in both tissue types by approximately 90% (mucosa; GSK con - 262% [196%, 443%], GSK W/ Carb - 119% [85%, 207%], full thick; GSK con - 326% [202%, 208%], GSK W/ Carb - 120% [87%, 153%]). Brefeldin A (10μM), an inhibitor of vesicular transport-mediated ATP release, had no effect on GSK-induced (TRPV4-activated) ATP release from both tissue types (mucosa; GSK con - 236% [192%, 357%], GSK W/ Bref A - 228% [94209 264%], full thick; GSK con - 206% [149%, 277%], GSK W/ HC - 223% [178%, 276%]). These findings suggest that the observed ATP is released via conductive pathways, with little or no involvement of vesicular transport.

Figure 3 – 15. Effect of inhibitors of various ATP release pathways on GSK1016790A-stimulated ATP release from GP bladder tissue. A: Carbenoxolone (100μM) significantly reduces GSK-induced ATP release in both mucosal and full thickness strips. n=9. B: Brefeldin A (10μM) has no effect on GSK-stimulated (TRPV4-activated) ATP release in both mucosa and full thickness strips. Data sets expressed as % medians [25%, 75%], n=5, p ≤0.05, Wilcoxon’s, GSK pre/post average vs. GSK W/ antagonist. Light grey bars: Averaged ATP release from pre and post control interventions of 1μM GSK. Dark grey bars: ATP release from middle drug intervention of 1μM GSK with a specific antagonist.
3.4.3.4. ROLE OF Ca²⁺ AND RELATED SIGNALLING IN TRPV4-MEDIATED ATP RELEASE

The dependence of TRPV4-mediated ATP release on influx of extracellular Ca²⁺ was explored (figure 3-16). A Ca²⁺-free modified Tyrode’s solution was used for this purpose (described in methods section 2.1.1.1), providing an extracellular environment free from Ca²⁺. The effect of 1μM GSK on ATP release is abolished in the absence of extracellular Ca²⁺ (mucosa; GSK W/Ca²⁺ - 202% [153%, 332%], GSK W/O Ca²⁺ - 111% [101%, 125%], full thick; GSK con - 198% [175%, 216%], GSK W/ Carb - 129% [93%, 159%])(figure 3-17: A). This tight dependence on extracellular Ca²⁺ demonstrates that urothelial TRPV4 functions mainly as a Ca²⁺ influx pathway, which was also observed in cultured urothelial cells under 4α-PDD (non-specific TRPV4 activator) stimulation (Gevaert et al., 2007).

Various other channels and signalling molecules were investigated in an attempt to determine the pathway underlying TRPV4-mediated ATP release. The Ca-activated Cl channel (CaCC) blocker NPPB (100μM) was also ineffective in reducing GSK-induced ATP release (mucosa; GSK con - 188% [143%, 255%], GSK W/ NPPB - 243% [219%, 345%], full thick; GSK con - 241% [191%, 291%], GSK W/ NPPB - 197% [156%, 327%])(figure 3-17: B). Genistein (100μM; selective tyrosine protein kinase inhibitor) suppressed GSK-induced ATP release to below basal levels in both tissue preparations (mucosa; GSK con - 221% [184%, 346%], GSK W/ Gen - 93% [75%, 119%], full thick; GSK con - 221% [191%, 409%], GSK W/ Gen - 93% [72%, 140%])(figure 3-16: C), suggesting a role for tyrosine protein kinases in either the downstream signalling pathway for TRPV4-mediated ATP release, or in the modulation of TRPV4 channels themselves.
Figure 3 – 16. Effects of a Ca2+-free extracellular environment and inhibitors of various signalling molecules and channels on GSK1016790A-stimulated ATP release from GP bladder tissue. A: Removal of Ca2+ from Tyrode’s superfusate significantly reduces GSK-stimulated ATP release, n=8. B: NPPB (100μM; inhibits Ca2+-sensitive chloride currents) has no effect on GSK-induced ATP release, n=6. C: Genistein (100μM; selective protein tyrosine kinase inhibitor) significantly reduces GSK-stimulated ATP release, n=8. All data sets expressed as % medians [25%, 75%], p values shown, * p ≤0.05, Wilcoxon’s, GSK pre/post average vs. GSK W/ antagonist or Ca2+-free Tyrode’s. Light grey bars: Averaged ATP release from pre and post control interventions of 1μM GSK. Dark grey bars: ATP release during middle drug intervention of 1μM GSK with a specific antagonist/Ca2+-free Tyrode’s.
3.4.3.4.1 TRPV4 ACTIVATION RAISES INTRACELLULAR $\text{Ca}^{2+}$

The ability of TRPV4 receptor activation to cause $\text{Ca}^{2+}$ influx was directly investigated in freshly isolated urothelial cells. Live-cell $\text{Ca}^{2+}$ imaging demonstrated a $\text{Ca}^{2+}$ rise in isolated urothelial cells from GP bladders in response to TRPV4 activation (figure 3-16: A). The averaged data from 7 bladders revealed that TRPV4 activation (GSK, 1μM) significantly raises $[\text{Ca}^{2+}]_i$ from resting levels (resting $\text{Ca}^{2+}$; 140.3nM [85.5 nM, 169.0 nM], GSK; 283.2 nM [249.4 nM, 356.2 nM]), providing further evidence for the importance of $\text{Ca}^{2+}$ in TRPV4-mediated signalling (figure 3-16: B). Thus, it is likely that extracellular $\text{Ca}^{2+}$ entry, resulting in increased intracellular $\text{Ca}^{2+}$, is involved in TRPV4-mediated ATP release.

Figure 3 – 17. Effect of GSK on intracellular $\text{Ca}^{2+}$ concentration. A. GSK (1μM) generates a $\text{Ca}^{2+}$ transient in a freshly isolated single urothelial cell from GP bladder. B. Averaged data; GSK (1μM) evokes a significant rise in $[\text{Ca}^{2+}]_i$. Data sets expressed as % medians [75%], n=7, p value shown, Wilcoxon’s, drug intervention vs. resting control.
3.4.3.5 ROLE OF EPITHELIAL SODIUM CHANNELS (ENAC)

As with TRPV4 channels, previous literature has also demonstrated a reduction in stretch-evoked ATP release with blockade of mechanosensitive ENaC channels from both the mucosa of whole isolated rat bladders and single urothelial cell preparations (Birder et al., 2003; Du et al., 2007). As such, the possibility of a relationship between the two channels was investigated with regard to TRPV4-mediated ATP release. Preliminary experiments using amiloride (1mM) to block ENaC channels during stimulation of ATP release with GSK, revealed a substantial release of ATP throughout the exposure of tissue to amiloride. As such, the effect of amiloride on basal ATP release was investigated (figure 3-18 A). Indeed, amiloride (1mM) significantly increased ATP release, with a delay of between 10-20minutes and peak release at 40minutes in both tissue preparations (peak release: mucosa; 287% [200%, 441%], full thickness; 371% [275, 517%])(table 10). Significant ATP release was lost after 90minutes. The antagonist study protocol was adapted accordingly, with a 90minute pre-control of amiloride before the 5minute GSK W/amiloride intervention (as opposed to the usual 10minute pre-control). This prevented interference from the amiloride-induced ATP release, such that any ATP release measured during the GSK W/amiloride period was attributable to TRPV4 activation and not due to amiloride.

Table 10 - Quartiles (median [25%, 75%]) of ATP release elicited by 1mM amiloride in bladder tissues (referring to figure 3 – 18 A graph).

<table>
<thead>
<tr>
<th>Tissue strip</th>
<th>Time of exposure to amiloride (1mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5min</td>
</tr>
<tr>
<td>ATP release</td>
<td></td>
</tr>
<tr>
<td>Mucosa</td>
<td></td>
</tr>
<tr>
<td>Full thickness</td>
<td></td>
</tr>
</tbody>
</table>

Amiloride (1mM) significantly attenuated, but did not fully suppress GSK-induced ATP release in mucosa (45% reduction) and full thickness (25% reduction) strips (mucosa; GSK con - 222% [193%, 277%], GSK W/ Ami - 164% [123%, 247%], full thick; GSK con - 224% [203%, 245%], GSK W/ Ami - 151% [121%, 227%])(figure 3-18 B). Benzamil, a more potent derivative of amiloride, was also used to provide further evidence for these findings (figure 3-18 C), as the non-specific effects of amiloride have been scrutinized. Preliminary findings showed that unlike amiloride, benzamil alone does not increase ATP release and as such, comparison of the targets of these two compounds may reveal the receptor/channel responsible for the ATP release observed with amiloride. Data suggests however that unlike amiloride, benzamil (100μM) does not have a suppressant effect, where a reduction in ATP
release was only observed in 2 of the 5 bladders assessed in mucosa and 3 of 5 in full thickness strips (figure 3-18 D) (mucosa; GSK con - 197% [153%, 235%], GSK W/ Benz - 151% [82%, 204%], full thick; GSK con - 196% [149%, 228%], GSK W/ Benz - 145% [107%, 219%]). Unfortunately, due to time constraints, only 5 bladders were assessed for benzamil, such that further repeats are needed to uncover any true trends. These findings suggest that ENaC channels may be partly involved in the TRPV4-mediated release of ATP, possibly through a cooperation of the two receptors. However, as amiloride and benzamil are non-selective and block a variety of Na\(^+\) channels, the implications of these findings will be explored fully in the discussion.

Figure 3 – 18. Effects of various Na\(^+\) channel blockers, employed to inhibit epithelial sodium channels (ENaC), on GSK1016790A-stimulated ATP release from GP bladder tissue. A: Amiloride (1mM) significantly increases ATP release from both tissue preparations with a 10-minute delay, a peak release at 40 minutes and a decline to near basal levels after 90 minutes. Data sets expressed as % medians [25%, 75%], n=13, * p ≤0.05, ** p ≤0.01, *** p ≤0.001, Wilcoxon’s, drug intervention vs. control (basal levels). B: Amiloride (1mM) attenuates GSK-stimulated (1μM) ATP release. Data sets expressed as % medians [25%, 75%], n=13, p values shown, Wilcoxon’s, GSK pre/post average vs. GSK W/amiloride. C: Benzamil (100μM) has no significant effect on GSK-stimulated ATP release, however due to time constraints, only 5 bladders were assessed. Paired data is shown in D, revealing individual trends of each repeat. For graph C, data sets expressed as % medians [25%, 75%], n=5, p values shown, Wilcoxon’s, GSK pre/post average vs. GSK W/benzamil. Graph B and C; Light grey bars: Averaged ATP release from pre and post control interventions of 1μM GSK. Dark grey bars: ATP release from middle drug intervention of 1μM GSK with 1mM Na\(^{2+}\) channel blocker.
3.4.4 ROLE OF TRPV4 IN STRETCH-EVOKED ATP RELEASE

The highly specific TRPV4 antagonist HC-067047 was used to investigate the role of TRPV4 in stretch-evoked ATP release from the GP mucosa. Mucosal and full-thickness tissue strips (5-6mm) were stretched to 150% of their original length for 30 seconds, and ATP release measured during and after the stretch (these parameters are based on preliminary work mentioned in section 2.5.1.1.3). A control experiment was performed to assess the viability and reproducibility of this protocol (figure 3-19). Strips were successively stretched in this manner 3 times, with a 1 hour recovery period between each (refer to 2.5.1.1.3 for full description of protocol). 150% stretch evoked significant release of ATP at 30 seconds and 1 minute after initial stretching, where ATP release subsided after 2 minutes.

![Graph showing ATP release during and after stretch](image)

Figure 3 – 19. Effect of 3 successive mechanical stretches on ATP release in GP bladder strips. Mechanical stretch evokes significant ATP release at 30 seconds and 1 minute in both tissue types during each stretch repeat. The ATP release appears to subside after 2 minutes. No significant differences were observed between the ATP released at 30 seconds and 1 minute during any of the 3 successive stretches (data sets compared using Mann-Whitney, e.g. mucosa; ATP release during stretch #1 30 seconds vs. mucosa; ATP release during stretch #3 30 seconds). Data sets expressed as % medians [25%, 75%], n=5, p values shown, Wilcoxon’s, time after stretch vs. control.
These dynamics of ATP release were reproducible for a second and third stretch. This experiment was repeated for 5 bladders, such that statistics yielding significant differences are not possible. However, as is shown, the ATP release at 30 seconds and 1 minute in both tissue strips during all 3 stretches have a p value of 0.0625 compared to controls. This information was sufficient to begin experiments using antagonists to assess the role of TRPV4 in stretch-induced ATP release.

To assess the involvement of TRPV4 in stretch-evoked ATP release, the same protocol used for the control experiment was followed, however TRPV4 was antagonised using HC-067057 (1μM) during the second stretch. Tissue stretch during inhibition of TRPV4 with HC-067047 still significantly raises ATP release from basal levels in both tissue preparations at 30 seconds and 1 minute (mucosa; 30sec - 197% [145%, 233%], 1min - 202% [150%, 214%], full thick; 30sec - 218% [174%, 221%], 1min - 127% [102%, 200%]) (figure 3-20 A). However, the stretch-evoked ATP release during TRPV4-inhibition is in fact significantly less than that evoked without inhibition at 30 seconds in both tissue preparations and at 1 minute also in full thickness (mucosa; stretch con 30sec - 361% [294%, 517%], Stretch W/ HC - 197% [145%, 233%], stretch con 1min - 292% [209%, 343%], Stretch W/ HC - 202% [150%, 214%], full thick; stretch con 30sec - 331% [244%, 489%], Stretch W/ HC - 218% [174%, 221%], stretch con 1min - 194% [137%, 255%], Stretch W/ HC - 127% [102%, 200%]) (figure 3-20 B). These values translate to approximately a 50% reduction in the stretch-evoked ATP release when inhibiting TRPV4 (mucosa; 30sec - 61% reduction, 1min - 46% reduction, full thick; 30s - 49% reduction, 1min - 70% reduction). This suggests that TRPV4 is partly responsible for the stretch-induced ATP release from the bladder mucosa.
Figure 3 – 20. Effect of TRPV4 antagonist HC-067047 on mechanical stretch-induced ATP release in GP bladder strips. A: Stretch-induced ATP release during inhibition of TRPV4 receptors with HC-067047 (second stretch during protocol). Mechanical stretch stimulates significant ATP release during TRPV4 receptor inhibition. Data sets expressed as % medians [25%, 75%], n=11, * p ≤0.05, Wilcoxon’s, time after stretch vs. control. B: Stretch-induced ATP release with (dark grey bars) or without (light grey bars) inhibition of TRPV4 (HC-067047; 1μM). Inhibition of TRPV4 receptors significantly attenuates stretch-induced ATP release in both tissue types 30seconds and 1minute after mechanical stimulation. At 2 and 3 minutes ATP release is close to basal control levels, with or without TRPV4 blockade. Data sets expressed as % medians [25%, 75%], n=11, p values shown, Wilcoxon’s, stretch W/O HC pre/post average vs. stretch W/ HC. Light grey bars: Averaged ATP release from pre and post stretch interventions. Dark grey bars: ATP release from middle stretch intervention with 1μM HC-067047. Refer to section 2.5.1.1.3 for protocol.
3.4.5 INFLUENCE OF TRPV4 ON BLADDER ROS PRODUCTION

Superoxide (O$_2^-$) production was measured in young GP full thickness bladder strips treated with either control Tyrode’s solution or 1μM GSK for 30 minutes at 37°C, assessed using lucigenin chemiluminescence. In the absence of added agents, basal ROS production in full thickness tissue lysates was minimal and did not differ between control and treated (1μM GSK) groups (MLU: Tyr con - 35 [30%, 55%], GSK - 46 [39%, 122%]) (figure 3-21). Addition of NADPH (100μM) significantly raised ROS production from basal levels in both groups, however no significance between groups was observed (MLU: Tyr con - 165 [118%, 222%], GSK - 270 [162%, 360%]). After addition of Tiron (10mM) ROS production was significantly suppressed from the NADPH treatment, but was still significantly greater than that of basal levels (MLU: Tyr con - 86 [57%, 154%], GSK - 88 [129%, 211%]). No difference between the control and treated group was observed after addition of Tiron.

![Figure 3-21. Measurement of reactive oxygen species (ROS): Effect of pre-treatment with 1μM GSK1016790A on ROS production in young GP bladder. A: Kinetics of ROS production over experimental time-course. 0-15 minutes; basal ROS production, 15-30 minutes; ROS production after additions of 100μM NADPH, 30-45 minutes; ROS production after addition of 5mM tiron (O$_2^-$ scavenger). Data expressed as % medians [25%, 75%], control n=10, GSK n=10. B: Median light units (MLU) averaged across the 15 minute period of each treatment. No significant difference in ROS production was observed between groups during any treatment. NADPH (100μM) significantly enhanced ROS production from basal levels in both tissues (**). ROS production in presence of Tiron was significantly less than during NADPH treatment (††), but still significantly greater than basal levels (**). Data sets expressed as % medians [25%, 75%], control n=10, GSK n=10, * p ≤0.05, **/†† p ≤0.01, Wilcoxon’s, control tissue basal vs. control tissue NADPH.]
Addition of Tiron (superoxide [O\textsubscript{2}\textsuperscript{-}] scavenger) enabled the proportion of ROS composed of O\textsubscript{2}\textsuperscript{-} to be calculated. This revealed that activation of TRPV4 receptors by pre-treatment with GSK significantly enhances O\textsubscript{2}\textsuperscript{-} production in bladder tissue lysates (percentage increase from control: 136% [114%, 317%])(figure 3-22). TRPV4-induced O\textsubscript{2}\textsuperscript{-} production may effect bladder signalling and alter oxidative stress levels with further downstream implications.

![Figure 3 – 22. Measurement of reactive oxygen species (ROS): Effect of pre-treatment with 1µM GSK1016790A on ROS production in young GP bladder. A: Paired data - shows difference in O\textsubscript{2}\textsuperscript{-} production between control and GSK-treated full thickness bladder strips from each bladder investigated. n=10. B: Normalized data - O\textsubscript{2}\textsuperscript{-} production in control tissue from each bladder is normalized to 100% and O\textsubscript{2}\textsuperscript{-} production in GSK-treated strips expressed as % change from control. Pre-treatment with 1µM GSK as opposed to control Tyrode’s solution stimulates significantly greater O\textsubscript{2}\textsuperscript{-} production. Data expressed as % medians [25%, 75%], n=10, * p ≤0.05, Wilcoxon’s, pre-treatment 1µM GSK vs. pre-treatment control Tyrode’s.](image)
3.5 DISCUSSION

This chapter identified the presence and expression of TRPV4 in the bladder and explored the effects of specific TRPV4 activation on mucosal ATP release and bladder contractility. Using the specific agonist GSK1016790A, the study has demonstrated a reproducible TRPV4-induced ATP release from the mucosa, but not from denuded smooth muscle, indicating the source of ATP and functional importance of this receptor in the bladder. In addition, the basic pathway underlying TRPV4-induced ATP release has been elucidated and further potential consequences of TRPV4 activation have been explored.

3.5.1 IDENTIFICATION AND TISSUE LOCALIZATION OF TRPV4

TRPV4 has previously been shown in urothelial tissue sections from mice and rats (Gevaert et al., 2007) by immunohistochemistry, and in the mouse mucosa and DSM by western blotting (Thorneloe et al., 2008; Yamada et al., 2009). The receptor has also been reported in isolated urothelial cells from human bladders in a small number of samples (Janssen et al., 2011). In GP bladder however, TRPV4 expression has only been suggested by using functional experiments by observations of TRPV4 agonist-induced whole-cell currents in isolated urothelial cells, where no conclusive studies using western blotting and IHC exist in current literature (Xu et al., 2009). In addition, detailed tissue distribution of this receptor in the bladder remains unclear. In this study, TRPV4 expression was demonstrated by IHC and western blotting in both human mucosa and GP bladder tissues with detailed information on tissue distribution.

Immunohistochemistry demonstrated TRPV4 expression in the urothelium, suburothelium and DSM of GP bladder tissue, with highest expression in urothelium. Results from western blotting provides further proof for TRPV4 expression in both mucosa and DSM, with significantly higher expression in the mucosa. Together, these data identify the urothelium as the predominant site of TRPV4 expression in the bladder. To our knowledge, this is the first study to show expression of TRPV4 in GP bladder using these techniques, also indicating the tissue localization. TRPV4 expression in human mucosal biopsies was also confirmed using these techniques, corroborated by evidence from the aforementioned literature.
3.5.2 FUNCTIONAL CHARACTERIZATION OF TRPV4 IN THE BLADDER

ATP release induced by specific TRPV4 activation has been previously demonstrated in a variety of intact tissues (Shahidullah et al., 2012) and isolated cell types (Egbuniwe et al., 2014; Mihara et al., 2011; Silva and Garvin, 2008), as well as in cultured urothelial cells (Kullmann et al., 2009). TRPV4 has also been strongly implicated in stretch-induced ATP release from the bladder mucosa by previous studies observing either a reduced stretch-induced ATP release from TRPV4−/− whole isolated bladders (Gevaert et al., 2007), or abolished stretch-induced ATP release from isolated urothelial cells using a broad TRP channel blocker (ruthenium red)(Mochizuki et al., 2009). TRPV4-induced bladder contractions have also been demonstrated in tissue strips and whole perfused bladders from mice using specific TRPV4 agonists (Birder et al., 2007; Gevaert et al., 2007; Thorneloe et al., 2008). However, the effect of specific activation of TRPV4 on ATP release using more physiologically relevant bladder preparations, especially in native urothelium and urothelium-intact bladder tissue remains to be assessed.

3.5.2.1 SELECTIVE ACTIVATION OF TRPV4

Specific activation of TRPV4 by GSK1016790A evokes a reproducible, significant ATP release from the bladder mucosa. In GP full thickness bladder strips, the ATP release is accompanied by a strong contraction during first exposure to GSK, however this contractile phenomenon is non-reproducible with subsequent exposures.

There is no correlation between the quantity of ATP release and magnitude of contraction, implying the two phenomena are relatively independent. This is supported by TRPV4 activation in denuded smooth muscle strips, where no ATP release is observed but a contraction of similar magnitude to that in full thickness strips is still evoked. This provides evidence for functional expression of TRPV4 receptors in the detrusor smooth muscle (DSM), which are able to elicit contractions through a mechanism independent of urothelial TRPV4 receptors and ATP release. This is supported by previous literature, which demonstrates separate functional roles for TRPV4 in both the urothelium (inducing ATP release [Birder et al., 2007; Gevaert et al., 2007]) and the DSM (direct contraction of smooth muscle [Thorneloe et al., 2008]), which have been shown to induce bladder overactivity in vivo. All observations together suggest that GSK-induced contractions result mainly from direct action on the DSM and not an indirect consequence of GSK-induced ATP release. Our findings also suggest that the majority of released ATP originates from the mucosa, as
no ATP release is observed in denuded DSM strips. However, communication between the mucosa and DSM may be necessary for ATP release from sources other than the mucosa.

Shown both here and in a previous study, contractions stimulated by GSK are of similar magnitude to those induced by carbachol and are sustained over a period of a few hours (Thorneloe et al., 2008). This shows that by its large magnitude and sustained contraction TRPV4 is a potent inotropic regulator. On the other hand, unlike GSK-induced ATP release, contractions cannot be generated for a second time after a recovery period of 1 hour. Again, this can probably be attributed to the independent effects of GSK on the mucosa (ATP release) and directly on the DSM (contraction). It may be that the TRPV4 receptors involved in ATP release have transient dynamics, defined by the specific cellular environment and signalling pathways involved, such that they are returned to a resting state in less than 1 hour. However, it is likely that TRPV4 receptors responsible for direct contraction of the DSM remain in an activated long open state for longer in order to achieve the sustained contraction observed, resulting in refractory state and hence an inability to elicit a second contraction in the timeframe defined by this study. TRPV4 receptors have indeed been shown to elicit the sustained proinflammatory effects of other receptors such as proteolytic-activated receptor 2 in DRG neurones (Poole et al., 2013). Desensitisation is another possibility.

GSK was also successful in evoking ATP release from human bladder biopsies of healthy mucosal tissue from aging (>65YOA) male patients. Out of the 10 biopsies assessed, only one showed a contraction in response to TRPV4 activation, where this was the only biopsy with fully-intact smooth muscle (full thickness strip). This behaviour of intact human bladder provides a strong indication that, with the reproducible ATP release, human bladder responds to GSK in the same way as GP tissue, supporting use of GP tissue as a relevant and translatable additional model for investigation of TRPV4 in this study.

### 3.5.2.2 SELECTIVE BLOCKADE OF TRPV4

Antagonism of TRPV4 reduced intrinsic ATP release from unstimulated mucosal sheets, supporting a role for this receptor in maintaining basal release of ATP from the urothelium. This observation suggests that TRPV4 channels are opened under resting conditions, likely by endogenous activators such as mediators, temperature, pH changes and even small degree of stretch. This effect was only observed in separated mucosal sheets and not in intact full thickness tissue, thus indicating a urothelium-specific phenomenon. This finding lends weight to the physiological importance of this receptor in urothelial function.
Antagonism of TRPV4 in human mucosal biopsies also reduces basal ATP release, despite following a longer superfusion. This supports human relevance of this phenomenon.

These initial experiments confirm the presence of TRPV4 and establish a functional role for these receptors in both urothelial ATP release and direct DSM contraction in the models used for this study.

### 3.5.2.3 IMPLICATIONS OF TRPV4-MEDIATED ATP RELEASE

As previously described, ATP is a critical signalling molecule in the bladder and as such, has a strong influence on correct bladder function. Not only can ATP influence bladder contraction through sensitization of bladder afferents (Rong et al., 2002), but it can also alter urothelial function and modulate interstitial and smooth muscle cells through purinoceptors (Elneil et al., 2001; Liu et al., 2009; Vlaskovska et al., 2001). It is well documented that TRPV4 may be involved in stretch-induced ATP release from the mucosa and therefore likely plays an important role in physiological bladder function. However, studies have identified an augmented basal ATP release from aging bladder mucosa (Sui et al., 2014) and augmented stretch-induced ATP release from the urothelium of pathological rat (Birder et al., 2003) and human (Yan Sun et al., 2001) bladders with interstitial cystitis. This augmented release has been shown to enhance sensation of pain through purinoceptor activation, however the responsible mechanism underlying this enhanced ATP release has not been identified. As this ATP is indeed capable of activating nociceptive purinergic receptors under these conditions, it is feasible to assume this ATP is just as capable of modulating purinoceptors present on downstream effectors that also express these receptors. It has indeed been shown that ATP released from the urothelium can affect DSM contraction in a paracrine manner, suggesting urothelially derived ATP release is sufficient to reach and influence activity of deep effector tissues including smooth muscle, sensory nerve endings and interstitial cells, contributing to sensory modulation (Sui et al., 2014). TRPV4 activity is therefore exceptionally important, as the receptor has the ability to significantly influence bladder function through ATP release. As such, it is also possible that TRPV4 may contribute to the augmented ATP release observed during bladder pathologies and aging, and in doing so influence the progression of bladders into such pathological states. This highlights the importance of further investigation of this receptor, as modulation of TRPV4 and its associated signalling may provide a novel tool for regulating bladder pathologies and sustaining correct bladder function in older individuals.
3.5.3 ORIGIN OF TRPV4-INDUCED ATP RELEASE

The mucosa was identified as the main source of TRPV4-induced ATP release. Denuded smooth muscle does not release ATP in response to TRPV4 activation, at least not without an intact mucosa, as synergy between the two tissues in full thickness bladder may grant smooth muscle-derived ATP release through various inter-tissue signalling. Raw data revealed that the quantity of ATP released in response to specific TRPV4 activation from mucosa was significantly greater per gram than full thickness bladder preparations. This implies the smooth muscle tissue in the full thickness strips is effectively redundant regarding ATP release, as when present, it reduces the ATP release per gram tissue. Together, these results provide strong evidence to suggest that the vast majority of ATP released in response to TRPV4 activation originates from the mucosa. As such, the ATP release observed from full thickness tissue shown in the figures of this study can also be attributed to the mucosa, without contribution from the underlying tissues. However, if there were a significant difference between the release of ATP from the mucosa and the release of ATP from full thickness tissue in response to a specific stimulant, this would imply the underlying tissues in the full thickness strip are influencing ATP release. That being said, no differences were observed between mucosal and full thickness strips in this study.

Basal ATP release from denuded smooth muscle was negligible. Mucosa and full thickness tissue released ATP without stimulation, where per gram, basal release from mucosa alone was significantly higher than intact full thickness tissue. These findings imply that the mucosa is the source of intrinsic basal ATP release.

3.5.4 PATHWAY FOR ATP RELEASE AND THE UNDERLYING SIGNALLING

Stretch-induced ATP release from the urothelium is facilitated by a variety of pathways including vesicular exocytosis, conductive pathways (connexin and pannexin channels) and various ATP carrier proteins (Wang et al., 2005). General stretch-induced ATP release from isolated urothelial cells requires mobilization of intracellular Ca\(^{2+}\) and to a lesser extent extracellular Ca\(^{2+}\) entry, and is facilitated by vesicular transport (Birder et al., 2003). Mochizuki et al. also demonstrated that ATP release from isolated cells induced specifically by TRPV4 is also tightly dependent on extracellular Ca\(^{2+}\) entry. TRPV4-induced ATP release in other tissues also requires extracellular Ca\(^{2+}\) influx and is hemichannel-mediated (Shahidullah et al., 2012), with one study specifically identifying pannexin-1 as the responsible channel (Seminario-Vidal et al., 2011) and as such, these may also underlie the
TRPV4-induced ATP release in bladder mucosa. Currently however, the full pathway underlying TRPV4-induced ATP release remains unclear.

3.5.4.1 VESICULAR OR CONDUCTIVE RELEASE & THE ROLE OF Ca\(^{2+}\)

Results suggest that TRPV4-induced ATP release from the mucosa is facilitated by conductive pathways, as demonstrated by a near-complete abolition of ATP release (90% reduction) by the connexin/pannexin inhibitor carbenoxolone, with no significant reduction by the vesicular transport inhibitor brefeldin A. Removal of extracellular Ca\(^{2+}\) also abolished any observed ATP release and live-cell calcium imaging revealed a significant rise in intracellular Ca\(^{2+}\) upon activation of TRPV4 with GSK, both of which suggest that TRPV-induced ATP release is Ca\(^{2+}\)-dependent, relying on extracellular Ca\(^{2+}\) influx. This is expected, as TRPV4 is a Ca\(^{2+}\) influx pathway. However, various studies provide evidence that Ca\(^{2+}\)-dependent ATP release is facilitated by vesicular transport (Boudreault and Grygorczyk, 2004; Coco et al., 2003) and another that ATP release through conductive pathways occurs under low [Ca\(^{2+}\)](Stout et al., 2002), conflicting with this finding. These studies may be of little relevance here, as they were performed using astrocyte cells in culture, however they are currently the only studies to have investigated the underlying mechanisms of cell-swelling induced Ca\(^{2+}\)-dependent ATP release. The results from this study are however, supported by others, which show that TRPV4-mediated ATP release from intact porcine lens epithelium requires extracellular Ca\(^{2+}\) influx and is hemichannel-mediated (Shahidullah et al., 2012), with another study in airway epithelial cells specifically identifying pannexin-1 as the responsible channel (Seminario-Vidal et al., 2011). These studies are therefore of greater relevance as they were performed in both whole tissue and cells from epithelia closely related to bladder epithelia, and assessed TRPV4-induced ATP release specifically. As such, taken together with results from this study, there is strong evidence supporting a Ca\(^{2+}\)-dependent (extracellular influx), hemichannel-mediated mechanism for TRPV4-induced ATP release from the urothelium. A very recent and interesting study indicated that pannexin-1 hemichannels facilitate P2Y\(_6\) receptor-mediated ATP release from the rat urothelium in vivo, and confirmed urothelial pannexin-1 expression by confocal microscopy (Timóteo et al., 2014). These pannexin-1 channels may therefore also be utilized in the pathway underlying TRPV4-induced ATP release.

3.5.4.2 Ca\(^{2+}\)-ACTIVATED CHLORIDE CHANNELS

To follow from this, various other signalling molecules and channels postulated to be involved in the underlying pathway were assessed. The CaCC blocker NPPB was used to
evaluate the possible role for these channels, as limited previous literature suggests that
ATP release through hemichannels is associated with activation of Cl⁻ channels (Cotrina et al., 1998). Blockade of CaCCs had no suppressive effect on TRPV4-induced ATP release, providing reassuring evidence that these channels are not involved in the pathway.

### 3.4.5.3 ROLE FOR PROTEIN TYROSINE KINASES

The potential role of protein tyrosine kinases was also evaluated using the inhibitor genistein, as again, these enzymes have been implicated in the modulation of both TRPV4 and ATP release. The first report of TRPV4 regulation by PTK was by Xu et al. who elegantly demonstrated that hypotonic stress results in rapid phosphorylation of TRPV4 by src-family tyrosine kinases (SFKs) at the tyr-253 residue (Xu et al., 2003). This residue is conserved across all species and in the same study, was shown to be vital for hypotonicity-induced TRPV4 channel activity. A further study demonstrated a crucial role for a newly identified N-terminal tyrosine residue in the activation of TRPV4 by mechanical stress and hypotonic cell swelling, but not for activation by the synthetic ligand 4αPDD. Again, SFK-mediated phosphorylation of this site was induced by such stimuli and was a necessary initial step before TRPV4 could elicit its specific responses (Wegierski et al., 2009). Yet another study showed that hypotonic stress-induced ATP release from aortic endothelial cells requires PTK-mediated phosphorylation of an unidentified protein of 110kDa. TRPV4 has a molecular weight of 98kDa and phosphorylation commonly shifts this by about 6kDa (Winkler et al., 1993), meaning the responsible protein could indeed be TRPV4, which has been identified in aortic endothelium (Watanabe et al., 2002b), however, this is simply a logical stipulation that requires much further investigation. Taken together, these studies provide strong evidence that mechanical stress first induces PTK-mediated phosphorylation of TRPV4, which is a necessary prerequisite for channel activity in response to such stimuli. The mechanisms by which PTKs recognise hypotonic and mechanical stress remain unclear, however it is well documented that mechanical stress up-regulates expression of PTKs (Zheng et al., 2004) and these kinases are involved in the hypotonic-stress response in many cell types (Edwards and Kapadia, 2000; Lepple-Wienhues, 1998; Xu et al., 2003), eliciting efflux of various solutes from the cell as a result (Musch et al., 1999).

Indeed, our findings indicate that PTK activity is necessary for agonist-induced TRPV4-mediated ATP release from the mucosa. Although phosphorylation is not necessary for TRPV4 activation by 4αPDD (Wegierski et al., 2009), the mechanism by which GSK activates TRPV4 to cause ATP release remains unclear and as such, the necessity for PTK-mediated phosphorylation for GSK-induced channel activity is possible. In aggregate, our
findings with those of others provide substantial evidence that PTK-mediated phosphorylation is an important initial step for TRPV4-mediated responses. This reveals another step in the pathway underlying TRPV4-induced ATP release.

3.4.5.4 INTERACTION WITH ENAC CHANNELS

For some time, mechanosensitive epithelial sodium channels (ENaC) have been implicated in stretch-evoked ATP release in the bladder. Previous studies have demonstrated suppression of urothelially-derived, stretch-evoked ATP release using the ENaC channel blocker amiloride, by as much as 100% in one study (Du et al., 2007) and 70% (by hypotonic swelling) in another (Birder et al., 2003). As such, we investigated the possible interplay between TRPV4 and ENaC channels in TRPV4-mediated ATP release, as it is possible that TRPV4 may elicit a proportion of its effect through secondary activation of ENaC channels. Amiloride was successful in suppressing GSK-induced ATP release in both mucosal and full thickness strips, providing evidence for this cross-talk. However, this was not replicated by the more potent derivative of amiloride, benzamil, and although only 5 bladders were assessed, no obvious trend was observed in individual data sets, where GSK still evoked ATP release in the presence of benzamil in 60% of bladders assessed. As benzamil is more potent, it can be used at lower concentrations, activating target molecules with higher specificity. Indeed, at the concentrations used in this study, benzamil has been shown to reduce mechanosensitivity, indicating a specific effect on ENaC and degenerin channels (Deg, also mechanosensitive) (Page et al., 2007), as its other targets are not mechanosensitive. As the concentrations required for amiloride to efficiently block ENaC channels affect such a variety of other channels (TRPP3, TRPA1, ASIC, Na⁺/H⁺ exchanger, non-selectively inhibits mechanogated membrane-ion channels, urokinase-type plasminogen activator [see methods table 2 for citations]), conclusions drawn from amiloride-based experiments cannot be confidently specific to ENaC channels. Of these additional targets, TRPA1 and ASIC channels are expressed in the urothelium (shown in rat (Streng et al., 2008) and human and rat (Kullmann et al., 2009; Sánchez-Freire et al., 2011) respectively). Evidence suggests that TRPA1 channels in the urothelium act to sense toxic and irritant substances, with no reported function in mechanosensation or ATP release to date. However, ASIC channels are implicated in both mechanosensation in the colon (Page et al., 2005) and ATP release from the rat urothelium (Sadananda et al., 2009). This suggests that in this case, the target for amiloride could be either ENaC or ASIC channels, as both appear to play roles in mechanosensation and ATP release. As such, assessing the effect of benzamil in additional bladders and investigating the effect of ASIC channel
blockers (e.g. gadolinium) may allow the responsible receptor to be determined. Our results have successfully identified that a proportion of TRPV4-induced ATP release requires the activity of another unknown receptor that is blocked by amiloride, with likely candidates being ENaC and ASIC channels. Previous literature (mentioned at the beginning of this segment) has suggested ENaC channels for this role and as such, ENaC channels are currently the more likely candidate. If such a cross-talk exists, where ENaC channels are responsible for a proportion of the ATP release elicited by TRPV4 activity, it may be possible to modulate urothelial ATP release with higher control, manipulating only a portion of the ATP release. In this way, symptoms of OAB could be controlled, with little interference on normal bladder function.

Superfusion with amiloride alone generated significant release of ATP from both the mucosa and full thickness bladder, with a 10 minute delay, peak release at 40 minutes and return to basal levels after 60-90 minutes. This agrees with findings in previous literature (Ferguson et al., 1997). Again, benzamil had no such effect and as such, the responsible molecule may be identified by comparison of the targets of each drug. However, the ATP release generated by amiloride may be multifactorial, owing to the multiple targets of amiloride or it may be that the high concentration of amiloride used (1mM) activates a greater proportion of ENaC channels than benzamil at 100μM, eliciting an unknown effect, factors all of which need careful consideration. In summary, amiloride seems to evoke ATP release when used alone, however attenuates the ATP release evoked by stretch or TRPV4 activation, effects likely elicited through ENaC channel blockade. As such, direct inhibition of ENaC channels could indeed exacerbate symptoms of OAB through amplified ATP release, however careful manipulation of the signalling underlying its stretch-related function could provide some level of pharmacological control. This provides evidence that TRPV4 and its related signalling may be a more suitable and reliable target for intervention of OAB symptoms.

### 3.4.6 STRETCH-INDUCED ATP RELEASE: A PROPORTION MEDIATED BY TRPV4

Various studies have provided strong evidence to suggest that TRPV4 plays a pivotal role in stretch-dependent ATP release. A decreased stretch-evoked ATP release has been demonstrated in whole isolated bladders from TRPV4-null mice, however this model cannot confirm the tissue source of ATP release (Gevaert et al., 2007). Mochizuki et al. provided further evidence with suppression of stretch-evoked ATP release from single cultured urothelial cells using a TRPV4 knockout model and broad TRP antagonists (Mochizuki et al., 2009). This study endeavoured to provide data of more physiological relevance, and as
such, to further investigate the channels responsible for physiological stretch-induced ATP release and the proportional roles each plays, the effect of specific TRPV4 antagonism on in vivo stretch-induced ATP release was investigated. A mechanical stretch of mucosal and full thickness strips to 150% of their original length for 30 seconds generated significant release of ATP 30s and 1 minute after initial stretch. This effect was significantly attenuated by inhibition of TRPV4 using the highly specific antagonist HC-067047. However, the stretch-induced ATP release was still significant in the presence of HC-067047, with an approximate 50% suppression. This provides novel evidence for the role of TRPV4 in stretch-evoked ATP release, also indicating the involvement of one or more other stretch-sensitive receptors. As ENaC channels have been strongly implicated in stretch-evoked ATP release, it would be interesting to assess the effect of ENaC channel blockade, and more so the effect of simultaneous TRPV4/ENaC channel blockade using this stretch model on stretch-evoked ATP release. This may reveal the proportional contributions of the various mechnosensative channels implicated in this role.

3.4.7 TRPV4-INDUCED ATP RELEASE – A POSSIBLE MECHANISM

The experiments performed have revealed a substantial proportion of the signalling pathway underlying TRPV4-induced ATP release and taken with previous findings, provided sufficient evidence to confidently postulate a comprehensive underlying mechanism. For full deliberation, differences between the agonist-induced and the more physiologically relevant stretch-induced pathways must be considered. The postulated mechanism for stretch-induced ATP release mediated by TRPV4 activation is as follows; 1. Mechanical stretch activates SFKs, which phosphorylate TRPV4 at tyr-253, permitting channel activity 2. TRPV4 mediates Ca\(^{2+}\) influx, raising intracellular \([\text{Ca}^{2+}]\); Ca\(^{2+}\) directly activates pannexin-1 channels, which mediate both the serosal and apical release of ATP 3. ATP activates P2Y receptors on neighbouring urothelial cells, in turn activating further pannexin-1 channels via Ca\(^{2+}\) entry, propagating and amplifying ATP release throughout the urothelium 4. Serosally released ATP is available to stimulate downstream effector tissues such as smooth muscle, interstitial and suburothelial cells and sensory nerve endings expressing purinergic/purine receptors, possibly resulting in sensory modulation (figure 3-23). ENaC channels have been included in this mechanism, as preliminary evidence suggests they may mediate a small proportion of TRPV4-induced ATP release. Although the underlying mechanisms remain unknown, it is an appealing thought that the SFKs that activate TRPV4 also activate ENaC channels, however ENaC channels are predominantly activated by serine proteases, so this is unlikely (Rossier and Stutts, 2009).
As previously described, this study and others provides strong evidence for steps 1, 2 and 4. Pannexin channel activity and evidence supporting its viability as the key facilitator of ATP release in this pathway has only been documented in very recent years. Our study demonstrated abolition of TRPV4-induced ATP release with carbenoxolone, implying a connexin or pannexin hemichannel as the pathway for ATP release. Connexin expression in the bladder has been previously described, with Cx26 localized to the urothelium, Cx43 to the lamina propria and strong expression of both Cx43 and Cx45 in the DSM (Ikeda et al., 2007). This narrows the possibilities down to Cx26 or a pannexin channel, however Cx26 is closed by cytoplasmic Ca\(^{2+}\) (Lopez et al., 2013) and is not modulated by phosphorylation like other connexins (Lampe and Lau, 2004; Traub, 1989). As such, the Ca\(^{2+}\) influx facilitated by TRPV4 will neither directly nor indirectly (for example, through calmodulin and CaMKII signalling) activate Cx26 allowing ATP release from urothelial cells. Novel evidence supporting a role for pannexin-1 channels in ATP release from various cell types and indeed the urothelium has recently been published (Iglesias et al., 2009; Locovei et al., 2006a). Timóteo et al. identified pannexin-1 channels in the urothelium and showed them to be functional, facilitating P2Y\(_6\)-receptor induced ATP release and that this is responsible for UDP (specific P2Y\(_6\) agonist)-induced OAB (Timóteo et al., 2014). Interestingly, they found that pannexin-1 is expressed throughout the urothelium and is not limited to one cell layer, a pattern similarly identified for TRPV4 in the current study, suggesting possible cooperation between these receptors. Further studies have revealed that pannexin-1 can be activated by Ca\(^{2+}\) and that this is likely a direct effect of the ion, rather than intermediate Ca\(^{2+}\) signalling (Locovei et al., 2006b). Evidence also suggests that Ca\(^{2+}\) activation of pannexin-1 is a result of raised intracellular Ca\(^{2+}\), as the channel does not respond to extracellular Ca\(^{2+}\) (Bruzzone et al., 2005). Unlike connexins, pannexin-1 therefore presents a novel calcium-activated conductive ATP release pathway, meaning Ca\(^{2+}\)-dependent ATP release does not need to be exclusive to vesicular release, as previously thought. This is in line with our findings, where previous studies and ours show decreased (Ca\(^{2+}\)-dependent) ATP release with the pannexin/connexin channel blocker carbenoxolone. Finally, a study in primary airway epithelial cells provided evidence that TRPV4-mediated ATP release induced by membrane stretch is achieved through pannexin-1 channels, directly linking the two (Seminario-Vidal et al., 2011). Taken together, the current evidence supports a role for pannexin-1 channels in facilitating Ca\(^{2+}\)-dependent, stretch-evoked ATP release from the urothelium, mediated by TRPV4.

The pathway for specific GSK-induced (agonist-induced) ATP release, as opposed to stretch-induced, would follow the same steps, varying only in the initial stage. GSK is
thought to activate TRPV4 either by direct activation, acting as a synthetic ligand, or by recruiting previously inactive receptors. In either case, the mechanism would bypass the SFK stage, where GSK would activate TRPV4, either directly or through recruitment of new receptors, in both cases eliciting Ca\textsuperscript{2+} influx thereby initiating the ATP release mechanism. However, findings from this study indicated that PTK activity is still necessary for GSK-induced ATP release. As discussed, TRPV4 phosphorylation by SFKs appears to be a necessary prerequisite for channel activity. If all PTKs are inhibited, as was done in this study, the proportion of basal phosphorylated TRPV4 receptors able to be activated may be reduced, resulting in the reduced agonist effect observed. Therefore, although the agonist-induced ATP release pathway for TRPV4 may not involve the same initial SFK step described for the stretch-induced pathway, it may still require a basal level of SFK activity to prime the receptors (through phosphorylation) for activation by GSK. It is highly likely the direct contractile effect of GSK on the DSM is elicited simply by Ca\textsuperscript{2+} influx, activating the contractile machinery through the common Ca\textsuperscript{2+}-dependent mechanism.

The summarised findings from this study, taken with those from supporting literature, define a plausible and highly evidenced mechanism for stretch-evoked, TRPV4-mediated ATP release. This provides a variety of targets and possibilities for controlling stretch-evoked ATP release during bladder filling, which may contribute to OAB syndrome and thus, pharmacological intervention of this pathway may serve useful in alleviation of OAB symptoms. Pannexin-1 channel inhibitors may be the most promising targets, as these channels seem to be the prominent pathway for both stretch (TRPV4)-induced and purinoceptor-induced ATP release from the urothelium. These would supress any NANC-mediated contractions that emerge during various pathological bladder states, also alleviating ATP-related pain, while leaving the physiological muscarinic contractile component fully intact for normal bladder function. Pannexin-1 expression has been demonstrated in the urothelium of rats (Beckel et al., 2015), however there is currently no report of its expression in other bladder tissues or in human bladders. If indeed, pannexin-1 is localized to the urothelium in human bladders, this would not only make targeting pharmacological agents simple, but also reduce side effects, making pannexin-1 channels highly appealing candidates for therapeutic intervention of OAB.
Figure 3 – 23. Postulated mechanism for stretch-induced, TRPV4-mediated ATP release from the urothelium. The urothelium receives sensory input in the form of stretch (as bladder fills) and this activates Src-family tyrosine kinases (SFKs)[1], which phosphorylate TRPV4 at tyr-253, permitting channel activity. Active TRPV4 mediates Ca$^{2+}$ influx [2], raising intracellular [Ca$^{2+}$]; Ca$^{2+}$ directly activates pannexin-1 channels [3], which mediate both the serosal and apical release of ATP. Both serosally and apically [5] released ATP activates P2Y receptors on neighbouring urothelial cells (autocrine action), in turn activating further pannexin-1 channels, propagating and amplifying ATP release throughout the urothelium [4]. Serosally released ATP is available to stimulate downstream effector tissues such as smooth muscle, interstitial and suburothelial cells and sensory nerve endings expressing purinoceptors (paracrine action)[6], possibly resulting in sensory modulation. ENaC channels responsible for a small proportion of TRPV4-induced ATP release are activated by an unknown mechanism, triggering subsequent ATP release [7]. Agonist-induced ATP release by GSK bypasses stage [1], directly activating TRPV4, initiating the downstream pathway by eliciting Ca$^{2+}$- influx [2]. Created using public access images from (Servier Medical, 2015)
3.4.8 INFLUENCE OF TRPV4 ON BLADDER ROS PRODUCTION

ROS production in the bladder has previously been linked to detrusor overactivity, through modulation of the cyclooxygenase pathway in C-fibre afferents in rats (Chien et al., 2003; Masuda et al., 2008). Interestingly, TRPV4 has been linked to ROS production, where receptor activation in human coronary artery strips increased the mitochondrial production of superoxide for physiological vasodilation (Bubolz et al., 2012). As such, lucigenin-enhanced chemiluminescence was employed to assess the potential of TRPV4 to produce ROS in the GP bladder. TRPV4 activation in full thickness bladder strips significantly enhanced superoxide production to 136% of control. As ROS production was enhanced upon addition of NADPH, this suggests an NADPH-oxidase (NOX) enzyme is responsible. TRPV4 activation induced Ca\(^{2+}\) influx and as NOX-5 is the only subtype activated by Ca\(^{2+}\) this is the likely subtype mediating TRPV4-induced ROS production (Bánfi et al., 2004). As both receptors are localized to the cell membrane, it is possible local increases in [Ca\(^{2+}\)] could activate NOX5 receptors in close proximity to activated TRPV4. NOX5 expression has not yet been confirmed in the GP bladder and as such, evaluation of this is necessary before postulations can be made and may provide interesting novel data. This increase in oxidative stress may result in bladder inflammation, detrusor overactivity through hyperactive bladder afferent signalling, or indeed contribute to the aging bladder phenotype. Wegierski et al. also showed that ROS significantly up-regulate the phosphorylation of TRPV4 in the presence of SFKs, possibly enhancing the activity of TRPV4 further (Wegierski et al., 2009). TRPV4 channels may therefore contribute to overactive bladder not only though ATP release, but also through stimulation of afferent signalling via ROS, indicating that TRPV4 has the potential to significantly influence pathological bladder states.
CHAPTER 4: TRPV4 RECEPTOR EXPRESSION AND FUNCTION IN THE AGING BLADDER
4.1 INTRODUCTION

It is well-known that bladder dysfunction increases markedly with age, with urinary urge incontinence affecting up to 13.3% of males and 30.3% of females of age 40 and above in western countries, accompanied by a huge financial cost (Milsom et al., 2014). Despite this, the underlying mechanisms have yet to be fully established, as they appear to be highly complex and multifactorial. Many studies have endeavoured to investigate this phenomenon, where the emergence of a NANC-component to contraction (Bayliss et al., 1999), changes in the purinoceptor signalling and afferent pathways controlling bladder function (Daly et al., 2014), and increased basal and stretch-evoked ATP release (Kumar et al., 2010; Sui et al., 2014) are some of the factors identified in aging bladders associated with the progression of overactive bladder.

The previous chapter identified an important functional role for TRPV4 receptors in ATP release from the mucosa of both GP and native human bladders, elucidated the underlying mechanisms and revealed their potential to influence ROS production. Both of these outputs have the potential to directly alter bladder function and as such, any changes in the behaviour of TRPV4 that may result from aging may contribute, in part, to overactive bladder. Previous studies in humans have revealed the cellular localization of urothelial TRPV4 (Janssen et al., 2011), however the only functional studies associated with humans that exist used heterologously expressed human TRPV4 in cultured HEK cells (Everaerts et al., 2010c). As such, the aim of this study was to investigate any changes in the expression and function of TRPV4 receptors that occur with aging in bladder strips from both humans and GPs, providing more physiologically relevant findings. Any changes observed may reveal that this receptor is not only important for physiological bladder function, but may also be involved in mediating overactive bladder and promoting other age-related bladder pathologies.

In addition, as purinoceptors have previously been shown to both mediate ATP release from the mucosa (Sui et al., 2014) and display altered receptor expression with aging/pathology (Daly et al., 2014; Moore et al., 2001; Tempest et al., 2004), the effect of aging on purinoceptor-mediated ATP release and their potential to influence TRPV4 mediated ATP release was also investigated.
4.2 SUMMARY

The objectives for investigating the possible changes to TRPV4 receptor expression and function associated with aging are as follows:

1. EXPRESSION STUDIES:
   
a. Compare the expression levels of TRPV4 receptors in the urothelium and suburothelium between young and aging GP bladders using quantitative immunohistochemistry.

   b. Compare the expression levels of TRPV4 in separated mucosa and smooth muscle between young and aging GP bladders using quantitative western blotting, identifying any tissue-specific changes.

2. IN VITRO FUNCTIONAL EXPERIMENTS USING FUNCTIONAL ORGAN BATH TECHNIQUE:
   
a. Compare the effect of TRPV4 activation on ATP release and bladder contractions in young and aging GP bladder preparations.

   b. Investigate the effect of co-activation of TRPV4 and P2Y receptors on ATP release and bladder contractions in young and aging GP bladder preparations.

   c. Investigate the effect of objective 2b on both normal and overactive human bladder biopsies.

3. FURTHER IN VITRO STUDIES:
   
a. Use P2Y\textsubscript{2} knockout mice to identify the purinoceptor subtypes responsible for any co-dependent effects observed during objective 2.
4.3 METHODS

The functional experiments performed in this chapter followed the protocols described in section 2.5.1 of methods. For a full description of other techniques used, please refer to the relevant methods section (detailed in text or figure legend where applicable).

4.3.1 EFFECT OF PRE-ACTIVATION OF ONE RECEPTOR GROUP ON ACTIVITY OF THE OTHER

The following experiments were designed to examine the relationship between TRPV4 and P2Y receptors in section 4.4.2.2.6 of this chapter. The concept of experiments (referring to step 2 in figure 4-1) was to first activate one receptor group (e.g. TRPV4), then also activate a second group (e.g. P2Y₂/P2Y₄) to determine whether the second can raise ATP release beyond that already being stimulated by the first. In this way it is possible to determine the dynamics of the relationship between two receptor types regarding ability to release ATP i.e. do they use the same mechanism. The ‘antagonist studies’ protocol was followed (figure 2-4), where the antagonist step was replaced with either of the agonists, for example when assessing pre-treatment with GSK the protocol was as follows (figure 4-1):

1. Control sol's 5min UTP Control sol's
2. Control sol's 5min GSK 5min UTP+GSK 5min GSK Control sol's
3. Control sol's 5min UTP Control sol's

Figure 4 – 1. Receptor relationship studies protocol. Schematic of work flow carried out for receptor-relationship studies. The ‘antagonist studies’ protocol was followed, where the antagonist step was replaced with an agonist (step 2, GSK). Then a second agonist (UTP) was introduced to determine whether activation of a second receptor group could enhance ATP release beyond that of the first agonist. ATP release was measured throughout the protocol in the same manner as the antagonist studies. Steps 1 and 3 act as control to ensure tissue is responsive (regarding ATP release) to the agonist under investigation.

For example, if UTP can stimulate ATP release during step 1 and 3, but pre-activation of tissue with GSK prevents this UTP-induced ATP release, this implies some shared mechanism for ATP release, allowing the relationship between the two receptors to be deliberated.
4.4 RESULTS

4.4.1 ALTERED EXPRESSION OF TRPV4 IN THE AGING BLADDER

This section of the study was performed to determine whether aging affects the tissue localization or overall expression levels of TRPV4 in the bladder. IHC allowed observations between the urothelium and suburothelium to be made, while western blotting provided a more robust method of quantification, but only between separate mucosa and smooth muscle tissue.

4.4.1.1 EFFECT OF AGING ON TISSUE LOCALIZATION OF TRPV4 IN GP AND HUMAN BLADDER TISSUES BY IHC

The distribution of TRPV4 receptors throughout the bladder was assessed in young and aging GP full thickness and human mucosal cryosections and examined using confocal microscopy in the same manner as described in the previous chapter (section 3.4.1.1). 9 young GP bladders, 7 aging GP bladders and 4 human bladder mucosa biopsies (taken from position of healthy tissue in male patients >65 years old with suspected or known tumours) were examined. Representative images for aging GP are shown in figure 4-2 and those for young GP and human are shown in the previous chapter. All groups showed clear expression of TRPV4 in the urothelium and suburothelium. The smooth muscle was assessed in 3 young and 3 aging GP bladders and 1 human biopsy (which had smooth muscle attached), TRPV4 expression was observed in all. TRPV4 staining seems to be more evident in the urothelium, with weaker expression in the suburothelium and weaker still in the smooth muscle. To assess this, staining was quantified by measuring pixel density of images and calculated per cell, providing final values of TRPV4 expression as ‘mean cell fluorescence’ (MCF) for each tissue type. The median values for mean cell fluorescence represented in figure 4-3 are shown in table 11.

Higher TRPV4 expression was observed in the urothelium compared to the suburothelium in all 7 aging GP bladders and 8 of the 9 young GP bladders. TRPV4 expression was similar between the urothelium and suburothelium in healthy, aging human tissue. The difference in expression levels between the two tissues seems to be greater in aging than young GP bladder, with a greater significant difference, suggesting there may be a shift in expression with age in favour of the urothelium. This method allowed differences between the urothelial and suburothelial expression to be identified, which is not possible with western blotting. However, comparison between age groups using this technique was not possible as some
sections of each group were examined on different days under different conditions, such that expression on different tissue sections on the same slide could be compared, but those examined on different days could not. Therefore, a protocol using western blotting was performed allowing comparison of TRPV4 tissue expression between age groups.

![Figure 4](image)

Figure 4 – 2. Representative images demonstrating expression of TRPV4 in aging GP bladder tissues. Cryo-sections (10μm) of aging GP bladder tissues at x40 magnification. TRPV4 is labelled with Alexa fluor 568 (red) and nuclei with To-PRO3 (Cy5; blue). Column A: Brightfield images demonstrating general morphology and tissue distribution. Column B: Secondary control; no primary antibody (Ab) added, ensuring no unspecific binding of secondary Ab to sample. Column C: Positively stained sections depicting expression and distribution of TRPV4 in the urothelium (green bar), suburothelium (pink bar), smooth muscle and vasculature. High TRPV4 expression is observed in the urothelium with some expression in the suburothelium. Images from each row (tissue type) were taken from the same ‘area’ of tissue on sections cut directly after one another, thus providing a reliable comparison between control and positive staining. These images were taken from sections of 1 of 7 aging GP bladders examined.
Table 11 - Quartiles (median [25%, 75%]) of MCF of TRPV4 in various tissues (referring to figure 4 – 3 graph).

<table>
<thead>
<tr>
<th>Tissues type</th>
<th>Young GP</th>
<th>Aging GP</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urothelium</td>
<td>2393 [1928, 2393]</td>
<td>2789 [2318, 4865]</td>
<td>2708 [2263, 3074]</td>
</tr>
<tr>
<td>Smooth muscle</td>
<td>1624 [611, 2098]</td>
<td>1665 [1258, 2098]</td>
<td>1347</td>
</tr>
</tbody>
</table>
4.4.1.2 EFFECT OF AGING ON RELATIVE TISSUE EXPRESSION OF TRPV4 IN GP BLADDER TISSUES BY WESTERN BLOTTING

The effect of aging on TRPV4 expression was assessed in separated mucosal and smooth muscle bladder whole-tissue lysates by western blot, run against rat brain lysate (RBL) positive control tissue (recommended by Ab suppliers). The representative blot in Figure 4-4 A shows clear bands at the same molecular weight (kDa) as the positive control, thereby confirming bands correctly represent TRPV4 in these samples. At first glance, it is clear the expression of TRPV4 is greater in aging tissue than young. To reliably confirm this, expression levels were quantified by densitometry and relative expression between tissues of each age group compared (figure 4-4 B).

Figure 4 – 4. Detection and quantification of TRPV4 in young & aging GP bladder. A: Representative blot of TRPV4 (98kDa, probed using Abcam primary Ab) and β-actin loading control (42kDa) (RBL: rat brain lysate Muc: mucosa, SM: smooth muscle). Membrane was cut at 55kDa (dashed blue line) and each half probed with the appropriate Abs. B: Quantified data of TRPV4 expression by densitometry. Each TRPV4 band is normalized to its corresponding β-actin loading control. Samples run in duplicate and averaged. Aging tissues have significantly higher TRPV4 expression than young. Aging mucosa has significantly greater expression than aging smooth muscle, young tissue does not. Data sets are expressed as % medians [25%, 75%], n=5, ** p ≤0.01, Mann-Whitney, Young vs. Aging or Aging vs. Aging.
Quantification of relative band intensity by densitometry revealed a significantly greater median TRPV4 expression (8 fold) in aging mucosa than young (arbitrary units normalised to loading control - aging; 42.9 [25.7, 75.6], young; 5.8 [2.1, 14.3]) and (16 fold) in smooth muscle (aging; 13.5 [10.1, 28.3], young; 0.8 [0.6, 3.4]). Additionally, TRPV4 expression in the mucosa was significantly greater compared to smooth muscle in aging but not in young bladders.

Taken with the confocal data, it appears that TRPV4 expression is drastically altered with age. Western blotting showed a distinct increase in TRPV4 expression in the mucosa and smooth muscle of aging bladders, and confocal images revealed that the higher TRPV4 expression observed in the urothelium compared to the suburothelium was even greater and more profound in aging tissue. These results suggest that TRPV4 expression is increased in the urothelium and smooth muscle, but not the suburothelium with age.

4.4.2 ALTERED FUNCTION OF TRPV4 AND PURINOCEPTORS IN THE AGING BLADDER

As aging drastically alters the expression of TRPV4 in the bladder, their function may also be affected, changes in which could contribute to age-related bladder dysfunctions. Purinoceptors have also previously been shown to mediate ATP release from the mucosa (Sui et al., 2014) and display altered receptor expression with aging/pathology (Daly et al., 2014; Moore et al., 2001; Tempest et al., 2004). As such, the effect of aging on both TRPV4 and P2Y receptor-mediated ATP release and bladder contraction was assessed. The effect of activating both receptors simultaneously was also investigated to determine whether they have a synergistic role in mucosal ATP release and whether this is also affected with aging. For this purpose, a carefully designed protocol was followed (section 2.5.1.1.2 – interaction protocol) where each n represents a tissue strip that was mounted within the organ bath and subjected to 5 drug interventions with a 1hour recovery period between each.

4.4.2.1 EFFECT OF PURINOCEPTOR AND TRPV4 ACTIVATION ON FUNCTIONAL OUTPUTS IN THE GP BLADDER

4.4.2.1.1 YOUNG GP MODEL

UTP (100μM) was used for purinoceptor activation as this agonist specifically activates P2Y₂ and P2Y₄ at this concentration and has previously been shown to induce reproducible ATP release from the GP mucosa (Sui et al., 2014). Very weak mucosal contractions were
observed in 33% of strips examined when challenged with UTP and none with GSK. GSK (1μM) evoked a large contraction in full thickness tissue which was significantly greater than that evoked by UTP (Force μN/mg tissue - GSK; 246.2 [175.1, 668.8], UTP; 3.6 [2.2, 4.0]) (median data figure 4-5 A, example traces figure 4-6). Subsequent drug interventions were not capable of eliciting a further contraction for reasons previously discussed when assessing reproducibility of GSK-induced contractions using control experiments (section 3.4.3.1). Significant ATP release was generated by all interventions from both tissue preparations (see table 12), aside from the post-GSK and post-UTP interventions in full thickness where significant release was lost (figure 4-5 B). This is likely attributable to drift in tissue function over the period of the experiment, where these post-interventions were performed to identify this. ATP release generated by UTP was not significantly different to GSK. ATP release generated by mucosa was not significantly different to full thickness tissue with any intervention. ATP release generated by UTP+GSK was significantly greater than that by GSK or UTP in mucosa and greater than GSK (but not UTP) in full thickness. This data will be compared to that from aging GP tissue and further analysed later.

Table 12 - Quartiles (median [25%, 75%]) of ATP release and force elicited by stated interventions in young GP bladder tissues (referring to figure 4 – 5 B graph).

<table>
<thead>
<tr>
<th>Tissue strip</th>
<th>100μM UTP (pre)</th>
<th>1μM GSK (pre)</th>
<th>100μM UTP + 1μM GSK</th>
<th>1μM GSK (post)</th>
<th>100μM UTP (post)</th>
</tr>
</thead>
</table>
Figure 4 – 5. Effect of UTP and GSK1016780A on bladder functional outputs in young GP bladder. Young GP bladder strips challenged with stated drug interventions in sequence from left to right (for full protocol refer to sections 2.5.1.1.1 and 2.5.1.1.2). A: Median force of mucosal and full thickness (FT) contractions generated by drug interventions. No contractions observed in mucosa with any agonist (confirmed by traces), a large contraction evoked in FT with the first exposure to GSK, where this contractile effect was lost (significantly less) in the following two exposures, including the joint intervention (UTP+GSK). The contraction generated by 1μM GSK (TRPV4 activation) was significantly greater than 100μM UTP (P2Y agonist). Note Log2 Y axis for clear visualization. B: Corresponding median ATP release from same bladder strips. All drug interventions significantly increase ATP release from basal levels in both mucosa and FT, apart from in FT during post controls. No significant difference in ATP release is observed between mucosa and FT for any drug intervention. In mucosa, the joint intervention (UTP+GSK) triggered significantly more ATP release than individual pre-UTP or pre-GSK interventions (significance not shown on graph). Additionally, ATP release triggered from FT by the joint intervention was significantly higher than the individual pre-GSK intervention (not shown). For both graphs: data expressed as % medians [25%, 75%], n=9 (pre UTP, pre GSK, UTP+GSK), n=8 (post UTP, post GSK), Wilcoxon’s, graph B *p<0.05, drug intervention vs. control (basal levels).
Small contractions were observed in 35% of the mucosal strips examined when challenged with UTP and in 50% when challenged with GSK. GSK (1μM) evoked a large contraction in full thickness tissue which was significantly greater than that evoked by UTP (Force μN/mg tissue - GSK; 485.4 [252.6, 814.4], UTP; 7.9 [2.5, 34.87])(figure 4-7 A). Interestingly, the subsequent drug intervention of UTP+GSK was capable of eliciting further contractions in 65% of the full thickness strips, although these were weaker (UTP+GSK; 22.25.4 [1.5, 174.5]). Significant ATP release was generated by all interventions from both tissue preparations (see table 13), however, as with young GP, no significant ATP release was observed in full thickness during post GSK and UTP interventions (figure 4-7 B). ATP release generated by UTP was not significantly different to GSK, nor was the ATP release generated by these interventions different between mucosa or full thickness tissue. However, the ATP release generated by UTP+GSK was significantly greater in mucosa than full thickness. ATP release generated by UTP+GSK was significantly greater than that by GSK or UTP in mucosa and greater than GSK (but not UTP) in full thickness.

Table 13 - Quartiles (median [25%, 75%]) of ATP release and force elicited by stated interventions in aging GP bladder tissues (referring to figure 4 – 7 B graph).

<table>
<thead>
<tr>
<th>ATP release (as % of averaged controls)</th>
<th>Tissue strip</th>
<th>100μM UTP (pre)</th>
<th>1μM GSK (pre)</th>
<th>100μM UTP + 1μM GSK</th>
<th>1μM GSK (post)</th>
<th>100μM UTP (post)</th>
</tr>
</thead>
</table>

Figure 4 – 6. Contractile responses of GP tissue strips to GSK and UTP. Record of contraction induced by 1μM GSK (A) and 100μM UTP (B) in mucosal and full thickness bladder strips. GSK evokes a strong, sustained contraction in full thickness tissue, compared to the weaker, transient contractions evoked by UTP. Agonists evoked negligible contractions in mucosal strips.
Figure 4 – 7. Effect of UTP and GSK1016780A on bladder functional outputs in aging GP bladder. Aging GP bladder strips challenged with stated drug interventions in sequence from left to right (for full protocol refer to sections 2.5.1.1.1 and 2.5.1.1.2). A: Median force of mucosal and full thickness (FT) contractions generated by drug interventions. Agonists generated negligible contractions in mucosa (confirmed by traces). First exposure to GSK generated a large contraction in FT, where this effect was weakened (significantly less, not shown) in the following two exposures, including the joint intervention (UTP W/ GSK). Note Log2 Y axis for clear visualization.

B: Corresponding median ATP release from same bladder strips. All drug interventions significantly increase ATP release from basal levels in both mucosa and FT to a similar level, apart from in FT during post controls. The joint intervention (UTP+GSK) triggered significantly more ATP release from the mucosa than from FT. The ATP release triggered from mucosa by the joint intervention was significantly higher than the individual pre-UTP or pre-GSK interventions (significance not shown on graph). Additionally, the ATP release triggered from FT by the joint intervention was significantly higher than the individual pre-GSK intervention. For both graphs: data expressed as % medians [25%, 75%], n=11 (pre UTP, pre GSK, UTP+GSK), n=7 (post UTP, post GSK), Wilcoxon’s, graph B *p<0.05, drug intervention vs. control (basal levels).
4.4.2.1.3 ORIGIN OF UTP-INDUCED ATP RELEASE

In the previous chapter the mucosa was identified as the origin of TRPV4-induced ATP release, supported by strong evidence. Figure 4-8 displays raw data of ATP release induced by UTP in the form of ρMoles released per gram tissue per minute, from the experiments in this section (refer to figure 4-7 B ‘pre-100μM UTP’ for normalized data). This allows the actual quantities of ATP released from each tissue preparation to be compared, providing an indication of the origin of ATP release. UTP significantly increased ATP release from mucosa and full thickness strips, as shown previously with normalized data (ρmoles/g tissue/min: mucosa - con; 88 [47, 162], UTP; 253 [185, 492], full thick - con; 16 [13, 37], UTP; 88 [45, 172]). Here we observe significantly greater ATP release per gram from mucosa compared to full thickness strips (significance not shown in figure). As with GSK, this implies that the DSM and other underlying tissues present in the full thickness strip add no further ATP release to that produced by the mucosa, as normalizing to tissue mass effectively reduces ATP release per gram of tissue, indicating these underlying tissues are redundant regarding ATP release. As such, data suggests that UTP-induced ATP release also originates from the mucosa. This implies that all UTP and GSK-induced ATP release observed in these experiments is from the mucosa, with no contribution from underlying tissues.

Figure 4 – 8. UTP-induced ATP release from young GP mucosa and full thickness bladder preparations: Raw data. Graph of raw data representing pmoles of ATP released per gram tissue per minute. UTP significantly enhanced ATP release from mucosal and full thickness young GP bladder strips. The UTP-induced ATP release from mucosa was significantly greater per gram than that from full thickness (not shown). Basal ATP release per gram was also significantly greater from mucosa than full thicknessss (not shown). ATP release expressed as pmoles/g tissue/min medians [25%, 75%], young GP n=8, aging GP n=11, p values shown, Wilcoxon's, drug intervention vs. control. Mucosa compared to full thickness using Mann-Whitney.
4.4.2.1.4 COMPARISON OF YOUNG AND AGING BLADDER OUTPUTS: CONTRACTILITY

UTP evoked minimal contractions in full thickness tissue, whereas GSK evoked strong contractions of similar magnitude in both age groups (Force μN/mg tissue – young FT; 246.2 [175.1, 668.8], aging FT; 485.4 [252.6, 814.4]). Interestingly, second exposure to GSK (during the joint UTP+GSK intervention) generated contractions in 65% of aging full thickness strips, where half of these were strong (>100μN/mg tissue) and half weaker (>20μN/mg tissue), compared to 9% in young tissue, which were all weak (aging; 22.1 [1.5, 174.5], young; 1.6 [0.4, 2.2])(figure 4-9). Again, this is likely attributable to the higher expression of TRPV4 in the smooth muscle of aging bladder and a direct effect of GSK on the abundant receptor population in this tissue.

Figure 4 – 9. Contractions generated by UTP and GSK1016790A in young and aging full thickness bladder strips. This data is taken from the previous young and aging contraction graphs (graphs A in figures 4-5 and 4-7) and organised to allow clear comparison between age groups. Note Log2 Y axis for clear visualization. No difference between contractions generated by pre-UTP, pre-GSK, post-GSK or post-UTP. The GSK+UTP intervention generated significantly greater contractions in aging GP FT strips than young. Young; pre-UTP, pre-GSK, UTP+GSK n=9, post-GSK, post-UTP n=8. Aging; pre-UTP, pre-GSK, UTP+GSK n=11, post-GSK, post-UTP n=7. Data sets expressed as % medians [25%, 75%], * p ≤0.05, Mann-Whitney, young GP vs. aging GP.
There was no significant difference in the quantities of ATP released from the mucosa per gram of tissue in response to TRPV4 activation between age groups (pmoles/g tissue/min: mucosa - con; 47 [37, 63], GSK; 105 [45, 379], full thick - con; 50 [27, 155], GSK; 88 [59, 217]) (figure 4-10). Basal ATP release was also similar.

Figure 4 – 10. GSK-induced ATP release from young and aging GP mucosa: Raw data. Graph of raw data representing pmoles of ATP released per gram tissue per minute. GSK significantly enhanced ATP release from mucosal strips in both age groups. The quantity of ATP released per gram was similar between groups for both basal and GSK-induced release. ATP release expressed as pmoles/g tissue/min medians [25%, 75%], young GP n=8, aging GP n=11, p values shown, Wilcoxon’s, drug intervention vs. control. Mucosa compared to full thickness using Mann-Whitney.

There was no significant difference in ATP release observed between age groups for any intervention in either mucosal or full thickness tissue (figure 4-11). However, to assess whether TRPV4 and purinoceptors interact to alter the ATP release in some way, the data was analysed further. The ATP released during the pre-UTP intervention was averaged with that of the post-UTP intervention. This provides values that take the drift in tissue function over the course of the experiment into account, allowing comparison of this intervention with the GSK and UTP+GSK intervention. Without this, a decrease in ATP release observed during the third intervention could be wrongly interpreted as an effect of the intervention,
rather than due to the drift in tissue function. This was also applied to the pre and post-GSK interventions. Finally, the ATP released during the averaged UTP intervention was manually combined with that of the GSK interventions and compared to the UTP+GSK intervention (figure 4-12).

Figure 4–11. Effect of UTP and GSK1016780A on ATP release from young compared to aging GP bladder tissues. These graphs allow for clear comparison of ATP release between young and aging bladder mucosa and full thickness (FT) strips triggered by the stated drug interventions (data is taken from ATP release graphs (B) in figures 4-5 and 4-7 but arranged for comparative purposes). A: Pre-100μM UTP intervention – increased ATP release in both mucosa and FT from both age groups. No difference between age groups (n=9 young, n=11 aging). B: Pre-1μM GSK intervention – increased ATP release in both mucosa and FT in both age groups. No difference between age groups (n=9 young, n=11 aging). C: 100μM UTP + 1μM GSK intervention – increased ATP release in both mucosa and FT in both age groups. No difference between age groups (n=9 young, n=11 aging). D: Post-1μM GSK intervention – increased ATP release in mucosa but not FT in both age groups. No difference between age groups (n=8 young, n=7 aging). E: Post-100μM UTP intervention – increased ATP release in mucosa but not FT in both age groups. No difference between age groups (n=8 young, n=7 aging). For all graphs: data expressed as % medians [25%, 75%], data sets compared to control (basal ATP levels) using Wilcoxon’s, *p<0.05, young compared to aging using Mann-Whitney test.
In young GP mucosa, activating TRPV4 and P2Y2/P2Y4 simultaneously, evokes the same proportional increase in ATP release as activating them individually and combining the two (manually combined individual activations: 482% [415%, 633%], simultaneous activation: 440% [343%, 727%]). This implies their joint effect is additive, suggesting they have no interaction and mediate ATP release via different mechanisms. However in the aging mucosa, activating the receptors simultaneously evokes significantly higher levels of ATP release, than the combined release from separate activation, implying their joint effect is potentiative here (manually combined individual activations: 468% [358%, 606%], simultaneous activation: 886% [309%, 1050%]). This suggests that some underlying interaction between the two receptor types emerges in the aging group, causing amplification of the ATP signal beyond that of their combined separate effects. It could be that a novel synergistic effect between these two receptors activates another ATP release pathway, or it may be that one receptor is now capable of sensitizing the other, rendering it more active or indeed activating it in a secondary manner. The fact that this phenomenon was not observed in full thickness tissue indicates the site of interaction was at mucosa but not smooth muscle.

Figure 4 – 12. Additive or potentiating effect of co-stimulation of ATP release using UTP (100μM) and GSK1016790A (1μM) in young and aging GP bladder tissue. Light grey bars: ATP release from Pre and post control interventions (figures 4-5 and 4-7) of UTP (and GSK separately) were averaged, then this average for UTP-induced ATP release was manually combined with that of GSK. This represents the total (sum of) ATP release evoked by these mechanisms when activated separately. Dark grey bars: represent the total ATP released when UTP and GSK are used simultaneously. In young GP tissues and aging FT, the combined ATP release is very similar to simultaneous, with no significant difference between the two, suggesting the effect of simultaneous stimulation is additive. In contrast, simultaneous activation in aging GP mucosa generated significantly more ATP release than combined ATP release from independent activation with these agonists. This suggests the effect of simultaneous stimulation is potentiating in aging GP mucosa. Data sets expressed as % medians [25%, 75%], p value shown, data sets compared using Mann-Whitney.
4.4.2.2 EFFECT OF PURINOCEPTOR AND TRPV4 ACTIVATION ON FUNCTIONAL OUTPUTS IN VARIOUS MOUSE MODELS

In order to identify the responsible purinoceptor subtype/s and dynamics underlying the apparent relationship between purinoceptor signalling and TRPV4 in the aging GP mucosa, young WT, young P2Y₂ knockout (P2Y₂KO) and aging P2Y₂KO mouse bladders were subjected to the same experimentation as above. As transgenic GPs are not permitted, these transgenic mice provide robust models for investigating the responsible subtypes.

TRPV4 expression was not evaluated in these mice as it has been well established in previous literature (Gevaert et al., 2007; Mochizuki et al., 2009; Yamada et al., 2009). Confirmation that knockout mice were void of P2Y₂ receptors was performed prior to the commencement of this study.

4.4.2.2.1 YOUNG WT MOUSE BLADDER

No contractions were observed in mucosa during any intervention. Both UTP (100μM) and GSK (1μM) evoked contractions in full thickness tissue, where those of GSK were significantly greater (Force μN/mg tissue - GSK; 193.5 [105.5, 607.5], UTP; 62.74 [25.12, 148.1]) (figure 4-13 A). The post UTP intervention was also capable of evoking a contraction of similar magnitude to that of pre UTP (Post UTP; 56.2 [7.9, 118.7]), however post GSK was not (Post GSK; 3.8 [-0.5, 14.04]). The joint intervention evoked contractions of similar magnitude to pre and post UTP (UTP+GSK; 81.84 [2.3, 239.3], and as post GSK could not simulate a contraction, the contractions observed during the joint intervention are likely attributable to the effect of UTP. Significant ATP release was generated by all interventions from both tissue preparations (see table 14), aside from post-UTP in both tissue preparations (figure 4-13 B). As with GP tissue, this is likely attributable to drift in tissue function over the period of the experiment. ATP release generated by UTP was not significantly different to GSK. ATP release generated by mucosa was not significantly different to full thickness tissue with any intervention. ATP release generated by UTP+GSK was significantly greater than that by GSK or UTP in mucosa and full thickness. This data will be compared to that from young P2Y₂KO and aging P2Y₂KO tissue and further analysed later.
Table 14 - Quartiles (median [25%, 75%]) of ATP release and force elicited by stated interventions in young WT mouse bladder tissues (referring to figure 4–13 B graph).

<table>
<thead>
<tr>
<th>Tissue strip</th>
<th>100μM UTP (pre)</th>
<th>1μM GSK (pre)</th>
<th>100μM UTP + 1μM GSK</th>
<th>1μM GSK (post)</th>
<th>100μM UTP (post)</th>
</tr>
</thead>
</table>

Figure 4–13. Effect of UTP and GSK1016780A on bladder functional outputs in young WT mouse bladder. WT mouse bladder strips challenged with stated drug interventions in sequence from left to right (for full protocol refer to sections 2.5.1.1.1 and 2.5.1.1.2). A: Median force of mucosal and full thickness (FT) contractions generated by drug interventions. No contractions observed in mucosa with any agonist (confirmed by traces). All interventions (except post-GSK) generated contractions in FT tissue, where that generated by 1μM GSK (TRPV4 agonist) was significantly greater than 100μM UTP (P2Y agonist). The contractions generated by pre- and post-UTP and UTP+GSK were all of similar magnitudes. B: Corresponding median ATP release from same bladder strips. All drug interventions significantly increase ATP release from basal levels in both mucosa and FT to a similar level, apart from post-UTP. The joint intervention (UTP+GSK) generates significantly higher ATP release than pre-UTP or pre-GSK alone in both mucosa (shown on graph) and FT (not shown). No significant difference in ATP release is observed between mucosa and FT. For both graphs: data expressed as % medians [25%, 75%], n=8, Wilcoxon’s, graph B *p<0.05, drug intervention vs. control (basal levels).
4.4.2.2 YOUNG P2Y₂KO MOUSE BLADDER

As with WT mouse, no contractions were observed in mucosa during any intervention. Both UTP (100μM) and GSK (1μM) evoked contractions in full thickness tissue, where those of GSK were significantly greater (Force μN/mg tissue - GSK; 167.2 [86.5, 198.6], UTP; 47.4 [27.2, 75.9]) (figure 4-14 A). The post UTP intervention was also capable of evoking a contraction of similar magnitude to that of pre UTP (Post UTP; 28.2 [6.9, 84.9]), however post GSK only evoked a contraction in 2 of the 8 strips (Post GSK; 9.1 [3.0, 47.7]). The joint intervention evoked contractions of similar magnitude to pre and post UTP (UTP+GSK; 56.9 [25.5, 139.9]), and as post GSK only stimulated a contraction in 25% of strips, these contractions are likely attributable to the effect of UTP. Significant ATP release was generated by all interventions from both tissue preparations (see table 15 and figure 4-14 B). ATP release generated by UTP was not significantly different to GSK. Unlike WT mouse, ATP released in response to the joint intervention (UTP+GSK) was not significantly different from those triggered by pre UTP or pre GSK, where all interventions generated a similar median release. ATP release generated by mucosa was not significantly different to full thickness tissue with any intervention. This data will be compared to that from young P2Y₂KO and aging P2Y₂KO tissue and further analysed later.

Table 15 - Quartiles (median [25%, 75%]) of ATP release and force elicited by stated interventions in young P2Y₂KO mouse bladder tissues (referring to figure 4 – 14 B graph).

<table>
<thead>
<tr>
<th>Tissue strip</th>
<th>100μM UTP (pre)</th>
<th>1μM GSK (pre)</th>
<th>100μM UTP + 1μM GSK (post)</th>
<th>1μM GSK (post)</th>
<th>100μM UTP (post)</th>
</tr>
</thead>
</table>
Figure 4 – 14. Effect of UTP and GSK1016780A on bladder functional outputs in young P2Y$_2$ KO mouse bladder. Young P2Y$_2$ mouse bladder strips challenged with stated drug interventions in sequence from left to right (for full protocol refer to sections 2.5.1.1.1 and 2.5.1.1.2). A: Median force of mucosal and full thickness (FT) contractions generated by drug interventions. No contractions observed in mucosa with any agonist (confirmed by traces). All interventions generated contractions in FT tissue, where that generated by 1μM GSK (TRPV4 agonist) was significantly greater than 100μM UTP (P2Y agonist). The contractions generated by pre- and post-UTP and UTP+GSK were all of similar magnitudes B: Corresponding median ATP release from same bladder strips. All drug interventions significantly increase ATP release from basal levels in both mucosa and FT to a similar level, with no loss of effect in post-interventions. No significant difference in ATP release is observed between mucosa and FT or between different drug interventions. For both graphs: data expressed as % medians [25%, 75%], n=8, Wilcoxon’s, graph B *p<0.05, drug intervention vs. control (basal levels).
4.4.2.2.3 AGING P2Y₂KO MOUSE BLADDER

Unfortunately, it was only possible to maintain 5 P2Y₂KO mice for the time necessary to reach ‘aging’ criteria (>10 months old), however these 5 provided an initial indication of any age-dependent changes to TRPV4 and purinoceptor-mediated bladder outputs.

Again, no contractions were observed in mucosa during any intervention. Both UTP (100μM) and GSK (1μM) evoked contractions in full thickness tissue, however these were not significantly different, unlike with the other 2 groups where GSK-evoked strong contractions (Force μN/mg tissue - GSK; 123.9 [35.61, 146.0], UTP; 55.3 [41.3, 62.9])(figure 4-15 A). Post UTP evoked weak contractions (Force μN/mg tissue >25, <80) in 3 of the 5 full thickness strips assessed; with lower magnitudes to those of pre UTP (Post UTP; 25.3 [2.8, 63.3]) and post GSK also only evoked contractions in 3 of the 5 strips, and these were weaker still (>15, <40)(Post GSK; 15.7 [2.0, 34.4]). The joint intervention evoked contractions of similar magnitude to pre and post UTP (UTP+GSK; 36.5 [9.4, 50.6]), and as such, these contractions are likely attributable to the effect of UTP. ATP release was generated by all interventions from both tissue preparations (see table 16 and figure 4-15 B), where an increase in ATP release from basal levels was observed in at least 4 of the 5 strips assessed for each intervention. Only 5 bladders were assessed, such that statistics are weak and therefore, these results only provide an indication of trends. As with young P2Y₂KO, median ATP release appears to be similar during all interventions, however the variations in ATP release are much larger here. As with all mouse models investigated, relative ATP release between mucosa and full thickness are also similar.

<table>
<thead>
<tr>
<th>Tissue strip</th>
<th>100μM UTP (pre)</th>
<th>1μM GSK (pre)</th>
<th>100μM UTP + 1μM GSK</th>
<th>1μM GSK (post)</th>
<th>100μM UTP (post)</th>
</tr>
</thead>
</table>

Table 16 - Quartiles (median [25%, 75%]) of ATP release and force elicited by stated interventions in Aging P2Y₂KO mouse bladder tissues (referring to figure 4 – 15 B graph).
Figure 4 – 15. Effect of UTP and GSK1016780A on bladder functional outputs in aging P2Y$_2$ KO mouse bladder. Aging P2Y$_2$ mouse bladder strips challenged with stated drug interventions in sequence from left to right (for full protocol refer to sections 2.5.1.1.1 and 2.5.1.1.2). A: Median force of mucosal and full thickness (FT) contractions generated by drug interventions. No contractions observed in mucosa with any agonist (confirmed by traces). All interventions generated weak contractions in FT tissue. Note, Y-axis for figures 4-13, 4-14 and 4-15 have the same scale for quick comparisons. B: Corresponding median ATP release from same bladder strips. All drug interventions stimulated ATP release from the tissue with a p value of 0.0625, therefore indicating significance would likely be reached with further n numbers. These data provide an indication of these functional outputs in aging mice for comparison with the other two previous groups. For both graphs: data expressed as % medians [25%, 75%], n=5, Wilcoxon’s, drug intervention vs. control (basal levels).
4.4.2.2.4 COMPARISON OF BLADDER OUTPUTS BETWEEN MOUSE GROUPS: CONTRACTILITY

Mucosal contractions were not evoked by any intervention in any of the mouse groups investigated and as such, no comparison is necessary. However, contractions of varying magnitude were elicited by drug interventions in full thickness strips from each group (figure 4-16). Those evoked by pre UTP (100μM) were of similar magnitude for all groups (median force ≈60μN/mg tissue). The contractions evoked by pre GSK (1μM) were not significantly different between groups, however these appear to be weaker in aging P2Y2KO mice. The contractions evoked by UTP+GSK were similar in all groups, where UTP is the agent responsible for these contractions (discussed previously for each group). Post GSK evoked negligible contractions and post UTP evoked contractions of similar magnitude to other UTP interventions and these were not dissimilar between groups.

Figure 4 – 16. Contractions generated by UTP and GSK1016790A in young WT, young P2Y2 KO & aging P2Y2 KO mouse full thickness bladder strips. This data is taken from the previous contraction graphs from the various mouse models (graphs A in figures 4-13, 4-14 and 4-15) and organised to allow clear comparison between groups. No difference observed between groups for contractions generated by drug interventions. However, WT tissue seems to contract with greater magnitude during drug interventions than P2Y2 mice and the aging P2Y2 tissue seems to only be capable of weak contractions. Young WT; n=8, Young P2Y2 KO n=8, Aging P2Y2 KO n=7. Data sets are expressed as % medians [25%, 75%], * p ≤0.05, compared using Mann-Whitney.
COMPARISON OF BLADDER OUTPUTS BETWEEN MOUSE GROUPS: ATP RELEASE

There was no significant difference in ATP release observed between mouse models for any intervention in either mucosal or full thickness tissue, apart from during the joint intervention (figure 4-17). UTP+GSK evoked significantly higher ATP release in young WT than in young P2Y2KO mucosa and full thickness tissue, highlighting this as the key difference between these 2 groups (relative ATP release during UTP+GSK - WT mucosa; 652% [505%, 1039%], P2Y2KO mucosa - 301% [190%, 351%], WT full thick; 481% [425%, 660%], P2Y2KO full thick - 402% [235%, 460%]). The data was then analysed in the same way as for the GP comparison (see section 4.4.2.1.5), where the ATP released during the joint intervention (UTP+GSK) is compared to the manually combined ATP release from the separate UTP and GSK interventions.

As with young GP mucosa, the effect of activating TRPV4 and P2Y2/P2Y4 simultaneously in young WT mouse mucosa, evokes the same proportional increase in ATP release as activating them individually and combining the two (manually combined individual activations: 533% [473%, 659%], simultaneous activation: 652% [505%, 1039%])(figure 4-18). Again, this implies the effect of activating these receptors simultaneously is additive, suggesting they have no interaction and mediate ATP release via different mechanisms.

This was also observed in young WT mouse full thickness tissue. However, knockdown of P2Y2 in young mouse mucosa causes this additive effect to be lost, where the ATP released during simultaneous activation is significantly less than that of the manually combined releases, and is similar to that of each individual UTP or GSK intervention (manually combined individual activations: 538% [467%, 691%], simultaneous activation: 301% [190%, 351%], averaged pre/post UTP interventions: 347% [300%, 446%], averaged pre/post GSK interventions: 235% [176%, 250%]). This effect caused by knockdown of P2Y2 was also observed in young P2Y2KO full thickness tissue (manually combined individual activations: 1017% [935%, 1538%], simultaneous activation: 402% [235%, 460%], averaged pre/post UTP interventions: 538% [350%, 747%], averaged pre/post GSK interventions: 264% [185%, 333%]).

As P2Y2 is absent, these results imply that P2Y4 and TRPV4 mediate ATP release via the same mechanism and therefore when activated simultaneously, their signalling pathways compete for this mechanism and as such, can only elicit an ATP release equaling that of
either of their individual effects only. The ATP release in aging P2Y$_2$ tissue is highly variable, a phenomenon previously observed in aging GP tissue also (Sui et al., 2014), and with the low n numbers, this makes comparisons and conclusions difficult here. Due to these variations, many additional aging P2Y$_2$KO bladders must be assessed before reliable conclusions can be drawn, as currently the data is insufficient to reveal any trend. These results suggest that P2Y$_2$ is necessary for the additive ATP release observed with simultaneous P2Y and TRPV4 activation. The large variations in the ability to release ATP in individual mice in older age group may reflect the ageing process.
Figure 4 – 17. Effect of UTP and GSK1016780A on ATP release from various mouse models. These graphs allow for clear comparison of ATP release between young WT, young P2Y2 KO and aging P2Y2 KO mouse bladder mucosa and full thickness (FT) strips, triggered by the stated drug interventions (data is taken from ATP release graphs (B) in figures 4-13, 4-14 and 4-15, but arranged for comparative purposes). A: Pre-100μM UTP intervention – increased ATP release in both mucosa and FT from young WT (YWT), young P2Y2 KO (YP2Y2) and aging P2Y2 KO (AP2Y2) bladders. No difference between groups. B: Pre-1μM GSK intervention – increased ATP release in mucosa and FT from all groups. No difference between groups. C: 100μM UTP + 1μM GSK intervention – increased ATP release in both mucosa and FT from all groups. This simultaneous drug intervention generated significantly greater ATP release from YWT mucosa and FT than YP2Y2 (data sets compared using Mann-Whitney test, p value shown). D: Post-1μM GSK intervention – increased ATP release in mucosa and FT from all groups. No difference between groups. E: Post-100μM UTP intervention – increased ATP release in mucosa and FT in YP2Y2 tissue only. No difference between age groups. Significance was not reached in AP2Y2 for any intervention as it was only possible to assess 5 bladders. All graphs: data expressed as % medians [25%, 75%], (WT young; n=8, P2Y2 KO young; n=8, P2Y2 KO aging; n=5) Wilcoxon’s, *p<0.05, drug intervention vs. control (basal levels). All age groups compared using Mann-Whitney test e.g. WT mouse – young vs. P2Y2 mouse – aging.
Additive or potentiating effect of co-stimulation of ATP release with UTP (100μM) and GSK1016790A (1μM) in young WT, young P2Y2 KO & aging P2Y2 KO mouse bladder tissues. Light grey bars: ATP release from Pre and post control interventions (figures 4-13, 4-15 and 4-15) of UTP (and GSK separately) were averaged, then this average for UTP-induced ATP release was manually combined with that of GSK. This represents the total (sum of) ATP release evoked by these mechanisms when activated separately. Dark grey bars: represent the total ATP released when UTP and GSK are used simultaneously. In young WT and aging P2Y2KO tissues, the combined ATP release is similar to simultaneous in both tissue preparations, with no significant difference between the two, suggesting the effect of simultaneous activation is additive. In contrast, simultaneous activation in young P2Y2KO tissues generated significantly lower ATP release than combined ATP release from independent activation of these receptors. This suggests the mechanism underlying the ATP release pathway triggered by TRPV4 or P2Y activation in this model may be shared. Young WT; n=8, Young P2Y2 KO n=8, Aging P2Y2 KO n=7. Data sets are expressed as % medians [25%, 75%], data sets compared using Wilcoxon’s, p value shown.
4.4.2.2.6 EFFECT OF PRE-ACTIVATION OF ONE RECEPTOR GROUP ON ACTIVITY OF THE OTHER

The following experiments were designed to further examine the relationship between TRPV4 and P2Y receptors and identify the nature of their cooperative properties that allow modulation of ATP release during simultaneous activation. The experimental design is described in section 4.3.1 of this chapter.

4.4.2.2.6.1 EFFECT OF PRE-ACTIVATION OF P2Y RECEPTORS ON TRPV4-MEDIATED ATP RELEASE: WILD TYPE MOUSE

Both pre and post controls of GSK significantly enhanced ATP release from mucosa and full thickness strips (figure 4-19). After 1 hour recovery, UTP significantly enhanced ATP release, after which GSK was also introduced, which significantly enhanced ATP release above that generated by UTP, and when GSK was removed leaving only UTP, ATP release dropped significantly to similar levels during initial UTP (% ATP from basal control: mucosa – UTP pre-activation; 291 [214, 437], UTP+GSK; 446 [410, 508], UTP post-activation; 258 [193, 339], full thick; - UTP pre-activation; 317 [237, 433], UTP+GSK; 502 [422, 666] UTP post-activation; 351 [324, 401]). This suggests that TRPV4 and P2Y2/P2Y4 receptors can mediate ATP release independent of the activity of the other in WT bladder.

4.4.2.2.6.2 EFFECT OF PRE-ACTIVATION OF P2Y RECEPTORS ON TRPV4-MEDIATED ATP RELEASE: P2Y2KO MOUSE

It was only possible to assess 5 bladders here, and as such data provide good initial inferences. As with WT mouse, both pre and post controls of GSK enhanced ATP release from mucosa and full thickness strips (figure 4-20). Again, after 1 hour recovery, UTP enhanced ATP release, after which GSK was also introduced. However in this case, GSK did not raise ATP release beyond that already generated by UTP in either tissue preparation (% ATP from basal control: mucosa - UTP pre-activation; 492 [351, 549], UTP+GSK; 619 [321, 684], full thick; UTP pre-activation; 649 [311, 1100], UTP+GSK; 619 [337, 1338]). This suggests that TRPV4 relies on the same mechanism as P2Y4 receptors for ATP release, as P2Y2 is knocked down here. Despite only 5 bladders being assessed, all interventions enhanced ATP release from control levels in all 5 bladders (where direct calculation of the probability gives significant p values of 0.0313), and GSK only raised ATP beyond that of UTP in 40% of bladders here, compared to 90% in WT, providing strong evidence for this conclusion.
4.4.2.6.3 EFFECT OF PRE-ACTIVATION OF TRPV4 RECEPTORS ON P2Y-MEDIATED ATP RELEASE: P2Y₂KO MOUSE

To confirm the above finding, the same protocol was followed but the order of agonists was switched, such that TRPV4 receptors were pre-activated, followed by subsequent, additional activation of P2Y₄. This determines whether P2Y₄ receptors solely rely on the same mechanism as TRPV4 for ATP release, or are able to utilize a second mechanism when their favoured, original mechanism is already being utilized by TRPV4 signalling. These experiments were performed in bladder strips taken from the same 5 P2Y₂KO mice as used for the previous experiment.

Both pre and post controls of UTP enhanced ATP release from mucosa and full thickness strips (figure 4-21). Following 1 hour recovery, GSK enhanced ATP release, after which UTP was also introduced. As with the previous experiment, UTP was incapable of raising ATP release beyond that already generated by GSK in either tissue preparation (% ATP from basal control: mucosa - UTP pre-activation; 302 [227, 372], UTP+GSK; 348 [229, 455], full thick; - UTP pre-activation; 206 [213, 342], UTP+GSK; 225 [209, 480]. This suggests that TRPV4 and P2Y₄ receptors rely solely on the one mechanism for ATP release.

Figure 4 – 19. Effect of GSK (TRPV4 agonist) on ATP release after pre-treatment with UTP (P2Y agonist) in young WT mouse bladder. A: Mucosal tissue strips. B: Full thickness tissue strips. Both pre- and post-control interventions of 1μM GSK increased ATP release from basal levels (control). During the middle ‘intervention’ period, 100μM UTP increased ATP release from control; introduction of GSK within this period caused a significant rise in ATP release above that already stimulated by UTP, followed by a significant decrease of ATP upon removal of GSK (‘UTP+GSK’ compared to ‘UTP’ using Wilcoxon’s). Data expressed as % Medians [25%, 75%], n=8, Wilcoxon’s, *p<0.05, **p<0.01, drug intervention vs. control (basal levels).
Figure 4 – 20. Effect of GSK (TRPV4 agonist) on ATP release after pre-treatment with UTP (P2Y agonist) in young P2Y2 KO mouse bladder. A: Mucosal tissue strips. B: Full thickness tissue strips. Both pre- and post-control interventions of 1μM GSK increased ATP release from basal levels (control). During the ‘intervention’ period, 100μM UTP increased ATP release from control; however introduction of GSK within this period did not cause a significant rise in ATP release above that already stimulated by UTP (‘UTP+GSK’ compared to ‘UTP’ using Wilcoxon’s). Data expressed as % Medians [25%, 75%], n=5, Wilcoxon’s, drug intervention vs. control (basal levels).

Figure 4 – 21. Effect of UTP (P2Y agonist) on ATP release after pre-treatment with GSK (TRPV4 agonist) in young P2Y2 KO mouse bladder. A: Mucosal tissue strips. B: Full thickness tissue strips. Both pre- and post-control interventions of 100μM UTP increased ATP release from basal levels (control). During the ‘intervention’ period, 1μM GSK increased ATP release; however introduction of UTP within this period did not cause a significant rise in ATP release above that already stimulated by GSK (‘GSK+UTP’ compared to ‘GSK’ using Wilcoxon’s). Data expressed as % Medians [25%, 75%], n=5, Wilcoxon’s, drug intervention vs. control (basal levels).
In order to further dissect the pathways and receptor subtypes responsible for mucosal ATP release, the role of P2Y<sub>6</sub> was investigated. The P2Y<sub>6</sub> agonist UDP (20μM) evoked ATP release from mucosal and full thickness strips from WT mouse bladders with similar effectiveness to UTP (% increase in ATP release from basal levels: mucosa – UTP; 426 [300, 638], UDP; 334 [208, 573] full thick – UTP; 458 [265, 944], UDP; 256 [185, 521])(figure 4-22 B). UTP evoked contractions in full thickness tissue whereas UDP did not (force μN/mg tissue: UTP; 63 [23, 217], UDP; 5 [1, 21])(figure 4-22 A). Neither agonist evoked contractions in mucosa (force μN/mg tissue: UTP; 29 [15, 36], UDP; 9 [6, 19]. This suggests a functional role for P2Y<sub>6</sub> in urothelial ATP release.

To confirm the P2Y receptor subtypes activated by UTP, the effect of P2Y<sub>6</sub> blockade on UTP-mediated ATP release was evaluated using the P2Y<sub>6</sub> specific antagonist MRS2578 (MRS)(figure 4-23). MRS (10μM) had no effect on UTP-mediated ATP release (% increase in ATP release from basal levels: mucosa – UTP; 417 [283, 480], UTP W/ MRS; 342 [166, 641] full thick – UTP; 323 [253, 664], UTP W/ MRS; 250 [173, 386]). This indicated that none of the ATP release evoked by UTP is mediated by P2Y<sub>6</sub>, confirming the effect of UTP is likely attributable to P2Y<sub>2</sub> and P2Y<sub>4</sub> only.
Figure 4 – 22. Effect of purinoceptor agonists on ATP release and contractile activity in WT mouse bladder tissues. A: Median force of mucosal and full thickness (FT) contractions generated by either UTP (P2Y$_2$/P2Y$_4$ agonist, 100µM) or UDP (P2Y$_6$ agonist, 20µM). Agonists generated negligible contractions in mucosa (confirmed by traces). UTP evoked contractions in full thickness tissue whereas UDP did not. B: Both UTP and UDP evoked significant ATP release from mucosa and full thickness tissue with similar effectiveness. For both graphs: data expressed as % medians [25%, 75%], n=7, Wilcoxon's, *p<0.05, **p<0.01, drug intervention vs. control (basal levels). Effect of UTP compared to effect of UDP using Mann-Whitney.

Figure 4 – 23. Effect of P2Y$_6$ blockade on UTP-mediated ATP release from WT mouse bladder tissues. Blockade of P2Y$_6$ receptors using MRS2578 (10µM) had no effect on UTP-mediated ATP release from mucosa or full thickness tissue strips. For both graphs: data expressed as % medians [25%, 75%], n=7, Wilcoxon's, *p<0.05, **p<0.01, UTP pre/post average vs. UTP W/ MRS.
4.4.2.3 EFFECT OF TRPV4 AND PURINOCEPTOR ACTIVATION ON FUNCTIONAL OUTPUTS IN THE NORMAL AND OVERACTIVE AGING HUMAN BLADDER

The previous experiments assessing the individual and joint effects of P2Y and TRPV4 receptor activation on bladder ATP release were also applied to both normal and overactive human bladder mucosa from older patients (normal: Male, >65, OAB: Female >45). Unfortunately, only 4 OAB biopsies were collected and as such, these results provide only an indication of mucosal behaviour in overactive bladders.

4.4.2.3.1 BASAL ATP RELEASE

Interestingly, a higher basal ATP release was observed in mucosa from overactive bladders. Figure 4-24 shows the basal ATP release during the first 3 interventions performed on each biopsy. This was approximately 10-fold higher in OAB positive samples during the first intervention (pmolar ATP release: OAB -ve; 48 [109, 23], OAB +ve; 463 [448, 808]), and 15-fold higher during the second 2 (pmolar ATP release: intervention 2 - OAB -ve; 34 [17, 64], OAB +ve; 778 [612, 1174] intervention 3 - OAB -ve; 28 [14, 55], OAB +ve; 778 [296, 969]), presumably due to activation of tissue by the first intervention. As physiological basal ATP release in human tissue is minimal, a ten-fold increase in basal ATP release could have profound effects on bladder function and may be partly responsible for the overactive phenotype.

4.4.2.3.2 TRPV4-INDUCED ATP RELEASE

The effect of TRPV4 activation on ATP release was characterised in both cohorts. Figure 4-25 A shows comparison of the effect of GSK (1μM) on ATP release from normal and overactive bladder mucosa. Significant ATP release is generated with a delay in normal aging bladder mucosa, with onset at 3 minutes, rising until a peak at 10 minutes, after which the effect is lost. Comparatively, GSK appears to elicit ATP release after only 1 minute from OAB positive mucosa, where the effect is lost after 3 minutes. Specifically, GSK enhanced ATP release from basal levels in all 4 OAB samples at 1 and 3 minutes and at 5 minutes in 3 of the 4 samples. However, the median values for peak ATP release are lower in OAB samples (% ATP release from basal levels: OAB -ve - 1min; 132 [70, 157], 3min; 173 [132, 319], 5min; 214 [126,441], 10min; 276 [149, 467], 20min; 134 [97,236], OAB +ve - 1min; 197 [193, 217], 3min; 179 [132,228], 5min; 170 [105, 208], 10min; 135 [119, 137], 20min; 151 [118, 182]). Therefore, these data indicate that TRPV4 is able to elicit ATP release from
mucosa of both normal and overactive human bladder mucosa, however the dynamics of release may differ slightly, with an earlier but diminished release in OAB samples. Expressing the data in this way (as a percentage increase from basal levels) indicates the proportionate drug effect of GSK on ATP release is similar in both cohorts. However, expressing ATP release as pmoles/g tissue/min revealed that, like basal ATP release, the quantity of TRPV4-induced ATP release was much higher from the mucosa of overactive bladders (pmolar ATP release: OAB –ve – basal; 35 [24, 40], GSK; 320 [241, 433], OAB +ve - basal; 656 [479, 1254], GSK; 1072 [735, 1854])(figure 4-25 B).

Figure 4 – 24. Basal ATP release levels in human bladder mucosa biopsies. Mucosal biopsies from overactive bladders release significantly higher basal levels of ATP than bladders without OAB symptoms. Basal ATP was measured during the Tyrode’s pre and post control for the first 3 interventions on the day of each experiment and averaged, providing the values represented by bars. ATP release expressed as pmoles/g tissue/min medians [25%, 75%], OAB negative; n=11, OAB positive; n=4, Mann-Whitney, p values shown, OAB positive vs. OAB negative.
Figure 4 – 25. Effects of TRPV4 activation on aging human bladder biopsies with or without OAB symptoms. A: Effect of 1μM GSK on ATP release from mucosal bladder biopsies from patients without OAB symptoms (male, >65, n=10, light grey bars) compared to mucosal bladder biopsies with OAB symptoms (female, >45, n=4, dark grey bars). Only 4 OAB bladders were assessed such that significant statistics are not possible from this cohort (p values stated). However, data indicates significantly higher ATP release from non-OAB patients at 10minutes. Data sets expressed as % medians [25%, 75%]. OAB negative; n=10, OAB positive; n=4, * p ≤0.05, ** p ≤0.01, or p value stated, Wilcoxon’s, drug intervention vs. control. Data sets compared using Mann-Whitney, OAB negative vs. OAB positive. B: Effect of 1μM GSK on mucosal ATP release expressed as pmoles/g tissue/min as opposed to a percentage increase from basal levels. ATP release across the 20minute period of exposure to GSK was averaged. Data indicates that GSK-induced ATP release is higher in the mucosa from patients with overactive bladders compared to those without overactive bladders. As only 4 OAB patients were assessed, data provide only an indication of trend, where significance is not yet possible. ATP release expressed as pmoles/g tissue/min medians [25%, 75%]. OAB negative; n=10, OAB positive; n=4, Wilcoxon’s, p values shown, Basal vs. GSK. Comparison between groups is not possible due to low n numbers.
4.4.2.3.3 CO-ACTIVATION OF TRPV4 AND PURINOCEPTORS

The protocol designed to assess the effect of joint activation of P2Y and TRPV4 receptors was then applied (section 2.5.1.1.2 – interaction protocol). No contractions were observed, as these biopsies were composed of mucosal tissue only (small tears of smooth muscle were attached to a small number of biopsies, however this was not enough to elicit contractions) and as such, no graph of contractility is shown. Significant ATP release was generated by all interventions from normal mucosa (see table 17), however, due to low n numbers, ATP release from OAB samples was not significant (figure 4-26). However, ATP was elevated by all interventions in the OAB samples in all 4 of the biopsies, resulting in the p value of 0.125 shown. The ATP releases generated by different interventions were not significantly different from one another.

Table 17 - Quartiles (median [25%, 75%]) of ATP release and force elicited by stated interventions in aging GP bladder tissues (referring to figure 4 – 26 graph).

<table>
<thead>
<tr>
<th>ATP release (% of averaged controls)</th>
<th>Cohort</th>
<th>100μM UTP (pre)</th>
<th>1μM GSK (pre)</th>
<th>100μM UTP + 1μM GSK</th>
<th>1μM GSK (post)</th>
<th>100μM UTP (post)</th>
</tr>
</thead>
</table>
This data was then analysed in the same way as for the animal models, comparing the combined, separate effects of UTP and GSK with the simultaneous effect (figure 4-27). Referring to figure 4-27 B, in normal (OAB negative) human mucosa, activating TRPV4 and P2Y<sub>2</sub>/P2Y<sub>4</sub> simultaneously evokes the same proportional increase in ATP release as activating them individually and combining the two (manually combined individual activations: 509% [402%, 760%], simultaneous activation: 595% [331%, 1504%]). This was also observed in young GP and young WT mouse models and implies the effect of simultaneous receptor activation is additive, suggesting they have no interaction and mediate ATP release via different mechanisms. This was also observed in the 4 OAB positive biopsies assessed thus far (manually combined individual activations: 335% [244%, 378%], simultaneous activation: 322% [209%, 437%]). Interestingly, when observing the individual trends for each individual experiment (figure 4-27 A), 2 of the 11 normal biopsies examined showed a considerable increase in ATP release when the receptor groups were
activated together, similar to that of the aging GP. It is possible therefore, that these aging human bladders had similar properties to those of the aging GP, resulting in potentiated ATP release during simultaneous activation. This may identify a further age-related change in bladder properties that may result in altered function.

Figure 4 – 27. Effect of co-stimulation of ATP release with UTP (100μM) and GSK1016790A (1μM) in human bladder mucosa biopsies. For all graphs: UTP+GSK groups: ATP release from Pre and post control interventions (from figure 4-26) of UTP were averaged, then this average for UTP-induced ATP release was manually combined with the average of GSK. This represents the total (sum of) ATP release evoked by both of these mechanisms when activated separately. UTP W/ GSK groups: represent the total ATP released when UTP and GSK are used simultaneously. A: Individual paired data from each mucosal biopsy revealing the individual trend of each experiment. B: Averaged ATP release. No significant difference between ATP released from simultaneous drug intervention compared to combined single-drug interventions. OAB negative cohort; 9 male patients with suspected or known tumours, >65 years of age. OAB positive cohort; 4 female patients with OAB symptoms, >45 years of age. Data expressed as % medians [25%, 75%], Wilcoxon’s, *p<0.05, simultaneous drug intervention vs. combined single-drug interventions.
4.5 DISCUSSION

The purpose of this chapter was to identify any age-related changes in the expression or function of TRPV4 receptors in the bladder. Findings revealed that not only is TRPV4 expression up-regulated in the aging bladder, but a novel relationship with P2Y receptors appears to emerge, where co-activation generates an amplified ATP release from the mucosa.

4.5.1 TRPV4 RECEPTOR EXPRESSION IS INCREASED WITH AGING

Results from IHC of young and aging GP bladder revealed similar expression profiles in both age groups, with highest TRPV4 expression in the urothelium and lower levels in both the suburothelium and DSM. The bladder sections investigated for this purpose were examined on different days across a period of months, where confocal imaging settings were optimised for each set of sections (per bladder; 1x secondary control, 3x positive). As such, comparison of TRPV4 expression levels between age groups is not possible, as settings were altered to account for background and secondary control fluorescence. However, this technique provides important information regarding the tissue specific expression of TRPV4 across the bladder wall, which is not possible with other techniques such as western blotting. Following from this, western blotting was employed to reliably confirm any differences in the expression levels of TRPV4 between age groups. Indeed, results revealed a 8-fold higher expression in separated mucosa and 16-fold higher in DSM. Taken together, and with findings from the previous chapter, these results indicate that; 1. the relative expression of TRPV4 between tissues remains similar with aging (IHC; urothelium > suburothelium and DSM, western; mucosa > smooth muscle) 2. However, the overall expression levels are greatly increased. Other studies have identified plasticity in the expression of other key receptors in the bladder that occur with both age and pathology. Changes in expression of bradykinin and muscarinic receptors and connexin and Ca\(^{2+}\)-activated K and Cl channels have been identified in overactive and obstructed bladders, pathologies both strongly associated with age (Chopra et al., 2005; Haefliger et al., 2002; Li et al., 2008; Somogyi and de Groat, 1999). In addition, IC has been shown to increase the expression of purinoceptors in the feline (Birder et al., 2004) and human bladders (Tempest et al., 2004), where patients suffering from IC also exhibit augmented stretch evoked ATP release (Sun and Chai, 2006). A further study demonstrated changes in purinoceptor and muscarinic receptor expression (between 2-6 fold reduction) and altered urothelial and DSM function as a direct result of aging in the mouse bladder (Daly et al., 2014). These findings
highlight how sensitive the mucosa is to pathology and aging with respect to expression of key receptors. As such, the findings in this chapter are in agreement with this and provide further important evidence, reporting for the first time a significant increase in TRPV4 expression as a direct result of aging.

4.5.2 CO-ACTIVATION OF TRPV4 AND P2Y RECEPTORS – EMERGENCE OF A FUNCTIONAL LINK

4.5.2.1 YOUNG AND AGING GP MODELS

There is good evidence to suggest that the urothelium is the principal source of ATP in the bladder, the release of which is primarily induced by stretch during bladder filling (Ferguson et al., 1997; Kumar et al., 2010, 2004; Sui et al., 2014). This process is crucial for physiological bladder function and relies on a multitude of receptors and pathways. Both TRPV4 and P2Y receptors, among others, have been strongly implicated in this process. UTP-induced ATP release has been previously demonstrated in GP mucosal sheets (Sui et al., 2014) and from urothelial cell lines (Chopra et al., 2008; Mansfield and Hughes, 2014), and TRPV4-associated ATP release has been extensively evidenced both in the previous chapter and in the literature. Owing to the apparent importance of these receptors in urothelial ATP release, it was hypothesised that a functional relationship may exist between the two receptor groups, capable of influencing the properties of urothelial ATP release. As such, the effect of co-activation of these receptors was investigated in both young and aging bladders.

4.5.2.1.1 PURINOCEPTOR AND TRPV4 MEDIATED ATP RELEASE

Both GSK (TRPV4 agonist) and UTP (P2Y2/P2Y4 agonist) evoked ATP release from the bladder, the main source of which was identified as the mucosa. Aging had no effect on the ATP released from bladder tissues in response to either GSK or UTP individually, where the quantities of ATP released were similar. In both mucosa and full thickness tissue from young GP, co-activation of these receptors generated an additive ATP release (i.e. quantity of ATP released by co-activation = separate TRPV4-induced ATP + P2Y-induced ATP). This implies that both receptor groups utilize unrelated pathways for ATP release (figure 4-29 B), as there is no less ATP release than the combined individual releases, which would imply competition of the ATP release pathway, or no more ATP release beyond the combined, which would imply a co-dependent amplification of ATP release signal. Additionally, levels of ATP released through P2Y activation were similar to that of TRPV4, possibly implying the
actual mechanisms for release are similar for each receptor group, even if the pathways are mutually exclusive.

Co-activation in aging mucosa resulted in an amplified or potentiated ATP release, beyond that of the combined individual effects. This amplified release during co-activation was only observed in the mucosa, where the effect was additive in aging full thickness tissue. The difference between co-activated release and the combined individual releases was completely attributable to a markedly higher ATP release during the co-activation intervention and not due to lower ATP release from either of the individual interventions (confirmed by a significantly higher release during the co-activation/joint intervention in mucosa compared to full thickness). Co-activation also generated a higher ATP release in the majority of aging mucosal strips compared to young mucosal strips (figure 4-28). These results show that the individual or combined effects of P2Y and TRPV4 receptor activation are similar in aging and young GP bladder tissue, apart from when the receptors are activated together in separated aging GP mucosa, where a much higher ATP release is observed, identifying this particular situation as the trigger for amplified ATP release. This co-activated amplification of ATP release only occurred in aging tissue, implying the need for an age-dependent change in tissue function, possibly in the form of a functional interaction between the two receptor groups that emerges with aging. The amplified ATP signal was not observed in full thickness aging tissue, implying that the tissues underlying the mucosa possess some modulatory control over ATP release from the mucosa. A negative feedback mechanism for limitation of urothelial ATP release must exist in some form, as ATP can evoke further ATP release via P2Y receptors (ATP-induced ATP release) and therefore without negative feedback ATP release would be continually amplified. Indeed, AMP and adenosine, breakdown products of ATP, have both been shown to inhibit mucosal ATP release, suggesting this as the primary negative feedback mechanism (Dunning-Davies et al., 2013; Mansfield and Hughes, 2014). This feedback is therefore highly influenced by the ectonucleotidases responsible for breakdown of ATP to AMP and adenosine. Eight members of the ectonucleoside triphosphate diphosphohydrolase (NTPDase) family have been identified in the mouse bladder, where their expression is highly cell specific. NTPD1, NTPD2, NTPD3, NTPD8 and NT5E subtypes catalyze the step-wise conversion of extracellular ATP to adenosine. However, expression is not ubiquitous; NTPD1 in cells of the lamina propria and DSM, NTPD2 in lamina propria cells adjacent to the DSM, NTPD3 and 8 in the urothelium only, and NT5E exclusively in the DSM, with the localization of others unidentified (Yu et al., 2011). As such, coordination between these enzymes governs the availability of nucleotide ligands for purinoceptors. The activity of
these enzymes will therefore influence AMP and adenosine-mediated inhibition of ATP release. Perhaps ectonucleotidase expression and function is altered with age, as indeed a reduced econucleotidase activity has been demonstrated in human DO bladders (Harvey et al., 2002). In the young GP the subtypes present in the mucosa may be sufficient to modulate ATP release via breakdown of ATP to AMP and adenosine in the absence of underlying tissues. However, ectonucleotidase activity and expression may be altered in the aging GP, such that the subtypes present in the mucosa cannot produce the AMP and adenosine necessary for negative feedback of mucosal ATP release. This would result in the augmented ATP release observed in aging GP mucosa when the underlying tissues have been removed. However in-depth examination of the expression of enzyme subtypes in aging bladders is needed to support his hypothesis.

In aggregate, these findings suggest that a mechanism for excessive ATP release mediated by co-activation of P2Y and TRPV4 receptors develops in the aging bladder, which has the potential to come into effect if regulatory mechanisms, likely in the form of negative feedback pathways imposed by the underlying tissues, are disrupted. As previously discussed, the urothelium is highly susceptible to age-related factors such as inflammation, injury, pathological insult, hypoxia and ischemia, all of which can damage and alter urotheial signalling properties. As such, it is not unreasonable to presume that some of these age-related factors are capable of causing a malfunction in bladder signalling that would permit this exaggerated ATP release, possibly through altered ectonucleotidase activity, which would in turn have considerable consequences on bladder function. A potential mechanism is discussed in section 4.5.2.4.1 after the effects in mouse models have been considered.

Figure 4 – 28. ATP release evoked by co-activation of TRPV4 and P2Y receptors in young and aging GP mucosa. Dot plot showing individual ATP release values for each young and aging bladder assessed. A greater proportion of aging bladders released higher levels of ATP in response to co-activation than their younger counterparts, reflected by a higher median (black bars). Data expressed as individual data points with % medians (black bars).
4.5.2.1.2 PURINOCEPTOR AND TRPV4 MEDIATED CONTRACTIONS

Weak contractions were generated by UTP in approximately 35% of mucosal strips examined in both young and aging GP. A contractile effect has previously been demonstrated by UTP on mucosa and this was attributed to suburothelial myofibroblasts, which contain contractile proteins and express P2Y$_2$ receptors, thus enabling them to mediate contraction (Sui et al., 2014). If P2Y$_2$ is indeed the responsible subtype, results suggest P2Y$_2$ function in these cells is unaltered by age. TRPV4 agonist GSK was unable to evoke contractions in young mucosal strips, however generated weak contractions in 50% of aging mucosal strips. It is possible that TRPV4 expression is also up-regulated in suburothelial myofibroblasts with age and this results in emergence of a contractile function. TRPV4 is indeed expressed by myofibroblasts in the lungs and activity of these receptors is up-regulated under pathological conditions (pulmonary fibrosis), supporting this finding (Rahaman et al., 2014).

UTP is a weak agonist for P2X1 & P2X3 receptors and therefore any small contractions observed in full thickness tissue are likely in part due to direct activation of DSM via these receptors (Chen et al., 1995; Robertson et al., 1996). However, P2Y activation by UTP only evoked minimal contractions in full thickness bladder strips from young or aging GP bladders. It is likely a proportion of this is due to direct action of UTP on DSM and the remainder possibly due to a weak paracrine effect of UTP-induced urothlum-derived ATP, as agonist-induced urothelial ATP has been shown to be capable of eliciting a paracrine effect on DSM contractions (Sui et al., 2014). The contractions evoked by GSK are a result of direct action on DSM as previously discussed, possibly with a small contribution from the paracrine action of GSK-induced mucosal ATP. It is also important to consider that the urothelium may release further currently unidentified neurotransmitters that could also contribute in part to DSM contraction.

GSK evoked contractions of similar magnitude upon first exposure in both young and aging full thickness tissue. In young tissue, further exposures were incapable of evoking contractions. Interestingly however, second and third exposures to GSK in aging tissue evoked further, weaker contractions in the majority of strips. This is likely a consequence of the considerably higher expression of TRPV4. These surplus receptors may already be incorporated into the membrane, where a proportion remains inactivated by the first GSK intervention of enough numbers to elicit a second contraction upon second exposure to GSK. Alternatively, these excess receptors may be housed in vesicles beneath the membrane, as per their normal trafficking process (Everaerts et al., 2010a; Schwartzberg,
1998; Wegierski et al., 2006), and as such a greater number are available for rapid incorporation into the membrane for subsequent activation. This identifies a functional change in TRPV4 receptors in aging tissue, likely as a consequence of TRPV4 overexpression, which has the potential to directly alter DSM contractile responses. This increased population of TRPV4 receptors is therefore capable of eliciting contractions in quicker succession and this could indeed contribute to overactive bladder activity.

4.5.2.2 MOUSE MODELS

4.5.2.2.1 PURINOCEPTOR AND TRPV4 MEDIATED ATP RELEASE

Co-activation of TRPV4 and P2Y receptors was investigated further using young wild type, young P2Y_{2}KO and aging P2Y_{2}KO mice, allowing the involvement of P2Y_{2} and P2Y_{4} to be determined. Both GSK (TRPV4 agonist) and UTP (P2Y_{2}/P2Y_{4} agonist) evoked ATP release from the mucosa and full thickness strips in all models. Co-activation in young WT mice generated an additive ATP release reflecting that observed in the young GP. However, knockdown of P2Y_{2} significantly reduced the co-activated ATP release to levels reflecting those observed during separate challenge with either GSK or UTP, meaning the co-activation effect was significantly less than additive. The effect of co-activation was investigated in 5 aging P2Y_{2}KO mice, however these exhibited high variations in ATP release thus preventing any rational conclusions from being drawn.

The levels of ATP release evoked by UTP were similar in all models (~400% increase), as were those by GSK (~300% increase). However, co-activation in P2Y_{2}KO elicited significantly less ATP than in WT, highlighting this intervention as the determining factor accounting for the less-than additive effect observed. This implies that here, P2Y_{4} and TRPV4 may share the same mechanism for ATP release; during co-activation the receptors compete for a shared pathway, which becomes fully saturated, preventing ATP release beyond that of the individual effect of one receptor type (figure 4-29 B). As the WT model exhibits an additive effect in the presence of P2Y_{2}, this implies that P2Y_{2} either utilizes a different mechanism all together, or is capable of utilizing an additional pathway once the first (utilized by TRPV4 and P2Y_{4}) becomes saturated.

If it is indeed the case that TRPV4 and P2Y_{4} share a mechanism for ATP release and P2Y_{2} utilizes an alternative, it might be expected that UTP-induced ATP release would be greater in WT than P2Y_{2}KO mice. However, our data show the levels of ATP release to be similar in both models, with no significant difference between the two (data values shown in table 18).
Table 18 - Values (median [25%, 75%]) of ATP release as pmoles / g tissue / min evoked by 100μM UTP in WT and P2Y$_2$KO mouse mucosa (graph not shown).

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>P2Y$_2$KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>N number</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Minimum</td>
<td>38.33</td>
<td>82.28</td>
</tr>
<tr>
<td>25% Percentile</td>
<td>156.5</td>
<td>165.2</td>
</tr>
<tr>
<td>Median</td>
<td>373.5</td>
<td>235.1</td>
</tr>
<tr>
<td>75% Percentile</td>
<td>654.5</td>
<td>798.3</td>
</tr>
<tr>
<td>Maximum</td>
<td>1491</td>
<td>1015</td>
</tr>
<tr>
<td>Mean</td>
<td>477.3</td>
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<tr>
<td>Std. Deviation</td>
<td>460.6</td>
<td>346.2</td>
</tr>
<tr>
<td>Std. Error of Mean</td>
<td>162.8</td>
<td>109.5</td>
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It may be that P2Y$_2$ and P2Y$_4$ mediate ATP release cooperatively to prevent release exceeding physiological levels, or a purinoceptor-specific feedback pathway mediates a balanced release between the two receptors, despite each using a different pathway. Purinoceptors also display high plasticity in expression under varying conditions in many tissues (Dubyak et al., 1996), as well as the bladder (previously discussed). It may be that expression of functional P2Y$_4$ receptors is up-regulated in the P2Y$_2$KO mouse, sustaining the levels released in WT, however competition for the pathway by TRPV4 prevents any additive release during co-activation.

The involvement of P2Y$_6$ receptors in this mechanism was eliminated as blockade of these receptors had no effect on UTP-mediated ATP release. However, P2Y$_6$ activation by the agonist UDP did evoke ATP release from the mucosa. This has been demonstrated previously in anaesthetized rats, where the ATP release was mediated by pannexin-1 channels and indirectly increased voiding frequency via P2X3 receptors on suburothelial afferent nerves (Carneiro et al., 2014; Timóteo et al., 2014). As such, current findings suggest that P2Y$_2$, P2Y$_4$ and P2Y$_6$ are the purinoceptor subtypes responsible for urothelial ATP release. The involvement of additional P2Y subtypes is unlikely as there is currently no report of expression of other P2Y subtypes in the urothelium, aside from P2Y$_1$, which does not respond to UTP and does not evoke ATP release from the urothelium when activated (Mansfield and Hughes, 2014). Currently, P2X receptors are also not believed to contribute to urothelial ATP release and this is supported by findings from multiple studies (Mansfield and Hughes, 2014; Sui et al., 2014, 2006).

As P2Y$_2$ and P2Y$_4$ respond to ATP, it is likely they mediate ATP-induced ATP release from the urothelium. Results from this study imply that P2Y$_4$ is primarily responsible for ATP/UTP induced ATP release, and P2Y$_2$ functions as an alternate pathway for additional ATP.
release once others become saturated. As P2Y<sub>6</sub> preferentially responds to UDP only, the physiological function of P2Y<sub>6</sub>-mediated ATP release is less clear, as unlike ATP, it is yet to be established whether UTP and UDP are released from the bladder tissues for physiological function. However, findings from one study did support the endogenous release of UTP and UDP from the bladder and that this modulated the micturition reflex via P2Y<sub>6</sub> (Carneiro et al., 2014). Additionally, physiologically relevant levels of nucleosides and nucleotides, including UTP and UDP have recently been identified in human urine samples and as such, P2Y<sub>6</sub> may be activated by endogenous UDP, contributing to urothelial ATP release (Contreras-Sanz et al., 2012).

P2X receptors respond preferentially to ATP, and P2Y<sub>1</sub> receptors to adenine nucleotides. As these are the only other purinoceptor subtypes present in the urothelium, the effects of UTP and UDP observed in this study can be attributed to P2Y<sub>2</sub>, P2Y<sub>4</sub> and P2Y<sub>6</sub> with confidence. However, the agonists used (UTP, UDP) do lack subtype specificity to some degree, where the EC<sub>50</sub> values have been determined in cultured cells exhibiting different purinoceptor phenotypes to the cells present in the bladder preparations examined here. As such, there may be some crossover of effects of UTP and UDP on these subtypes that cannot be determined, making it difficult to definitively identify the receptor subtypes responsible for the respective observations. A novel P2Y<sub>6</sub> agonist PSB0474 (3-phenacyl-UDP) has recently been developed, which has a higher selectivity for the receptor than UDP (EC50=70nm, >500 fold selectivity) (El-Tayeb et al., 2006). This UDP derivative could be used in future studies for more definitive results.

4.5.2.2.2 PURINOCEPTOR AND TRPV4 MEDIATED CONTRACTIONS

Unlike GP bladders, no mucosal contractions were observed in any mouse model when challenged with UTP. For the WT, this is likely due to species differences, where subtype-specific expression levels and functional responsibilities of purinoceptors differ between the two animals. UTP-induced mucosal contractions would not be expected in P2Y<sub>2</sub>KO bladders, as it was suggested that these contractions were mediated by P2Y<sub>2</sub> expressing myofibroblasts. As with young GP, no contractions were generated by GSK in either of the young mouse models. Unlike aging GP however, no contractions were observed in aging mice, however only 5 bladders were assessed. It may be that further n numbers would reveal contractions in a similar proportion of bladders as observed in aging GP, or it may be that TRPV4 expression is not altered with age in mice and as such, this needs further investigation.
Contractions of similar magnitude were generated by UTP in full thickness strips from all 3 models. As discussed for GP tissue, this is likely in part due to direct action of UTP on DSM P2X3 receptors with a small contribution from a paracrine effect of mucosal ATP release. GSK evoked contractions in full thickness strips from all mouse models in a similar fashion to that observed in GP tissue. However, in the 5 aging mouse bladders examined there was no reproducible effect of GSK like that observed in aging GP and again, this may be due to an unchanged TRPV4 expression in aging mice.

4.5.2.3 FURTHER MOUSE STUDIES: DOES TRPV4 ACTIVITY LIMIT THAT OF P2Y RECEPTORS AND VICE VERSA?

The apparent interplay between TRPV4 and P2Y receptor signalling for ATP release was investigated. As discussed, it was hypothesised that TRPV4 and P2Y4 share a mechanism for ATP release, and P2Y2 is either able to utilize a further mechanism once the first becomes saturated, or indeed utilizes a different mechanism all together. Using P2Y2KO mice, it was found that if ATP release was induced by P2Y4 receptors slightly in advance of TRPV4, TRPV4 could not mediate additional ATP release above that already being generated by P2Y4. This was also found if TRPV4 receptors were activated in advance of P2Y4. Interestingly, if P2Y2 and P2Y4 were activated in advance of TRPV4 in WT bladders, TRPV4 was then capable of generating additional ATP release. This supports the previous hypothesis that P2Y2 is the subtype required to mediate additional ATP release, accounting for the additive effect observed in WT models.

4.5.2.4 SUMMARISED FINDINGS AND IMPLICATIONS FROM ANIMAL STUDIES

In aggregate, the substantial collection of findings from these animal studies permits reliable postulation of the changes that occur during aging that underlie the observed shift from additive to potentiated mucosal ATP release. TRPV4 and P2Y2/P2Y4 activation induced ATP release in both species, highlighting the similarity in basic receptor functions between these two models. TRPV4-induced contractions were again similar between species, however contractile responses to purinoceptor activation varied slightly, with strong contractions in mouse and minimal contractions in GP, indicating the presence of some differences that may need to be considered when using these models.
4.5.2.4.1 CO-ACTIVATION OF TRPV4 WITH PURINORECEPTORS ELICITS ADDITIVE OR POTENTIATIVE ATP RELEASE

Co-activation of TRPV4 and P2Y receptors in both young GP and WT mice generates an additive ATP release, the total of which reflects that of the sum of ATP release generated by each receptor group individually. This can imply that the two receptor groups utilize different, mutually exclusive mechanisms for ATP release, however in this case there appears to be a more complex relationship. Mouse studies identified the importance of P2Y2 receptors in this observed additive ATP release in young WT animals. Results suggest that TRPV4 and P2Y4 can generate ATP release independently; however P2Y2 is necessary for co-activated additive ATP release. Without P2Y2, co-activation results in a level of ATP release reflecting that of only one of the individual agonists, not the sum of both, with multiple experiments evidencing this phenomenon. This implies that TRPV4 and P2Y4 share the same mechanism for ATP release, and when activated simultaneously, compete for this, such that the total ATP release still reflects that of only one receptor group (figure 4-29 B). Following from this, a few possible theories for the additive and potentiated ATP release observed in other models can be drawn. Activation of TRPV4 generates ATP release via raised [Ca$^{2+}$], likely mediated by pannexin-1 channels as described in the previous chapter. Activation of P2Y4 alone generates ATP release via an unknown mechanism. It is possible this is partly mediated via pannexin-1 channels, as P2Y6 receptors have been shown to induce pannexin-1 mediated ATP release from the urothelium (Timóteo et al., 2014), likely via internal Ca$^{2+}$ release via the IP$_3$ / PLC signalling pathway (Iglesias et al., 2009), the standard signalling transduction for P2Y2, P2Y4 and P2Y6 receptors. Interestingly, activation of P2Y2 and P2Y4 generates similar levels of ATP release to P2Y4 alone. This implies either a negative feedback mechanism is in place (likely via inhibitory action of AMP and adenosine), or the receptors cooperate to mediate a balanced ATP release, either of which would prevent purinoceptor-induced ATP release beyond a certain level. Indeed in other systems, multiple P2Y subtypes function together to achieve a balanced output, where certain subtypes are necessary for function and others only contribute. This relative contribution can shift depending on the chemical environment or stimulatory factors, as is the case for platelet function (Daniel et al., 1998; Hechler et al., 1998). In this case therefore, it may be that P2Y4 is the predominant subtype responsible for UTP-mediated ATP release and P2Y2 may only contribute to a certain controlled level, if at all. However, once TRPV4-mediated ATP release is also initiated, the mechanism utilized by both TRPV4 and P2Y4 will become saturated and this may trigger a shift in the balance between P2Y2 and P2Y4 mediated ATP release in favour of P2Y2, utilizing a further mechanism and in doing so permitting the
additive ATP release observed (figure 4-29 A). Put simply, it may be that P2Y₄ is responsible for principal purinoceptor-associated release of ATP and P2Y₂ is necessary for additional release (amplification).

However, another possible theory is that in WT animals, P2Y₂ is the predominant subtype for purinoceptor-induced ATP release and utilizes a different, mutually exclusive mechanism to TRPV4, allowing additive release during simultaneous activation. However, knockout of P2Y₂ may induce purinoceptor plasticity, up-regulating functional expression of P2Y₄ receptors capable of inducing ATP release, to compensate for the lack of functional P2Y₂ receptors. However, these P2Y₄ receptors utilize the same ATP release mechanism as TRPV4, thus preventing additive ATP release during co-activation. As plasticity of purinoceptors and balance between subtypes for a shared function appears to be such an important characteristic of these receptors, this possibility cannot be ruled out. As such, comparative expression levels of P2Y₂ and P2Y₄ in both WT and P2Y₂KO mice must be determined for firm conclusions to be drawn.

Possibly the most interesting finding from these studies was the emergence of a potentiated ATP release from the aging GP mucosa during co-activation of P2Y and TRPV4 receptors. It is likely the mechanics underlying this phenomenon are highly complex, as it depends on both an unknown change that occurs with aging and separation from underlying tissues. Presence of the underlying tissues prevents this augmented ATP release and as previously discussed, this suggests the existence of a regulatory feedback mechanism originating from these supportive tissues, preventing augmented ATP release, even when a change or dysregulation in the mucosa emerges. The urothelium is susceptible to a variety of harmful age-related factors such as inflammation and pathological insult, all of which can have a dramatic impact on urothelial function. As such, it is highly likely that regulatory mechanisms exist in the supporting tissues as a safe-guard to correct any malfunctions resulting from such transformative factors. Therefore, it is possible that aging may disrupt these safe-guards also, resulting in uncontrolled release of ATP.

The mechanisms underlying the observed age-dependent enhancement of TRPV4/P2Y co-mediated ATP release are unclear. If the purinoceptor-mediated components identified in mouse are translated in GP it is likely that TRPV4 and P2Y₂ coordinate this enhanced ATP release through some synergistic functional interaction that develops in aging. A link between TRPV4 and P2Y₄ is less likely as findings suggest these receptors already share a limited mechanism, making a synergistic amplification unlikely. A functional interaction between P2Y₂ and the closely related TRPV1 channel has been previously described,
where co-expression of P2Y$_2$ and TRPV1 was identified and functional activation of TRPV1 was induced by ATP via P2Y$_2$ receptors in the absence of any other stimuli (Lakshmi and Joshi, 2005; Moriyama et al., 2003). This identified a functional interaction between the two receptors, permitting activation of TRPV1 via P2Y$_2$. As such, it is possible a similar interaction develops between P2Y$_2$ and TRPV4 in the aging mucosa, possibly mediated by the considerable increase in TRPV4 expression and high plasticity of purinoceptors. If TRPV4 receptors are functionally linked to P2Y$_2$ receptors, this could enable utilization of multiple ATP release pathways during co-activation, permitting excessive ATP release beyond physiological levels.
Figure 4 – 29. Postulated mechanisms for ATP release from urothelial cells evoked by simultaneous activation of TRPV4/P2Y2/P2Y4 in various animal models. A: Mechanism in young WT mice and GPs. Activation of TRPV4 alone (synthetic by GSK or physiological by stretch) induces ATP release mechanism ‘1’, likely mediated by ↑[Ca^{2+}]_i resulting in opening of pannexin-1 channels. Activation of P2Y receptors alone (synthetic by UTP, physiological by ATP [ATP-induced ATP release, pathological states etc]) induces ATP release via mechanism ‘1’ also, possibly with a balance between the two subtypes in favour of P2Y4. Simultaneous activation of all 3 receptor types induces an additive ATP release (the sum of TRPV4 activation alone + P2Y activation alone). Once shared mechanism ‘1’ utilized by both TRPV4 and P2Y4 becomes saturated, purinoceptor functional balance shifts to P2Y2, which utilizes additional pannexin-1 channels or an additional ATP release mechanism altogether (2), permitting additional ATP release. B: Mechanism in P2Y2 KO mice. Separate activation of either TRPV4 of P2Y4 evokes ATP release of similar proportions via shared mechanism ‘1’. As such, simultaneous activation saturates this shared mechanism and in this case, P2Y2 is absent preventing additional ATP release. C: Mechanism in aging WT GP. A functional interaction between TRPV4 and P2Y2 emerges. Separate activation of receptors generates ATP release via mechanism ‘1’ in the same way as young GPs. Simultaneous activation also evokes ATP as in young GPs, however here the functional interaction between a proportion of TRPV4 and P2Y2 receptors grants augmented ATP release possibly by heightened stimulation of current ATP release mechanisms (2, 3 - ⋆) or via activation of a novel unknown pathway (4) that emerges with aging. These postulations are evidenced by the findings from this study. Created using public access images from(Servier Medical, 2015).
The results suggest a mechanism where only a proportion of the TRPV4 receptors are functionally linked to P2Y₂ receptors, where activation of both is required for induction of additional ATP release pathways, granting excess release. A postulated mechanism is described in figure 4-29 C. However, as previously discussed it may simply be that ectonucleotidase expression patterns are altered in the aging bladder, such that subtypes critical for full degradation of ATP to AMP and adenosine are only expressed in the underlying tissues. Removal of the underlying tissues in aging bladders would therefore disrupt the negative feedback mechanism mediated by AMP and adenosine on ATP release, possibly resulting in the amplified ATP release observed. However, if this is the case, an augmented ATP release would also be expected with UTP alone, which was not observed, unless an additional purinoceptor-specific feedback mechanism is also present. As such, it is more likely the augmented ATP release observed in aging GP mucosa is attributable to a novel functional interaction between TRPV4 and purinoceptor signalling, however altered ectonucleotidase activity may contribute to some degree. However, evaluation of the expression and distribution of ectonucleotidase subtypes between the tissues of young and aging bladders may still prove interesting.

Simultaneous activation of TRPV4 and P2Y receptors can be achieved physiologically, where stretch activates TRPV4 and endogenous ATP will activate P2Y receptors. ATP can be supplied by parasympathetic nerves or from the urothelium itself and augmented basal and stretch-induced ATP release has been demonstrated in aging and pathological bladders (Birder et al., 2003; Sui et al., 2014; Yan Sun et al., 2001). It may be that this age/pathology-dependent increase in basal ATP release over-stimulates the purinoceptors, such that subsequent TRPV4 activation by stretch results in augmented ATP release, or it may be that the age/pathology-dependent augmented ATP release described in the literature is a result of the co-activated phenomenon identified in this study. Either way, the amplified ATP release identified in the current study will be capable of disrupting bladder function and promoting an overactive bladder state.

The hypothetical underlying mechanisms described here are based on the findings from this study and recent literature only. However, despite the mechanisms not being fully understood, this study has identified an age-dependent augmented mucosal ATP release associated with purine and stretch-detecting receptors. This phenomenon may be a significant contributing factor in the generation of overactive bladder and associated pathologies. As such, this may be an important area for further investigation, as gaining a full understanding of this phenomenon may reveal novel methods for therapeutic intervention.
4.5.2.5 CO-ACTIVATION IN HUMAN MUCOSAL BIOPSIES FROM PATIENTS WITH OR WITHOUT OVERACTIVE BLADDER

An important finding from human studies was that basal ATP release from the mucosa is considerably increased in patients diagnosed with overactive bladder. This is in agreement with a previous study that found increased basal and stretch-induced ATP release from mucosal bladder strips from patients with idiopathic detrusor overactivity (IDO), but similar levels in patients with neurogenic overactivity (NDO) (Kumar et al., 2010). Previous studies have demonstrated a decreased muscarinic (Datta et al., 2010) and increased P2X receptor expression (O’reilly et al., 2002) in overactive human bladders, which may influence this augmented basal ATP release. However currently, no studies have examined P2Y receptor expression in overactive human bladders, which may be of high importance as they are strongly associated with mucosal ATP release (Sui et al., 2014; Timóteo et al., 2014).

TRPV4 activation by GSK evoked ATP release from both normal and overactive mucosal sheets. The proportional increase in ATP release from basal levels was similar between cohorts, indicating the drug effect of GSK was similar. However, actual quantities of both basal and TRPV4-induced ATP release were much higher in mucosa from overactive bladders. It is likely therefore, that TRPV4 contributes in part to the amplified stretch-induced ATP release observed in mucosal strips from IDO bladders (Kumar et al., 2010). In addition, the onset, peak and offset of ATP release occurred more rapidly in mucosa from overactive bladders. This implies that TRPV4 receptors may be more responsive in OAB mucosa. If like in the GP, TRPV4 expression is increased in aging human mucosa, perhaps a greater proportion of receptors are sensitized prior to activation, for example, by PTKs, increasing their sensitivity and responsiveness to stimuli. This could contribute to the increased voiding frequency observed during OAB.

The effect of TRPV4/P2Y co-activation on ATP release was additive in both cohorts. However, there was greater variation in the normal aging cohort, with 20% of bladders showing an amplified ATP release similar to that observed in the aging GP. The effect was additive in the 4 overactive bladders assessed. However, these patients were younger (45-55) than the normal cohort (>65). This phenomenon may only occur in a low proportion of human aging bladders, however it may still be a significant factor in the progression of pathologies in such bladders. Further n numbers in both groups are needed to fully assess this. However, as this phenomenon was not observed in any of the 4 overactive bladders examined, it is unlikely this is a leading factor in overactive bladder, although it may contribute to or support the disorder in bladders that exhibit overactivity due to other causes.
To summarise, this study has established similarities in the mechanisms and receptor subtypes responsible for mucosal ATP release between rodents and humans and identified age-dependent changes in both the expression and behaviour of TRPV4. Both P2Y2/P2Y4 and TRPV4 activation evoke ATP release from the mucosa of GPs, mice and humans, demonstrating common functional characteristics between species. P2Y4 can evoke ATP release from mouse bladders independently, however P2Y2 appears to be necessary for amplification of ATP release beyond that of the apparent principal pathways. P2Y6 activation also evokes ATP release, meaning the urothelium can also respond to endogenous UDP, however the physiological relevance of this remains unclear. This indicates that different purinoceptor subtypes have different roles, which integrate in a complex manner to achieve an overall purinoceptor-mediated function in ATP release. A functional interaction between purinoceptors and TRPV4 appears to emerge with aging, characterized by an augmented mucosal ATP release upon co-activation of these receptors, which is not observed in younger animals. This may in part result from the significant increase in TRPV4 expression identified in aging bladders, promoting the development of a functional link between TRPV4 and purinoceptors. However, age-dependent changes in ectonucleotidase activity could also facilitate an augmented ATP release and must therefore be considered. This could provide an interesting area for future research.

The age-dependent changes in expression and function of receptors critical for bladder function identified here add to the growing number of age-related changes already identified in the urothelium. As bladder pathologies are strongly associated with age, it is evident that these changes contribute to age-related bladder dysfunction and may have profound implications on physiology and pathophysiology of the aging bladder. Equally, identifying the underlying mechanisms responsible for such changes may provide novel approaches for therapeutic intervention of bladder disorders.
CHAPTER 5. GENERAL DISCUSSION
5.1 SUMMARY AND INTERPRETATION OF PRINCIPAL FINDINGS

The primary aim of this project was to characterise the activity of TRPV4 in both physiological and pathophysiological/aging bladders and determine the therapeutic potential of this channel for OAB syndrome. For this purpose, two hypotheses were tested; TRPV4 significantly regulates urothelial function through urothelial ATP release; changes in TRPV4 channel activity occur in aging/overactive bladders that contribute to abnormal bladder function. This builds upon the work of previous studies that have identified the importance of TRPV4 in bladder function (Everaerts et al., 2010c; Gevaert et al., 2007; Janssen et al., 2011; Mochizuki et al., 2009; Thorneloe et al., 2008). The current studies clarified the mode of action of TRPV4 in native urothelium, elucidated the underlying mechanisms and identified age-dependent changes in channel function and expression.

5.1.1 CHARACTERIZATION OF TRPV4 RECEPTORS IN THE BLADDER

5.1.1.1 EXPRESSION OF TRPV4

TRPV4 expression has been previously identified by immunostaining and western blotting in the urothelium and DSM from mouse and rat (Gevaert et al., 2007; Janssen et al., 2011; Thorneloe et al., 2008; Yamada et al., 2009). TRPV4 expression has also been demonstrated by immunostaining in isolated human urothelial (Janssen et al., 2011). However, information regarding the specific expression profile of TRPV4 throughout the bladder tissues is lacking. In the current study, findings from immunostaining and western blotting in GP bladders revealed a predominant expression of TRPV4 in the urothelium. TRPV4 expression was also confirmed in 3 human mucosal biopsies by western blotting and in 4 by immunostaining; more importantly, in-vitro experiments demonstrated functional expression of TRPV4 in all biopsies examined. The effect of aging on TRPV4 expression was profound, where findings indicated that TRPV4 expression was up-regulated in the urothelium and DSM, but not the suburothelium of aging bladders. This is an important novel finding, as altered expression may alter the overall behaviour of TRPV4 in aging bladders, possibly impacting on bladder function. As such, TRPV4 expression in young and aging human mucosa should be assessed, as this may reveal a critical age-dependent change that could influence the age-related pathologies observed in human bladders.

The role of TRPV4 in normal bladder function has been previously explored by a collection of studies in animal bladders (Everaerts et al., 2010c; Gevaert et al., 2007; Mochizuki et al., 2009; Thorneloe et al., 2008). The collective findings from these provide direct evidence for
TRPV4 mediated ATP release from cultured urothelial cells and indirect evidence for this role in bladder function from in vivo TRPV4 knockout mice. These studies provide initial evidence for the importance of TRPV4 in bladder function and pathology. The present studies built upon this collection of research to establish the physiological role of this receptor using native urothelium and bladder tissue from both animal models and humans to determine the underlying mechanisms of TRPV4 function in the bladder.

5.1.1.2 TRPV4-MEDIATED UROTHELIAL ATP RELEASE

Multiple lines of evidence from the current study established a functional role for TRPV4 in urothelial ATP release. Selective activation of TRPV4 evoked a significant and reproducible release of ATP from the mucosa of human, guinea pig and mouse bladders, demonstrating common functionality of TRPV4 between species. As such, this serves as evidence that the TRPV4 activity observed in these animal models can be translated to humans with confidence. Mucosal strips released far greater quantities of ATP per gram of tissue than full thickness strips, suggesting the majority of ATP released by the bladder in response to TRPV4 activation originates form the urothelium. Removal of the mucosa resulted in near complete loss of GSK/TRPV4-induced ATP release, further indicating that the urothelium is the source of ATP release.

5.1.1.2.1 SELECTIVE BLOCKADE OF TRPV4

Antagonism of TRPV4 with HC-067047 completely abolished any GSK-induced ATP release, confirming selectivity of GSK regarding activity in the bladder. Additionally, antagonism with HC-067047 reduced basal ATP release in both young GP and human mucosa. This implies TRPV4 has a function in maintaining basal ATP release and is therefore activated by an endogenous ligand. Currently, EETs are the only endogenous activators of TRPV4 identified (Vriens et al., 2005; Watanabe et al., 2003b). EETs are mechano- and osmotransducing messengers thought to mediate the channels responses to such stimuli. However, EETs are involved in numerous other biological processes such as angiogenesis (Michaelis et al., 2005) and cell-cell communication (Popp et al., 2002), importantly between endothelial cells and smooth muscle cells (Fleming, 2001). As such, it is likely that EETs are still bioavailable in the bladder in the absence of mechanical/osmotic stimuli due to these other important processes and can therefore contribute to the basal TRPV4 activity observed. However, TRPV4 channel activity can also be enhanced by Ca\(^{2+}\)-CaM complex and IP\(_3\), which couple the membrane-bound receptor to intracellular Ca\(^{2+}\) stores (Garcia-Elias et al., 2008; Strotmann et al., 2003). As such, it has been postulated
that rises in $[\text{Ca}^{2+}]$, resulting from other physiological processes can also contribute to basal/spontaneous channel activity. SFKs can also sensitize TRPV4 channel activity and are also thought to mediate the channels responses to mechanical/hypotonic stimuli (Wegierski et al., 2009; Xu et al., 2003). SFKs are ubiquitously expressed and regulate a vast number of cellular processes such as cell motility, responses to extracellular growth factors, specialized cellular responses such as platelet aggregation and responses to oxidative stress (reviewed elsewhere [Parsons and Parsons, 1997; Schwartzberg, 1998]). As such, it is also likely that these are available for basal TRPV4 activation, possibly sensitizing the receptor for lower level stimulation. A role for urothelial TRPV4 in spontaneous bladder contractility has been previously demonstrated (Gevaert et al., 2007). Removal of the urothelium and knockout of TRPV4 resulted in reduced spontaneous activity, suggesting urothelial TRPV4 was responsible for this spontaneous activity, and not TRPV4 expressed in the DSM. The findings here support this, identifying a role for TRPV4 in basal ATP release from the urothelium, that may be involved in this TRPV4-dependent spontaneous activity observed previously.

5.1.1.2.2 FUNCTIONAL CONTRIBUTION OF TRPV4 TO STRETCH-INDUCED ATP RELEASE

Mechanical stretch also evoked a reproducible ATP release from the mucosa. The findings here demonstrated a 50% reduction in stretch-induced ATP release with TRPV4 blockade. This is not entirely in agreement with the literature, where previous studies showed a 90% reduction in stretch-evoked ATP release from freshly isolated urothelial cells (Birder et al., 2003) and a 100% reduction from rat tissue strips (using a protocol identical to the one in the current study) (Du et al., 2007) with the ENaC blocker amiloride. It is not feasible that ENaC channels are solely responsible for stretch-induced ATP release, as there is an extensive collection of studies evidencing a role for TRPV4 in this process. It is likely that both mechanosensitive channels contribute a proportion of this ATP release, where the respective contributions could be determined by individual and simultaneous blockade of each receptor. This could also identify proportional contributions from further mechanosensitive receptors yet to be identified in this process; for example, the stretch-activated channels which have been identified in urothelial cells (Wu et al., 2011). The mechanisms by which stretch activates TRPV4 channels have been studied previously and it is currently thought that the process requires activation of TRPV4 by EETs (possibly direct [Fernandes et al., 2008]) generated by the PLA$_2$ dependent endogenous signalling pathway (Watanabe et al., 2003b), or phosphorylation by SFKs (Wegierski et al., 2009; Xu et al.,
2003), or indeed both. However, the possibility of direct activation by mechanical force has not been excluded (Christensen and Corey, 2007) and therefore it is clear that further research is required to fully establish the mechanisms underlying stretch activation of TRPV4.

5.1.1.2.3 PATHWAYS FOR TRPV4-MEDIATED ATP RELEASE

The downstream mechanisms underlying TRPV4-induced ATP release were partly elucidated here. PTK activity was required for GSK-induced ATP release. This was not observed in a similar study using the TRPV4 agonist 4αPDD, however this is likely due to different activation mechanisms for each agonist. Activation by 4αPDD and heat both rely on tyrosine residue 555, which is not required for activation by cell-swelling and EETs, which rely on the PLA₂ pathway (Vriens et al., 2003). This highlights the various distinct mechanisms needed for activation of TRPV4 by different stimuli and therefore, it is possible that unlike 4αPDD, activation by GSK requires phosphorylation by SFKs, possibly sharing the same mechanism as activation by stretch.

This study found that GSK/TRPV4-induced ATP release is mediated majoritively by conductive pathways (90%), with little or no contribution from vesicular transport. The process was also tightly dependent on extracellular Ca²⁺ entry, the main action mediated by TRPV4 channels. This was further evidenced by live-cell calcium imaging, which identified a rise in [Ca²⁺] upon TRPV4 activation in freshly isolated GP urothelial cells. Blockers of Ca²⁺-activated chloride channels had no effect on GSK-induced ATP release, excluding them from the mechanism. The collective findings, supported by those from previous studies, suggest a mechanism whereby; stretch activates TRPV4 receptors via SFK-mediated phosphorylation of the channel; TRPV4 mediates Ca²⁺ influx raising [Ca²⁺]; this activates a Ca²⁺-dependent, hemichannel-mediated ATP release mechanism, likely through pannexin-1 channels (refer to section 3.4.7 for a more detailed discussion).

5.1.1.2.4 IMPLICATIONS OF TRPV4-MEDIATED ATP RELEASE: ROLES IN PHYSIOLOGY AND PATHOPHYSIOLOGY

The ATP release mediated by TRPV4 can influence the physiology of the bladder. It is currently thought that TRPV4 senses distension in the bladder during filling and communicates this to the underlying tissues as an ATP signal. This may be a fundamental process in the bladders overall ability to sense fullness, whereby the extent of bladder distention (equating to urine volume) is relayed to underlying afferent nerves via an ATP signal generated, in part, by TRPV4. These nerves continuously convey information regarding urine volume to the PMC for voluntary bladder control and perception of bladder
fullness, initiating urges to void at appropriate volumes (20-25mmHg[Häbler et al., 1990]), permitting correct periodic storage and voiding of urine. Interestingly, pain is perceived once certain harmful volumes are reached (30-50mmHg[Häbler et al., 1990]), mediated via ATP through P2X3 and P2X2/3 receptors on suburothelial sensory nerves (Burnstock, 1999; Cockayne et al., 2000; Vlaskovska et al., 2001). As such, urothelial TRPV4 may not only mediate volume-dependent pain perception in the bladder under physiological conditions, but may also induce pain and respond to inflammatory stimuli as a result of various pathologies and inflamed bladders. Not only this, but changes in the expression and function of TRPV4 that result from aging or pathology that have indeed been identified, may influence painful bladder. TRPV4-mediated nociception in the rat has been shown to be enhanced by PGE$_2$, a hyperalgesic inflammatory mediator (Alessandri-Haber et al., 2003). As such, inflamed bladders could enhance TRPV4 mediated nociception in the same manner, implicating TRPV4 in painful bladder pathologies. A fundamental role for TRPV4 in urothelial transduction of sensory information regarding bladder distension has therefore been identified, which may also have implications in bladder overactivity and pain.

5.1.1.3 TRPV4-MEDIATED DSM CONTRACTIONS

A role for TRPV4 expressed in the DSM has been identified both here and in previous studies (Thorneloe et al., 2008). TRPV4 activation evoked sustained contractions in intact bladder strips of similar magnitude to those evoked by the gold standard muscarinic agonist carbachol. These contractions were also observed in denuded DSM strips and therefore, it was concluded that direct activation of TRPV4 receptors in the DSM can cause DSM contractions, although additional small contractions could result from an indirect effect of ATP release. This supports findings from previous studies, identifying the presence of functional TRPV4 receptors in the DSM and highlighting the ability of TRPV4 to directly contract bladder muscle with a force equalling that of muscarinic activation, however the physiological significance of this remains to be established (Thorneloe et al., 2008). Whether these channels directly mediate physiological bladder contraction or not, these findings reveal the potential they have to do so, and as such, TRPV4 activity could indeed influence bladder pathologies associated with over-activity. Although nerve-mediated contractions in humans are mediated entirely through M$_3$ receptors under physiological conditions, with the possible emergence of a purinergic component in DO bladders, TRPV4 in the DSM may still influence bladder contractile activity. They provide a direct Ca$^{2+}$ entry channel into smooth muscle cells specialized to respond to raised Ca$^{2+}$ by eliciting contraction. Despite this, the study by Thorneloe et al found that Nifedipine suppressed, but did not abolish, GSK-induced...
DSM contractions, indicating the involvement of L-type Ca\textsuperscript{2+} channels in TRPV4-mediated contractions (Thorneloe et al., 2008). The results suggested that TRPV4 activation depolarises the membrane potential, activating L-type Ca\textsuperscript{2+} channels, which supply further Ca\textsuperscript{2+} for contraction, where the direct supply of initial Ca\textsuperscript{2+} through TRPV4 channels contributes to initial contraction. Additionally, TRPV4\textsuperscript{-/-} had no effect on DSM contractile responses to electrical field stimulation (EFS) \textit{in vitro}, indicating they do not contribute to nerve-mediated contractions and only possibly play a modulatory role on DSM contractile behaviour during normal micturition. The data from the present study takes it further from the findings in mice, and demonstrates that TRPV4 in the DSM is capable of evoking strong contractions across all major species, including human, proving that this receptor is a fundamental regulator of the DSM. However, its primary role in the DSM seems to be modulating smooth muscle contractility, possibly responding to bladder distension during filling, owing to its mechanosensitivity. As a role for TRPV4 receptors in basal ATP release has been identified here, it is possible they play a basal role in DSM also. These receptors may be activated at a basal level in the same way as described previously, for example, by raised [Ca\textsuperscript{2+}], resulting from other physiological processes, initiating low level contractile activity in isolated muscle bundles to maintain bladder tone during filling. They may also recognise stretch and initiate contractions in these isolated muscle fascicles during higher pressures of bladder filling to continue to provide tone until voiding. Despite the fact DSM TRPV4 receptors seem to only play a small modulatory role on DSM contractility under physiological conditions, the ability of such receptors to evoke cholinergic-like contractions has been identified. As such, these receptors have the potential to strongly influence bladder contractility if their function is altered, for example by over-stimulation or increased functional expression. More importantly, inflamed bladders may generate inflammatory mediators such as prostaglandins, change of pH and temperature that can activate TRPV4 receptors, highlighting one way in which these receptors may become overstimulated during pathology, influencing their function and therefore, potentially influencing bladder contractility.

Indeed, TRPV4 receptors have the potential to cause or influence bladder overactivity. Infusion of GSK into the bladders of WT mice induces bladder overactivity, believed to be mediated by both distinct roles of TRPV4, by causing urothelial ATP release, which sensitizes bladder afferents, and by directly contracting DSM (Thorneloe et al., 2008). The concentrations used were high (10\textmu M) and therefore may not be physiologically relevant, however, the results again indicate the potential importance of TRPV4 in bladder overactivity.
5.1.1.4 TRPV4-MEDIATED ROS PRODUCTION

The current study identified a further, potentially pathologic output of TRPV4, where TRPV4 activation appears to increase bladder superoxide (O$_2^-$) production. O$_2^-$ plays physiological roles in cellular signalling, for example in cell growth, where low levels stimulate cell proliferation (Pimentel et al., 2001). Stretch increases ROS production in cardiac myocytes (Pimentel et al., 2001) and selective TRPV4 activation initiates mitochondrial ROS production in human coronary artery strips for vasodilation (Bubolz et al., 2012). Our novel findings suggest that TRPV4 activation, possibly by stretch, induces ROS production for ROS-mediated physiological processes. However, ROS production in the bladder has previously been linked to detrusor overactivity, through modulation of C-fibre afferents in rats (Chien et al., 2003; Masuda et al., 2008). In addition, ROS significantly up-regulate the phosphorylation of TRPV4 by SFKs (Wegierski et al., 2009), presenting a positive feedback on TRPV4, where TRPV4-mediated ROS production enhances the receptors activity. A fine balance must therefore be achieved between the multitude of stimuli able to regulate the receptors activity (heat, osmolarity, mechanical stress, ligand activators, regulation by Ca$^{2+}$ and phosphorylation, ROS) for correct physiological function of the receptor. ROS overproduction is an important pathological process in inflammation, many chronic diseases and ageing. As such, any imbalance in these processes may alter receptor activity, and lead to increased ROS production which can aggravate bladder ageing and overactivity.

Collectively, current findings indicate a multifactorial role for TRPV4 in overactive bladder, where the channel can potentially enhance ATP release and ROS production and directly contract DSM, all of which can significantly influence bladder overactivity and also pain.

5.1.2 A ROLE FOR PURINOCEPTORS IN UROTHELIAL ATP RELEASE

A role for various purinoceptor subtypes in urothelial ATP release has previously been described by the research group in humans and GPs (Sui et al., 2014) and by other studies in rats (Carneiro et al., 2014). Purinoceptor-mediated mucosal ATP release was identified in the present study, where different subtypes appear to have different roles, providing an overall purinoceptor-mediated function. UTP-induced ATP release was conserved across all species examined, highlighting common functional characteristics between the animal and human urothelium. P2Y$_4$ appears to be the subtype responsible for principal purinoceptor-mediated ATP release, demonstrated with P2Y$_2$ KO, where P2Y$_2$ appears to function as a secondary pathway once others become saturated. Our findings from animal studies suggest that TRPV4 and P2Y$_4$ share an ATP release pathway and P2Y$_2$ utilizes an alternative. As such, P2Y$_4$ cannot contribute to ATP-induced ATP release during TRPV4
activation, as the pathway is already utilized (by TRPV4) and therefore P2Y₂ may function here to allow ATP-induced ATP release via an alternative pathway during TRPV4 activation. P2Y₆ is also capable of evoking ATP release from the mucosa, however as this subtype responds primarily to UDP, its physiological roles regarding ATP release are limited by endogenous UDP availability. The suggested roles are therefore as follows:

- **P2Y₄**
  - Principal subtype for purinoceptor-mediated ATP release in response to endogenous ATP/UTP
  - ATP-induced ATP release, aside from when TRPV4 is activated

- **P2Y₂**
  - Utilizes a different release pathway to P2Y₄
  - ATP-induced ATP release
  - Provides secondary route for purinoceptor-mediated ATP release once P2Y₄ pathway becomes saturated

- **P2Y₆**
  - Endogenous UDP-mediated ATP release.

For a full dissection of the results and the subsequent interpretations, please refer to the detailed discussion in chapter 4.

### 5.1.3 THE EFFECT OF AGING ON TRPV4 ACTIVITY

It is well established that bladder pathologies are highly associated with increasing age. As TRPV4 receptors have the potential to contribute to overactive bladder in a multifactorial manner, the current study explored the effects of aging on TRPV4 activity to determine if any association exists between age and altered channel behaviour, which may contribute to age-related bladder pathology. An increase in TRPV4 expression was identified in the mucosa (8 fold) and DSM (16 fold) of aging GPs compared to tissues of their younger counterparts. Despite this, TRPV4-mediated ATP release and DSM contractions were similar between age groups, indicating that although expression is increased, basic function of the receptor appears to be conserved. Unfortunately, it was not determined whether the increased number of TRPV4 receptors are functionally located to the cell membrane, or are held in vesicles below the surface in an inactive state, ready for incorporation into the membrane. The contractile effect of GSK was more reproducible in aging tissue compared to young. This suggests that at least a proportion of the surplus TRPV4 is held in vesicles, permitting a more rapid incorporation of fresh receptors into the membrane following first stimulation, allowing subsequent contractions to be generated more rapidly. However, this is
only applicable to the DSM and the location of surplus TRPV4 receptors in the urothelium remains to be determined. In addition, UTP- (P2Y$_2$/P2Y$_4$-) mediated ATP release was also similar between age groups. However, co-activation of P2Y$_2$/P2Y$_4$ with TRPV4 generated an augmented ATP release from mucosal strips that was only observed in aging bladders. The situation in aging full thickness tissue may be more complicated and presence of the underlying tissues may impose additional regulatory mechanisms on urothelial ATP release, or affect its breakdown. As the phenomenon was only observed in aging mucosa, it appears that a functional interaction between TRPV4 and P2Y receptors emerges with aging, generating augmented ATP release when activated simultaneously. As TRPV4 expression is greatly increased with age, and purinoceptor plasticity is such a common phenomenon induced by physiological processes, various pathologies and aging, it is possible a functional link between the two receptors develops as a result of these circumstances. To investigate this further, immunoprecipitation assays should be performed to determine whether the two receptors are co-expressed in the aging urothelium. This phenomenon was only observed in 20% of normal aging human bladders assessed and was not observed in any of the 4 overactive bladders. As such, this interaction is not a necessary prerequisite for overactive bladder, however it may emerge in a small proportion of aging human bladders and could therefore exacerbate, or contribute to the overactive bladder phenotype. Despite the mechanics underlying this phenomenon remaining elusive, this study has successfully identified the age-dependent emergence of an augmented ATP release mediated by a novel receptor interaction. This, together with the increase in expression, reveals yet another way in which TRPV4 can mediate bladder overactivity.

An increased basal and TRPV4-mediated ATP release was identified in overactive human mucosa. This is the first report that TRPV4-mediated ATP release is enhanced in overactive bladders. This provides strong evidence that the overactivity results, at least in part, from a considerably increased mucosal ATP release, and that all forms of ATP release, whether it be basal, receptor-mediated, or indeed stretch-mediated (Kumar et al., 2010) are increased during this pathology.

In summary, the studies here and others have revealed the fundamental physiological role of TRPV4 in urothelial ATP release, as well as potential roles in direct modulation of DSM contractility and ROS production. These studies have also revealed the immense potential TRPV4 has to mediate bladder overactivity, whether that be through increased functional expression, augmented urothelial ATP release, modulation of DSM contractility, ROS production, or through age-induced functional interactions with other receptors involved in urothelial ATP release.
5.1.4 NOVEL TARGETS FOR TREATMENT OF OVERACTIVE BLADDER

OAB is one of the most prevalent pathologies of the bladder, however currently, it is also one of the least understood and understudied. A large study evaluating a cohort of over 7 million patients diagnosed with OAB syndrome over a 45 year period found that 24.4% were treated with antimuscarinics and the remainder went untreated (Helfand et al., 2010). The poor efficacy and range of side-effects of current therapies including antimuscarinics, results in low patient compliance and presents an immediate need for novel therapeutics against the disorder.

5.1.4.1 TRPV4 AS A NOVEL TARGET AGAINST OVERACTIVE BLADDER

Various studies have demonstrated an increased functional bladder capacity and decreased frequency of voiding contractions by either pharmacological intervention or genetic disruption of TRPV4 in mice (Everaerts et al., 2010c; Gevaert et al., 2007). The study by Gevaert et al. also demonstrated reduced spontaneous contractions in explanted bladder strips from TRPV4−/− mice and this was attributed to the basal function of TRPV4 in the urothelium and not the DSM. As OAB is highly associated with an increase in spontaneous bladder contractions, this finding, with the others, first indicated that TRPV4 may be a promising target for OAB. Our findings provide the first evidence that TRPV4 receptors in the bladder are universal functional regulators across animals and humans, and in particular identifies a role for TRPV4 in stretch-evoked urothelial ATP release in physiologically relevant bladder strips and reveals age-dependent changes that may influence OAB, highlighting the multifactorial potential of TRPV4 to contribute to OAB. Importantly, reduced basal ATP release was achieved with selective blockade of TRPV4, further indicating the potential of this receptor as a drug target.

However, TRPV4 is widely expressed and is responsible for many important functions such as vasodilation in the vascular epithelia, regulation of ion exchange in the kidneys, ciliary beating in the airway epithelia, mechanosensation in the cochlea, neuronal communication and bone remodelling to name a few. As such, un-targeted blockade of these receptors by pharmaceutical agents may induce severe and wide spread side-effects. However, novel drug delivery systems (DDSs) targeting drugs to the bladder are currently under development, which appear promising (reviewed here [Zacche et al., 2015]). Systemic and oral administration of drugs requires large doses to achieve therapeutic concentration at the desired tissue target, increasing the likelihood of undesired side-effects through widespread effects of the drug. The anatomy of the bladder permits simple and direct delivery of drugs.
through a catheter under cystoscopy, which is relatively non-invasive and allows drugs to be easily targeted to the bladder, reducing possible side-effects. However, the continually changing urine volume and periodic voiding present difficulties, as these factors cause drugs to become diluted and periodically washed away. Additionally, the low permeability of the urothelium prevents efficacious diffusion of drugs into the bladder wall. Current treatment for OAB in cases where antimuscarinics are ineffective involves intradetrusorial injection of botox® and this is routine practice in many clinics (Kuo and Kuo, 2013). Repeated catheterization for this purpose is less well-tolerated by patients than for example, systemic or oral administration and therefore, novel DDSs are in development. These include nanocarriers, polymeric hydrogels and biodegradable and nondegradable intravesical devises. Nanocarriers can be formulated from lipids, proteins, polymers and metals and allow site-specific delivery of drugs to desired targets (Singh and Lillard, 2009). Liposomal delivery of drugs and uptake by the urothelium has been successfully demonstrated in the bladder (Rajaganapathy et al., 2015). A more bladder-specific method involves application of drug-containing polymeric hydrogels to the bladder wall, which allow sustained released of drugs for up to 24hours and are not removed by voiding (Tyagi et al., 2004). Intravesically administered drug delivery devices are also in development, which are inserted and remain in the bladder, allowing controlled and extended delivery of drugs over periods of days to weeks (Haupt et al., 2013; Tobias et al., 2010; Von Walter et al., 2009). These novel and viable DDSs highlight the ways in which drugs can be specifically targeted to the bladder and could therefore be applied to TRPV4 antagonists, avoiding the possible side-effects associated with widespread application of such drugs.

As TRPV4 receptors involved in bladder function are predominantly expressed in the urothelium, intravesicular delivery of drugs to this site would be more easily targetable, compared to targeting to the DSM, which would require diffusion of drugs over a greater distance and through many diffusion barriers. Our findings suggest TRPV4 is responsible for 50% of the stretch-induced ATP release involved in communication of intravesical pressure, a process fundamental for correct dynamics of bladder filling and voiding. Furthermore, blocking the receptor has no effect on nerve-mediated contractions, and hence normal miciturition reflexes. Therefore, normal bladder function would be preserved. TRPV4 also appears to contribute to spontaneous bladder contractions during filling (Gevaert et al., 2007) and indeed, blockade of this receptor has been shown to increase functional bladder volume. Additionally, results suggest that TRPV4 in the DSM regulates membrane potential, with little contribution to voiding contractions under physiological conditions, however, this DSM TRPV4 is very capable of eliciting overactivity and therefore likely contributes to the
disorder during pathology. Therefore, inhibition of this receptor for therapeutic purposes would likely reverse any pathological effects presented by DSM TRPV4 with little interference on other normal bladder processes. Overall, our current understanding of TRPV4 suggests that specific blockade could alleviate symptoms of OAB, while retaining physiological functioning of the bladder. This would be effective whether altered TRPV4 activity is partly responsible for the observed bladder overactivity or not. These points make TRPV4 an attractive novel target for treatment of OAB. However, the TRPV4−/+ mice studied by Gevaert et al. exhibited an incontinent phenotype, with increased non-voiding contractions resulting in increased urine spotting, despite reduced frequency of full voiding. Therefore, it appears that both complete knockout of TRPV4 function and greatly enhanced TRPV4 activity (Thorneloe et al., 2008) can both induce LUTS. As such, manipulating the degree of TRPV4 activity through controlled dosing of antagonists may permit precise modulation of bladder function. Combined with novel approaches to drug targeting, careful and controlled pharmaceutical modulation of TRPV4 activity appears to be a very promising novel approach for treatment of bladder overactivity.

5.1.4.2 PANNEXIN-1 CHANNELS: A FURTHER POTENTIAL TARGET

Very recent research has demonstrated a role for pannexin-1 channels in mediating both purinoceptor and TRPV4-induced ATP release in epithelial cells (Seminario-Vidal et al., 2011; Shahidullah et al., 2012), where the former has been demonstrated in the urothelium (Beckel et al., 2015; Timóteo et al., 2014). These proteins have only recently been identified and appear to play a role in cell-cell communication and passage of large molecules by forming active hemi-channels, which are closed by common gap-junction blockers (Locovei et al., 2006a). As such, our finding that carbenoxolone prevents TRPV4-mediated ATP release suggests the involvement of these channels here. These channels are widely expressed and have been identified in the eye, thyroid, prostate, kidney, bladder, brain and spinal cord of rats, where the most studied functions are formation of neuronal circuits and ATP release (reviewed elsewhere [Barbe et al., 2006]). As these channels appear to mediate many forms of urothelial-ATP release including that induced by both stretch and activation of multiple receptors, these may provide a further novel approach for treatment of overactive bladder through control of urothelial ATP release. They are expressed throughout the urothelium, however their expression in DSM has not yet been explored (Timóteo et al., 2014). If they are expressed exclusively in the urothelium, this will make the channels easily targetable and remove the possibility of other bladder-related side-effects, lending further positive features of these channels regarding pharmacologic modulation.
5.2 CRITIQUE OF METHODOLOGY

5.2.1 SPECIES EXAMINED AND TISSUE AVAILABILITY

The studies in this thesis were performed on both animal and human tissue, allowing comparison of the similarities and differences between native tissue and that from animals and providing human relevance.

A strength of this study was the use of GPs as the main animal model for research. These animals provide an excellent model for age-related translation studies as they exhibit many qualitative age-related changes also observed in humans. The bladders of these animals share more similarities to those of humans than other rodents and commonly exhibit overactivity with aging, lending them as useful tools for study of the bladder (discussed fully in methods section 2.2.1). However, the micturition patterns were not assessed in the GPs used, which could have been used to identify those with or without overactive bladder phenotypes. This may have been useful when interpreting the data, where overactivity may have been responsible for some of the higher variations in ATP release observed in aging bladders. This should be performed in future experiments.

Transgenic mice were also used as they offer an important research tool that other species cannot and as such, findings from these knockout animals were used to supplement the data from other models. Despite this, rodents do exhibit some important differences in bladder physiology to humans, such as a NANC component to contraction, and as such, findings from these studies should be interpreted with care, as any deviation from humans will have a reduced degree of relevance. Nonetheless, many similarities were observed between species, particularly for urothelial ATP release and detrusor smooth muscle contractions, the two key physiological functions of the bladder wall, where the drug effects of GSK and UTP were similar in humans, mice and GPs. This provides evidence that many of the regulatory mechanisms and functional characteristics of the urothelium are conserved between these species, justifying use of these animals to provide translatable information regarding bladder function.

Human samples provide direct information on any function under investigation and were hence included in this study. Evidences gained from the collected samples have permitted the first ever characterisation of basic mode of action of TRPV4 receptors in native human urothelial tissues. Unfortunately, a lower number of human biopsies were obtained than anticipated, especially for the OAB group, due to unforeseen circumstances out of the control of the research group. As such, data from the OAB group only provide indications of
behaviour of this tissue for comparison to the normal group, although observed differences, particularly in basal and TRPV4-mediated levels of ATP release, provide very interesting initial findings. It is expected that further n numbers will provide statistically significant conclusive findings. Low numbers of biopsies also meant that n numbers for IHC and western blotting analysis were limited. Once these techniques had been fully optimized, the available biopsies were split between techniques to provide as much data as possible regarding expression of TRPV4. Optimizing techniques first reduced the risk of failed experiments using these precious samples. Despite this, expression was confirmed in 3 of the 4 biopsies assessed using western blotting and relative tissue expression was examined in 4, providing sufficient data. With more samples, it would be interesting to compare TRPV4 expression levels between normal and overactive human bladders.

5.2.2 TECHNIQUE-SPECIFIC STRENGTHS AND WEAKNESSES

5.2.2.1 IHC AND WESTERN BLOTTING

These techniques were used in conjunction to provide a comprehensive expression profile for TRPV4, both within the tissues of the bladder and between young and aging bladders. Using both techniques allowed the limitations of each to be overcome. IHC allows specific expression between different tissue layers and the location of the receptors of interest to be examined and compared, which is not possible with western blotting. However, the confocal microscopy stage of IHC requires settings to be optimised per set of sections and an extensive number of sections were examined over a long period of time, making comparison of expression between age groups highly unreliable. Therefore, quantification of this technique only allows differences in relative expression to be evaluated within each section. For example, TRPV4 expression was significantly greater in the urothelium compared to the suburothlium in both young and aging GP bladders, however comparison between age groups was not possible due to the aforementioned varying parameters required to correctly examine each set of sections. This was overcome by using western blotting, where young GP tissues were run alongside aging GP tissues on the same gel, allowing relative levels of TRPV4 to be compared reliably. As age-groups were always run simultaneously, any experiment-specific changes in conditions would apply to both, meaning relative differences in expression were always reliable.

TRPV4 Abs have a reputation for low specificity, presenting another possible limitation. An anti-TRPV4 primary Ab supplied by Alomone Labs™ was used for IHC, and peptide controls demonstrated specificity. This Ab was also employed for western blotting and results were
then replicated using a further TRPV4 Ab from a different company, reinforcing any observations.

During western blotting, expression levels of the housekeeping protein GAPDH were initially used as loading control, which is typically recommended for this purpose due to its commonly consistent ubiquitous expression. However, levels were consistently higher in separated DSM lysates than mucosal lysates. This was quickly identified and attributed to the fact that tissues with increased energy demand (including smooth muscle) exhibit higher GAPDH expression, owing to the enzymes role in the glycolytic pathway (Barber et al., 2005). As such, β-actin was used for loading controls thereafter.

5.2.2.2 FUNCTIONAL ORGAN BATH EXPERIMENTS

The majority of work in this thesis was produced using the well-established organ bath technique, which has been extensively used to accurately measure contractility and ATP release from urinary bladder preparations (Burnstock et al., 1978; Ferguson et al., 1997; Sui et al., 2014). Physiological outputs exhibit a high degree of variation between individuals, which is to be expected, even between animals of the same species. This was accounted for when measuring ATP, where each strip served as its own internal control. Expressing data as a relative change from control revealed drug effects had low variability between animals, although the actual quantities of ATP released per gram may vary to a greater degree. To maximise data output from each animal bladder, the maximum number of experiments (determined by quantity of tissue available) were performed in succession directly after sacrifice. This meant that experiments were commonly performed over a 24hour period and as such, tissue integrity and drift in function over the experimental period is a factor that needed consideration. For animals, fresh strips were mounted for each set of interventions. Preliminary control experiments performed by the research group found that tissue function with regards to contraction and ATP release remains fairly unchanged even after 2 days incubation at 4°C. However, repeated challenge of tissue with drugs during each experiment (commonly 3-5 separate drug interventions) will have an effect on tissue function. This was controlled for by performing pre and post drug controls for each individual intervention, allowing drift in tissue function to be accounted for. During some of the longer experiments, where strips were challenged with 5 separate interventions, significant ATP release was lost during the fifth, and sometimes fourth intervention, highlighting the importance of these controls. Internal controls also eliminated the possibility that ATP release was a direct result of tissue damage and not drug intervention, however this could be definitively confirmed by
assessing lactate dehydrogenase activity, a marker of tissue damage. In some cases, to confirm tissue integrity at the end of experiments a hypotonic 70% Tyrode's : 30% \( \delta \text{H}_2\text{O} \) solution was used to evoke contraction and ATP release, indicating that the tissue remained responsive. However, the majority of interventions performed in this study were successful in evoking ATP release, in itself confirming tissue activity throughout the experimental period. For the purpose of comparing actual quantities of ATP release per gram of tissue, only experiments performed as the first intervention after sacrifice were included, to reduce differences in ATP release resulting from time-dependent tissue drift and repeated exposure to interventions.

Human biopsies were collected from clinics approximately 2 hours from the laboratory and as such, it was essential to confirm integrity of these samples before commencing extended periods of experiments. For this purpose, mucosal strips were initially challenged with carbachol, which evokes highly reproducible ATP release. If ATP release was confirmed, subsequent experiments were performed. Carbachol is a strong contractile agent and therefore if contractions were observed, this would confirm presence of underlying DSM, providing further information regarding the biopsy.

Initially, superfusate samples were frozen immediately after sampling for later evaluation of ATP content. However, variations in the time taken to freeze these samples could result in variations in data due to rapid hydrolysis of ATP. Additionally, this method required thawing of samples before ATP measurement could be performed, meaning samples measured later on in the procedure would have been thawed for longer, providing another source of error. As such, the protocol was improved early on, by measuring ATP content directly after sampling, eliminating any time-dependent variations.

It should be noted that the ATP release measured using this system is likely an underestimation due to the flow imposed by the setup and uncontrollable factors imposed by the tissue, such as ectonucleotidase activity and diffusion barriers, all of which will reduce the observed ATP release. However, this setup was used as it allows dynamic changes in ATP release in response to various interventions to be accurately measured.

5.2.2.2.1 MECHANICAL STRETCH EXPERIMENTS

For this purpose, tissue strips were mechanically stretched to 150% of their original length by careful adjustment of the force transducer fixation hooks. This magnitude of stretch could damage tissue strips, such that the ATP release attributed to stretch may be generated by tissue damage. Once again, tissue integrity should have been confirmed by measuring
lactate dehydrogenase activity, as this will have even greater implications on conclusions here than for the previously mentioned experiments.

5.2.2.3 MEASUREMENT OF ROS PRODUCTION

A method requiring low-temperature crushing of tissue in liquid nitrogen was used for this purpose. This is essentially the physical destruction of cells, a process necessary for isolation of subcellular organelles. This can damage organelles and will disrupt many cellular signalling processes, although crushing at these low temperatures will preserve enzymatic activities, which are heat liable. Literature on this topic is scarce, however this is a widely used and accepted technique for cell lysis, where custom made scientific pestle and mortars have been designed for the purpose. In this study, intact tissue samples were incubated in agonist for 30 minutes at 37°C, such that cellular signalling was retained, and the samples were then snap frozen in this state. Samples were then crushed in liquid nitrogen to retain enzymatic activity, thawed and ROS production in treated samples was compared to control samples. An increased ROS production was observed in treated samples, and the use of control tissue suggests this is a true phenomenon. However, this technique relies on the enzymes that are activated by TRPV4 and that are responsible for the increased ROS, remaining active throughout the experimental time course, making the findings dubious. Other methods for ROS detection exist, permitting changes in ROS to be observed in tissue sections and cell preparations using fluorescence microscopy (briefly described here [Bubolz et al., 2012]). These preparations can be incubated in agonist in the same way as before, with the addition of fluorescent dyes that probe for ROS. These techniques would allow measurement of TRPV4-induced ROS production, whilst retaining cellular signalling functions and thus the functional link between TRPV4 and the enzyme responsible for the ROS production. Incubation with various antagonists could also be employed to elucidate the underlying mechanisms. As such, our findings provide a preliminary indication of a role for TRPV4 in ROS production, where improved methods are needed to further evidence this.

5.2.3 SPECIFICITY OF TRPV4 RELATED DRUG COMPOUNDS

Agonists were used at concentrations that provide physiologically relevant responses, whilst maintaining a high level of specificity. The selectivity of GSK for TRPV4 was also experimentally demonstrated here. The specificity of HC-067047 has been previously demonstrated, with no effect on other TRP receptors up to concentrations of 5μM (Everaerts et al., 2010c), where the IC₅₀ (140nM) was determined in mouse urothelial cells, making this
value relevant and applicable to this study (Everaerts et al., 2010c). As such, the conclusions drawn using these compounds are reliable.

In summary, all possible appropriate measures were taken to provide data of the highest accuracy, representing the true functional output as closely as possible. The main limitation of this study was lack of human samples, however data from those provided has indicated important differences between normal and overactive bladders that will likely be confirmed with further biopsies.

### 5.3 CONCLUSIONS

The studies within this thesis have provided novel information about the functional roles of TRPV4 within the bladder and the underlying mechanisms. Changes in the receptors expression, activity and interactions with other fundamental receptors have also been identified. Key conclusions are listed below:

- TRPV4 is predominantly expressed in the urothelium, with lower level expression in the suburothelium and DSM.
- Selective TRPV4 activation evokes mucosal ATP release that is:
  - tightly dependent on extracellular Ca\(^{2+}\) entry.
  - mediated by conductive pathways, likely via pannexin-1 channels.
  - dependent on PTK activity.
- Selective blockade of TRPV4 reduces basal ATP release and a proportion of stretch-evoked ATP release from the mucosa.
- Selective TRPV4 activation evokes strong and sustained contractions via direct action on DSM.
- Expression is significantly increased in both the mucosa and DSM in aging bladders.
- The ATP release evoked by co-activation of TRPV4 with P2Y\(_2\)/P2Y\(_4\) is augmented in aging mucosa.
- Basal and TRPV4-mediated ATP release is higher in human mucosa from overactive bladders than from normal bladders.

Importantly, the results demonstrate the multifactorial means by which TRPV4 activity can potentially influence overactivity and thus, provide further rationale for therapeutic intervention of OAB through the pharmacological modulation this receptor.

Therefore, careful pharmacological modulation of this receptor may be a promising novel approach in the treatment of OAB syndrome, however further in vivo research is required.
5.4 FUTURE DIRECTION

5.4.1 MECHANISM UNDERLYING TRPV4-MEDIATED ATP RELEASE

The first results chapter of this thesis identified a role for TRPV4 in mucosal ATP release and partly elucidated the underlying mechanism. However, further experiments are required to establish the full mechanism.

It was proposed that pannexin-1 channels facilitate the ATP release, based on our finding using carbenoxolone (broad connexin/pannexin blocker) and the current literature. Therefore, assessing the effect of a pannexin-1 specific inhibitor, such as pannexin-1 mimetic inhibitory peptide \((^{10}\text{Panx})\) on TRPV4-mediated ATP release would confirm this. Patch clamp technique could then be used to determine whether \(\text{Ca}^{2+}\) directly activates pannexin-1 channels, which has been suggested, or if it is through an indirect second messenger. This would provide a fully comprehensive mechanism for TRPV-mediated ATP release.

5.4.2 CHANNELS RESPONSIBLE FOR STRETCH-INDUCED ATP RELEASE

Stretch experiments revealed that TRPV4 is partly responsible for stretch-evoked ATP release with a 50% contribution. Current literature using amiloride suggests ENaC channels are responsible for 90-100% of the stretch-evoked ATP release. To confirm the relative contributions definitively, the effect of benzamil, a more potent ENaC channel blocker and HC-067047 on stretch-evoked ATP release should be assessed individually and in combination. This may also indicate the involvement of other receptors not yet identified.

5.4.3 TRPV4-MEDIATED ROS PRODUCTION

An increased ROS production was identified in bladder tissue after challenge with GSK. This should be confirmed using whole tissue and single cell techniques by fluorescence microscopy, which retain cell signaling functions. The role of NOX5 in this ROS production should also be assessed by first determining the expression of NOX5 in the bladder and then through inhibition of the enzyme.
5.4.4 A ROLE FOR TRPV4 IN BASAL ATP RELEASE

A reduced basal ATP release was observed upon blockade of TRPV4. Further experiments could identify the endogenous activator responsible for basal TRPV4 activity, for example through blockade of likely candidates including EETs, Ca\(^{2+}\)-CaM and IP\(_3\).

5.4.5 TRPV4 EXPRESSION IN AGING AND PATHOLOGICAL BLADDERS

The second results chapter of this thesis identified an increased TRPV4 expression in aging GPs. This should also be examined in young and aging human bladders, which will be performed once further samples for each group have been obtained. It would also be interesting to compare the expression of TRPV4 in normal and overactive human bladders, which again will be done with further samples.

5.4.6 A FUNCTIONAL LINK BETWEEN TRPV4 AND P2Y RECEPTORS

The study also identified an increased mucosal ATP release in aging animals during co-activation of TRPV4 with P2Y\(_2\)/P2Y\(_4\), suggesting a functional interaction. Co-precipitation assays should be performed to confirm a physical link between the two receptors and also identify the responsible purinoceptor subtype.

The most important future work for this project is to continue to examine the effects of OAB on TRPV4 expression and TRPV4-mediated ATP release in human tissue.
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kinase-dependent tyrosine phosphorylation of TRPV4 on TYR-253 mediates its
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TRPV4 channels and an absence of capsaicin-evoked currents in freshly-isolated,

localizations of the transient receptor potential channels TRPV4 and TRPV1 in the


