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Variations between G protein-coupled receptor-mediated signalling pathways in the urinary bladder

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Variations between G protein-coupled receptor-mediated signalling pathways in the urinary bladder

Charlotte Phelps

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Associate Professor Christian Moro and Professor Russ Chess-Williams

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Abstract

One of the most common causes of lower urinary tract symptoms are a result of dysfunction to the bladder's ability to contract. In normal micturition, the detrusor smooth muscle responds to acetylcholine to facilitate voiding and as a result, the main pharmaceutical treatments target the muscarinic receptors. However, other receptors may also be involved in bladder contractions. Aside from the muscarinic receptors, stimulation of a variety of other receptors and systems in the urinary bladder tissue are known to cause strong and sustained contractions. These include the histamine (H1), 5-hydroxytryptamine (5-HT_{2A}, serotonin), neurokinin (NK2), prostaglandin (PGE2), and angiotensin-II (ATII) receptors. Any link to contractions provides a potential association with contractile disorders, such as overactive and underactive bladder. However, the mechanisms underlying contraction, including the second messengers and associated receptor pathways, are not fully understood. As such, investigating the contractile-mediating receptors and their downstream signalling pathways presents an area of interest.

This thesis aimed to investigate the G protein-coupled receptor systems (GPCRs) which are known to mediate strong contractions in urinary bladder tissue: muscarinic; histamine; 5-HT; NK; PGE2; and ATII, and to determine the signalling pathways underlying their identified responses. As age is often correlated with increased urinary tract symptoms, age-related alterations to the signalling pathways underlying receptor-mediated contractions within the detrusor smooth muscle were also investigated. To achieve these aims, isolated tissue baths containing adjacent strips of urothelium with lamina propria (U&LP), or detrusor smooth muscle, were used to observe the contractile activity in response to receptor activation with various agonists. The influence of extracellular calcium (Ca^{2+}), intracellular Ca^{2+}, and Rho kinase in receptor-mediated contractions of the urinary bladder was also assessed.

In response to agonist stimulation, muscarinic, histamine, 5-HT, NK, PGE2, and ATII receptors in both the U&LP and detrusor, demonstrated increases in baseline tension and spontaneous phasic contractile activity. The magnitude of influence of Ca^{2+} from extracellular sources varied between the receptors but was responsible for approximately 20-50% of the contractile activity of the six GPCRs in the U&LP. A prominent role of L-type Ca^{2+} channels in the cell membrane was identified in the U&LP for receptor-mediated contractions, due to a lack of any differences identified between responses after directly removing Ca^{2+} from the extracellular fluid or using an L-type Ca^{2+} channel antagonist, nifedipine. The contribution of Ca^{2+} from intracellular stores was also investigated. In the U&LP, histamine exhibited the only significant dependence on intracellular Ca^{2+}, which was responsible for 40% of the contraction. Alternatively, in the detrusor smooth muscle, intracellular Ca^{2+} was responsible for 38% of contractions to NKA and 35% of contractions to PGE2. Contractions induced by stimulating the muscarinic, histamine, 5-HT and ATII receptors were not significantly affected by inhibiting intracellular Ca^{2+}. The influence of Rho kinase was also investigated for this pathway's contribution to GCPR-mediated contractions. Both the U&LP and detrusor of juvenile and adult porcine urinary bladders had a strong dependence on Rho kinase for contraction, and the Rho kinase inhibitor Y-27632 abolished contractions for all six GPCRs assessed by approximately 50%.

Uncovering insights into the mechanisms underlying novel receptor systems in the urinary bladder may present novel therapeutic targets for future pharmacological therapies in the management of bladder disorders. This may also provide further understanding of the pathophysiology underlying bladder contractile disorders, such as underactive and overactive bladder.

Keywords: urinary bladder, urothelium, lamina propria, mucosa, detrusor, smooth muscle, spontaneous contractions, G protein-coupled receptor, calcium, Rho kinase.

Declaration by Author

This thesis is submitted to Bond University in fulfilment of the requirements of the degree of Doctor of Philosophy by Research.

This thesis represents my own original work towards this research degree and contains no material that has previously been submitted for a degree or diploma at this University or any other institution, except where due acknowledgement is made.

No artificial intelligence or generative AI text was used or employed in the drafting, writing or production of any aspect of this thesis or the works within.

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Declaration of Author Contributions

The following people contributed to the publication of work undertaken as a part of this thesis:

i. Charlotte Phelps (CP),

ii. Russ Chess-Williams (RCW),

iii. Christian Moro (CM).

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Research Outputs

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2. Phelps, C., Chess-Williams, R., Moro, C. (2023). The role of intracellular calcium and Rho kinase pathways in G protein-coupled receptor-mediated contractions of urinary bladder urothelium and lamina propria. *American Journal of Physiology – Cell Physiology, 324*(3), C787-C797. https://doi.org/10.1152/ajpcell.00441.2022

3. Phelps, C., Chess-Williams, R., & Moro, C. (2022). The dependence of urinary bladder responses on extracellular calcium varies between muscarinic, histamine, 5-HT (serotonin), neurokinin, prostaglandin, and angiotensin receptor activation. *Frontiers in Physiology, 13,* 1-9. https://doi.org/10.3389/fphys.2022.841181

4. Phelps, C., & Moro, C. (2022). Ageing and the bladder mucosa: A scoping review of recent animal model studies. *The Australian and New Zealand Continence Journal, 28*(4), 77-83. https://doi.org/10.33235/anzcj.28.4.77-83

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3. Phelps, C., Chess-Williams, R., & Moro, C. (2022, October 14). Future therapies for the treatment of bladder contractile disorders? *ASCEPT Special Interest Group Virtual National Symposium – Advances in Urogenital and Gastrointestinal Research.* Virtual conference.

4. Phelps, C., Chess-Williams, R., & Moro, C. (2021, November). Identifying novel mediators of contraction within the urinary bladder urothelium and lamina propria tissue layers. *Proceedings of the Australian Physiological Society 60th Diamond Jubilee Conference*. Gold Coast, Queensland, Australia.

5. Phelps, C., Chess-Williams, R., & Moro, C. (2021, July). Potential targets for underactive bladder treatments: Receptor-mediated contractions of the urinary bladder urothelium. *Physiology 2021.* United Kingdom, virtual conference.

6. Phelps, C., Chess-Williams, R., & Moro, C. (2021, October). Novel targets for the pharmaceutical management of bladder contractile disorders: Identifying mediators of contraction in the urinary bladder urothelium. *ASCEPT Special Interest Group Virtual National Symposium on Advances in Urogenital and Gut Research Symposium.* Virtual conference.

7. Phelps, C., Chess-Williams, R., & Moro, C. (2021, October). Extracellular ion channels for the induction of bladder urothelium and lamina propria contractions. *Bond University 2021 Pitching Research Competition.* Gold Coast, Queensland, Australia. **Winner of a $1000 prize for Overall Winner and a $500 prize for People's Choice.**

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2. Moro, C., Mills, K. A., & Phelps, C. (2024). The CRAFTS learning framework: equipping learners to create relevant, accessible, fun, tailored and scholarly activities in higher education. *Interactive Learning Environments*, 1-12. https://doi.org/10.1080/10494820.2024.2308100

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5. Michelle, M., Phelps, C., & Moro, C. (2023). Medical students as advocates for a healthy planet and healthy people: Designing an assessment that prepares learners to take action on the United Nations Sustainable Development Goals. *Medical Teacher, 45*(10), 1183-1187. https://doi.org/10.1080/0142159X.2023.2225721

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Education: A Randomized Controlled Trial. *Medical Science Educator, 32,* 1579–1586. https://doi.org/10.1007/s40670-022-01635-5

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15. Moro, C., & Phelps, C. (2022). Smartphone-based augmented reality physiology and anatomy laboratories. *Medical Education, 56*(5), 575-576. https://doi.org/https://doi.org/10.1111/medu.14756

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17. Stromberga Z., Phelps C., Smith J., Moro C. (2021). Teaching with disruptive technology: The use of augmented, virtual, and mixed reality (HoloLens) for disease education. In: Rea P.M. (eds) Biomedical Visualisation. *Advances in Experimental Medicine and Biology*, vol 1317. Springer, Cham. https://doi.org/10.1007/978-3-030-61125-5_8

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2. Moro, C., & Phelps, C. (2022, November). Incorporating planetary health concepts into physiology. *AuPS Scientific Meeting.* Hobart, Tasmania, Australia.

3. Moro, C., & Phelps, C. (2022, July). Should assessments match modern teaching methods within physiology? *The Australian and New Zealand Association for Health Professional Educators (ANZAPHE) Festival 2022.* Virtual conference.

4. Moro, C., & Phelps, C. (2021, December). Do assessments match modern teaching methods within today's curricula? *ASCEPT 2nd Virtual Education Symposium*. Virtual conference.

5. Moro, C., & Phelps, C. (2021, November). Equally engaging both face to face and online students during live lectures with interactive polling. *AuPS Scientific Meeting 2021*. Gold Coast, Queensland, Australia.

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8. Veer, V., Phelps, C., & Moro, C. (2021, April). Using 3D holographic technology (HoloLens) for asthma education in health sciences and medicine. *Future Physiology 2021*. United Kingdom, virtual conference.

9. Phelps, C., & Moro, C. (2021, April). Interactive quizzing in online classes: The suitability of Kahoot! for face-to-face and online delivery in health sciences and medical education. *Future Physiology 202*1. United Kingdom, virtual conference.

Ethics Declaration

Information on ethical procedures is stated within each relevant chapter.

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1. Chapter 3: Phelps, C., Chess-Williams, R., & Moro, C. (2022). The dependence of urinary bladder responses on extracellular calcium varies between muscarinic, histamine, 5-HT (serotonin), neurokinin, prostaglandin, and angiotensin receptor activation. *Frontiers in Physiology, 13,* 1-9. https://doi.org/10.3389/fphys.2022.841181 under the terms of the Creative Commons Attribution 4.0 International licence (CC BY 4.0 DEED).
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3. Chapter 5: Phelps, C., & Moro, C. (2023). Ageing and the bladder mucosa: A scoping review of animal model studies from the last five years. *The Australian and New Zealand Continence Journal, 28*(4): 77-83. https://doi.org/10.33235/anzcj.28.4.77-83 under the terms of the Creative Commons Attribution 4.0 International licence (CC BY 4.0 DEED).
4. Chapter 6: Phelps, C., Chess-Williams, R., & Moro, C. (2023). Ageing influences detrusor contractions to prostaglandin, angiotensin, histamine and 5-HT (serotonin), independent to the Rho kinase and extracellular calcium pathways. Scientific Reports, 13. https://doi.org/10.1038/s41598-023-44916-8 under the terms of the Creative Commons Attribution 4.0 International licence (CC BY 4.0 DEED).

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Abbreviations

5-hydroxytryptamine	5-HT
Angiotensin II	ATII
Adenosine triphosphate	ATP
Calcium	Ca^{2+}
Coexistent overactive-underactive bladder	COUB
Diacylglycerol	DAG
Dimethyl sulfoxide	DMSO
Detrusor overactivity	DO
Detrusor underactivity	DU
Guanosine diphosphate	GDP
G protein-coupled receptor	GPCR
Guanosine triphosphate	GTP
Interstitial cells of Cajal	ICC
Inositol trisphosphate	IP3
Lower urinary tract	LUT
Myosin light chain kinase	MLCK
Neurokinin-A	NKA
Protein kinase C	PKC
Phospholipase C	PLC
Overactive bladder	OAB
Prostaglandin E2	PGE2
Underactive bladder	UAB
Urothelium and lamina propria	U&LP

Chapter 1

Literature review

1.1 Anatomy of the urinary bladder

The urinary bladder is one of the primary structures comprising the lower urinary tract (LUT), along with the urethra and the prostate in males. This structure has a high degree of variability between genders, as a result of differences in pelvic anatomy and sexual functions. In both males and females, the urinary bladder is situated posteriorly to the symphysis pubis and anterior to the pelvic cavity. The area of the cavity occupied by the bladder is dependent on the volume of fluid within the vesical lumen (DeLancey et al., 2002). In females, the urinary bladder is closely related to the anterior vaginal wall and uterus (Figure 1-1), and in males is located superior to the prostate gland and directly anterior to the rectum (Figure 1-2). Due to its origination from the urogenital sinus, the bladder remains loosely associated with the anterior abdominal wall and the median umbilical ligament (Ramakrishnan & Eswara, 2020).

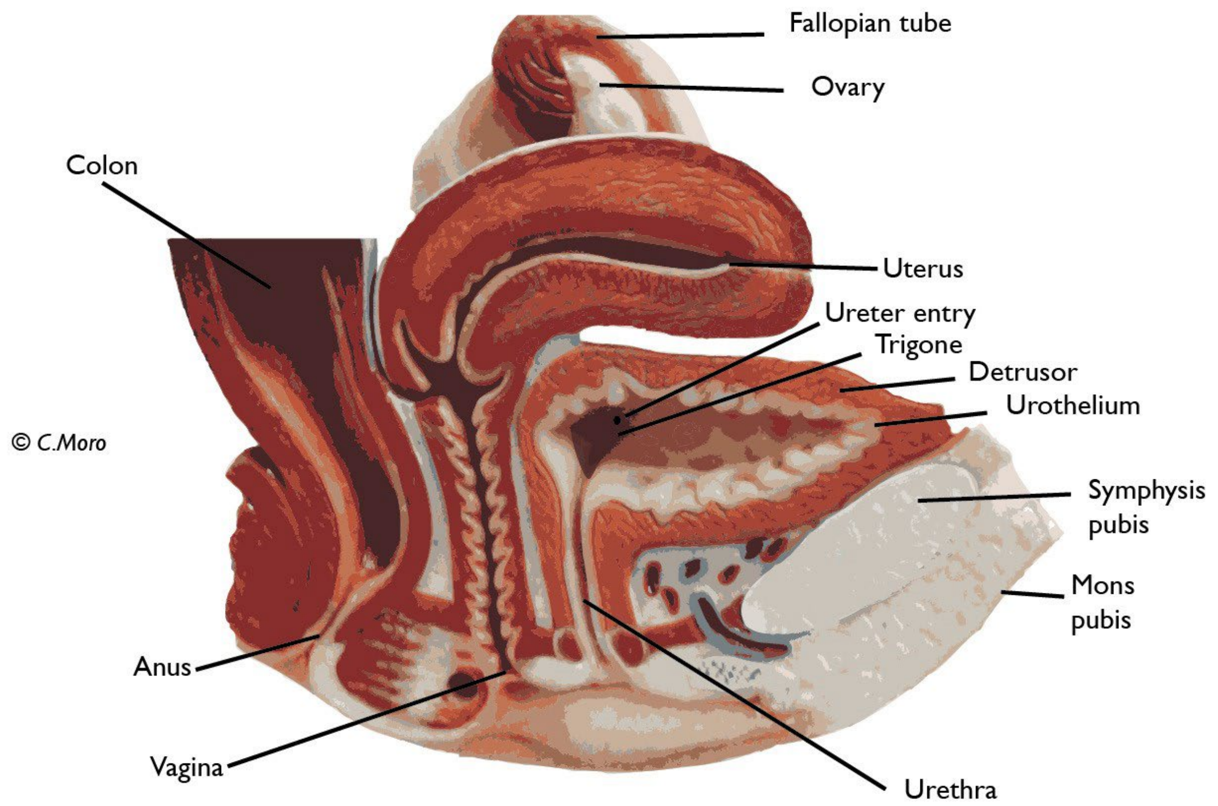

Figure 1-1: Depiction of female lower urinary tract anatomy and associated structures. Reproduced with permission by C Moro.

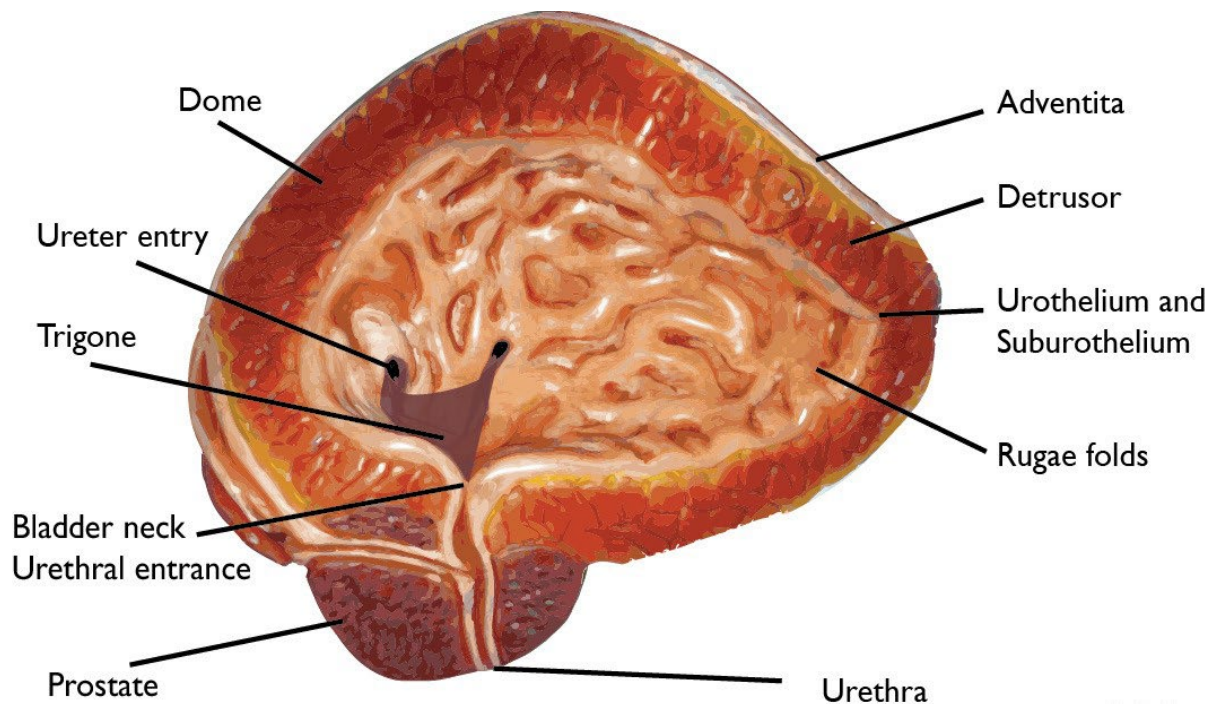

Figure 1-2: Depiction of male lower urinary tract anatomy. Reproduced with permission by C Moro.

The urinary bladder is a hollow organ that is round or oval shaped in its distended form and a flattened tetrahedron when empty. In the adult bladder, the volume of urine it can typically hold is 500mL (Shermadou et al., 2021). The anatomical position of the urinary bladder varies and is dependent on the volume of urine stored and the resulting degree of distention. When empty, it is situated entirely in the true pelvis, but as it fills it expands and becomes anterosuperior to the abdominal cavity and preperitoneal space (Brenner, 2019). It is interesting to note that in the neonate and infant, the bladder only lies partially in the pelvis, and it is not until six to eight years of age that it becomes truly pelvic (Lander & Newman, 2013).

The urinary bladder structure can be divided into three distinct regions:

- Dome: The dome forms the body of the bladder, which lays above the ureteral orifices. It consists of detrusor smooth muscle, which is bound by the urothelial layer on the inner surface and the adventitia on the exterior.
- Bladder neck: The lowest part of the bladder where the inferolateral surfaces meet the base. In the male, it lies directly on the prostate and in females the bladder neck and urethra lie in the connective tissue of the anterior vaginal wall.
- Trigone: Triangular-shaped region that is located at the base of the bladder. This structure anchors the terminal ureters to the bladder base, allowing the efflux of urine and preventing reflux back into the ureters. The three angles include the two ureteric orifices and the internal urethral orifice.

1.2 Urinary bladder wall

Four distinct tissue layers contribute to the structure of the urinary bladder wall. From the innermost luminal layer, these include the urothelium, lamina propria, detrusor smooth muscle, and adventitia (Figure 1-3) (Bolla et al., 2021). A specialised layer of epithelial tissue lines the bladder lumen, termed the urothelium. Directly beneath the basement membrane of the urothelium is the lamina propria, a layer of connective tissue with rich vasculature and nerve supply. A clearly defined basal lamina forms a distinct separation between the urothelium and lamina propria layers (Jost et al., 1989). The detrusor smooth muscle layer provides the bulk of the bladder wall and is protected by an external adventitia/serosa and an internal tight epithelial layer. The thickness of the bladder wall is negatively correlated with bladder volume (Fananapazir et al., 2018), with an average bladder wall thickness in normal males and females (mean ± standard deviation) of 3.35 ± 1.15 mm (0.7 to 7.6 mm, range) (Hakenberg et al., 2000). There are significant gender differences in bladder wall thickness, with an average thickness of 3.33 ± 0.08 in men and 3.04 ± 0.06 in women (Hakenberg et al., 2000). The thickness of the bladder wall is smaller in children compared to adults, and there is also a correlation in children between bladder wall thickness and body mass index or height (Uluocak et al., 2007). Further, age in adult populations appears to be a predictor of urinary bladder wall thickness (Anzia et al., 2021), and increases in thickness tend to be greater in males than females with increasing age (Hakenberg et al., 2000).

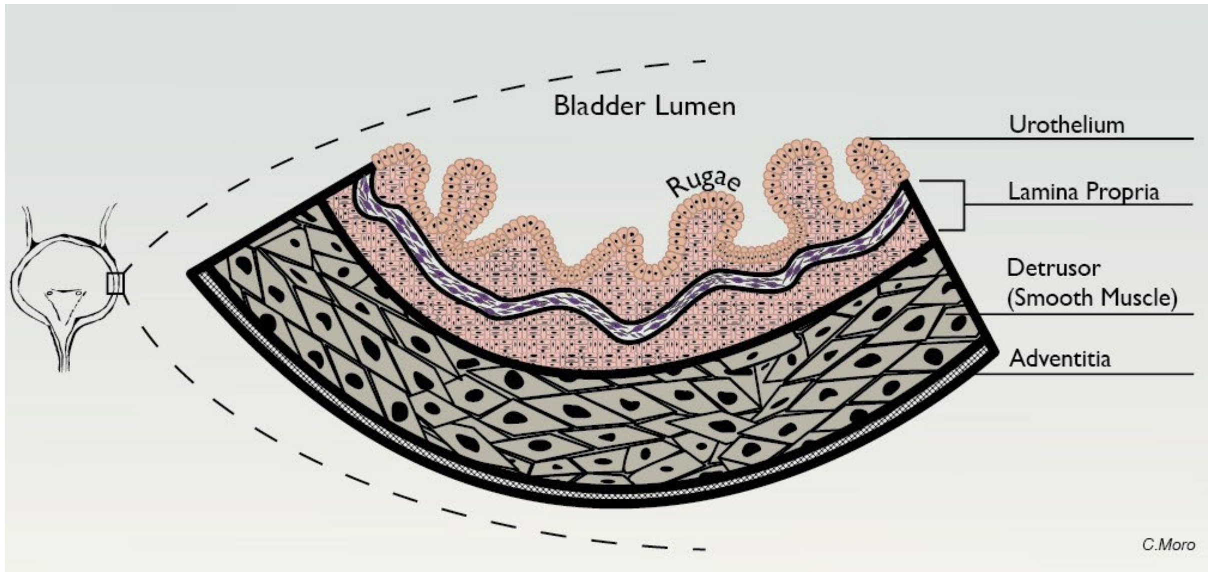

Figure 1-3: Illustration of the four distinct layers of the urinary bladder wall, including the urothelium, lamina propria, detrusor and adventitia. Reproduced with permission by C Moro.

1.2.1 *Urothelium*

Lining the lumen of the urinary bladder is the urothelium. It is a stratified epithelium, also classified as transitional epithelium, as the cell structure and arrangement is highly variant depending on the degree of stretch on the bladder wall. When empty, the urothelium is highly folded to form rugae, and the urothelial cells maintain a cuboidal structure. During the filling process, the rugae unfold to increase the internal surface area of the bladder (Li et al., 2023) and when fully distended, the urothelial cells flatten and transition to a squamous shape. Once believed to be pseudostratified in nature, the transitional urothelium is now accepted as stratified in nature due to its regular architecture (Jost et al., 1989). This layer of tissue exhibits one of the slowest turnover rates of all human epithelium, at a rate of 200 days in homeostatic conditions (Hicks, 1975; Jost & Potten, 1986). However, urothelial proliferation is accelerated in pathologic conditions, with complete restoration observed within 10 days (Lavelle et al., 2002; Romih et al., 2001). The bladder urothelium typically consists of 3-6 cell layers, and regardless of the number of cell layers, share the same basic design, including basal cells, intermediate cells, and the outermost umbrella cells in direct contact with the lumen.

Umbrella cells, also termed superficial cells, form a single layer of highly differentiated and polarised cells in contact with the lumen. In the empty bladder, umbrella cells maintain a cuboidal structure, however, when the bladder distends, the cells stretch and transition to a squamous morphology (Khandelwal et al., 2009). The umbrella cells are interconnected by tight junctions, forming the impermeable protective layer of the bladder wall to protect against water, protons, urea, and ammonia (Chang et al., 1994). Normal urine contains toxic substances, such as protamine sulfate and low molecular weight products, that are capable of injuring the bladder, which can lead to increased epithelial permeability, as indicated by potassium sensitivity, and in some cases develop cystitis symptoms (Rajasekaran et al., 2006).

Along with tight junctions, the impenetrable nature of the bladder wall is also maintained by the presence of desmosomes, specialised ion pumps, and a thick layer of glycosaminoglycans (National Research Council Subcommittee on Biologic Markers in Urinary Toxicology, 1995). The barrier function of the urothelium can be divided into three distinct regions: the apical barrier, lateral barrier, and basal barrier. The apical barrier is made up of urothelial plaque that covers approximately 90% of the surface and contains a group of transmembrane proteins called uroplakins to form a flexible layer (Jackson et al., 2020). In addition, the thick mucus layer of highly hydrophilic glycosaminoglycans at the apical surface of the urothelium absorb water micelles that attach to the sulfate polysaccharides and sandwich between the cells of the bladder surface to resist entry of harmful substances (Parsons et al., 1988). The lateral barrier consists of soft and tight junctions, and the basal barrier is made of a group of proteins including cadherin, claudins, and laminins (Klingler, 2016).

Deep to the umbrella cells are one or more strata of intermediate cells, whose nuclei assume intermediate degrees of shape and chromatin configuration (Jost et al., 1989). The thickness of the overall intermediate cell layer varies with the degree of distention, appearing to have fewer cells in the full bladder state. How this is achieved is not fully understood, however it may be explained by adjacent layers of intermediate cells sliding past one another during the filling stage (Khandelwal et al., 2009). The intermediate cell layer is also believed to contribute to the proliferation of urothelial cells during injury, as indicated by the presence of progenitor cells (Colopy et al., 2014). The basal cell layer of the urothelium is characterised by keratin 5-expressing basal cells (K5-BC). Homeostatic repair of the urothelium is attributed to the differentiation of K5-BCs into intermediate and superficial cells (Colopy et al., 2014). A single layer of undifferentiated basal cells forms contacts with the capillary beds at the basement membrane of the urothelium (Hossler & Monson, 1995).

Until recently, the bladder urothelium was characterised solely for its protection functions. There is now increasing interest in its signalling properties, as each urothelial cell type express a range of receptors that enable it to sense and respond to a variety of physical, chemical, and biological stimuli (Apodaca et al., 2007; Birder & de Groat, 2007). The urothelium is known to release various mediators, including adenosine triphosphate (ATP) (Ferguson et al., 1997), acetylcholine (Moro et al., 2011), nitric oxide (Birder et al., 1998), prostaglandins (Downie & Karmazyn, 1984), and possible nerve growth factor (Birder et al., 2007), which have excitatory or inhibitory actions on afferent nerves located close to or within the urothelium. The release of these mediators is controlled by the various receptors and ion channels (Ochodnický et al., 2012) expressed on urothelial cells, which respond to diverse stimuli from many sources including stretch and distention during bladder filling, nerve growth factor, chemokines, changes in pH due to inflammation, and acetylcholine, ATP or noradrenaline released from nerves and inflammatory cells (Merrill et al., 2016). The presence of an intact urothelium is associated with an increase of spontaneous contractile activity in whole bladder preparations (Akino et al., 2008), and the isolated urothelium and lamina propria is capable of developing spontaneous phasic contractions in the absence of any stimulation (Moro & Chess-Williams, 2012). Spontaneous contractions are enhanced by the urothelium, particularly if hypertrophied, for example in animals with neurogenic overactivity (Fry et al., 2012). This activity could be affected by the expression of receptors within the urothelial layer, for example, Kim et al. (2008) reported an increase in the expression of muscarinic and purinergic receptors in the urothelium of rats with detrusor overactivity induced by bladder outlet obstruction. On the other hand, Mansfield et al. (2005) reported a decline in the expression of muscarinic receptors in the normal ageing bladder, which could result in the development of underactive bladder symptoms.

1.2.2 *Lamina propria*

The lamina propria, also known as the submucosa or suburothelium, is a layer of loose connective tissue that is situated beneath the distinct basement membrane of the urothelium. Together with the urothelium, these two layers form the mucosa (Birder & Andersson, 2013), which will be referred to as the urothelium and lamina propria (U&LP) throughout this thesis. In the human urinary bladder, the lamina propria varies in thickness, ranging from 500µm in the fundus and 100µm in the trigone (Brenner, 2019). It has a rich vascular network, consisting of capillaries and lymphatic vessels, as well as structural matrix components, including elastic and collagen fibres. The extracellular matrix also contains various cell types, including myofibroblasts, endothelial cells, pericytes, adipocytes, and afferent and efferent nerve endings (Gabella, 2019). Underlying the connective tissue layer of the lamina propria and bordering the detrusor layer is the muscularis mucosae, a thin layer of musculature containing smooth muscle fascicles that has been identified in guinea-pig (Heppner et al., 2011), pig (Mitsui et al., 2020), and human bladder (Dixon & Gosling, 1983). However, compared to the muscularis mucosae in the intestines, this layer in the bladder is less well-defined and muscle fibres are irregularly arranged (Dixon & Gosling, 1983). There is increasing interest in the role of the muscularis mucosae in developing spontaneous contractions in the urothelium and lamina propria layers (Drake et al., 2018; Fry & Vahabi, 2016; Mitsui et al., 2020), which might function to prevent microvasculature stretching upon bladder wall distension during the storage phase (Lee et al., 2016).

One cell population of interest within the lamina propria are the myofibroblasts, which have similar characteristics to both smooth muscle cells and fibroblasts. The myofibroblasts, also described in the literature as interstitial cells, interstitial cells of Cajal (ICC), ICC-like cells, fibroblasts, or c-kit+ cells, have been identified in various tissues throughout the body,

including the gastrointestinal tract (GIT) (Sanders, 1996), testes (Holstein et al., 1996), urethra (Sergeant et al., 2000), and urinary bladder (McCloskey, 2010). A layer of these cells is particularly evident at the base of the urothelium (Andersson & McCloskey, 2014). They form a network interconnected by Cx43 gap junctions for cell-to-cell communication and are closely associated with afferent nerves and smooth muscle, acting as an integrating network for signals and responses throughout the bladder wall (Sui et al., 2002).

The primary role of myofibroblasts is in growth, repair, and wound healing (Powell et al., 1999), however, they have also been found to contribute to smooth muscle spontaneous activity (Brading & McCloskey, 2005; Sanders, 1996). Similar cells found in the GIT are known to be the origin of pacemaker signals underlying spontaneous activity (Sanders et al., 2006). In the urinary bladder, myofibroblasts have been shown to be involved in the modulation of detrusor smooth muscle contractile activity, suggested by strong spontaneous contractions in mucosa-intact tissue strips compared to mucosa-denuded tissue in the diabetic overactive rat bladder (Andersson & McCloskey, 2014). These cells in the lamina propria also express membrane receptors, such as M3 muscarinic receptors responsible for smooth muscle contraction (Grol et al., 2009; Mukerji et al., 2006), as well as prostaglandin E_2 receptors (EP1 and EP2) (Rahnama'i et al., 2010). It has also been suggested that myofibroblasts are involved in relaying sensory information to regions of the bladder that are poorly innervated (Nile & Gillespie, 2012). Their role in regulating spontaneous contractile activity is thought to be mediated by electrical and calcium transients across the lamina propria (Fry et al., 2012; Hashitani et al., 2004). In overactive bladder and detrusor overactivity presentations, there was an increased concentration of myofibroblasts and altered distribution, resulting in increased excitability of contractile bladder tissue (Juszczak et al., 2013). Therefore, of particular interest is the investigation of chemical mediators capable of mediating spontaneous contractile activity of

the U&LP, as there is increasing evidence that these contractions are capable of impacting the function of the underlying detrusor smooth muscle (Chakrabarty et al., 2019).

However, there remains no clear consensus about the presence of true myofibroblasts in the urinary bladder (Gabella, 2019; Neuhaus et al., 2018). Myofibroblasts are contractile and immunopositive for actin, vimentin, desmin, and myosin filaments, along with fibronexus junctions. In the urinary bladder, myofibroblasts stain for vimentin and α-smooth muscle actin, but not for desmin (Cheng et al., 2011). 3D-electron microscopy characterised two types of interstitial cells (IC) that may be present in the lamina propria: fibroblast type IC (fIC) and myoid type IC (mIC) (Neuhaus et al., 2018). The fICs had more pronounced cell bodies and thicker cellular protrusions, whereas the mICs possess a flat, sheet-like morphology. The mICs are thought to resemble myofibroblasts as both cells contain the same component of the fibronexus, which is a cell surface specialisation forming cell-to-matrix junctions (Eyden, 1993). The fibronexus consists of fibronectin filaments and actin microfilaments (Singer, 1979). More recently, single-cell RNA sequencing of human and rat bladder cells identified the presence of myofibroblasts between the urothelium and detrusor layers (Zhao et al., 2023). The authors also highlighted altered signalling and decreased cell-to-cell interactions of the myofibroblasts in aged bladders, which may have further implications during ageing (Zhao et al., 2023). The presence of contractile units within these cells makes them a potential mediator of bladder contractility and a potential target for future pharmaceutical therapies.

1.2.3 *Detrusor smooth muscle*

The detrusor layer forms the bulk of the bladder, consisting of thick interlacing bundles of smooth muscle cells arranged as a complex meshwork. The meshwork consists of three layers, including a middle circular layer, and an indistinct inner and outer layer of longitudinal muscle

bundles (Brenner, 2019). This orientation of muscle fibres provides the bladder with the ability to stretch in response to filling (Sam et al., 2021). Human detrusor cells are arranged in large muscle bundles that are composed of smaller sub-bundles that run in all directions (Brading, 1987). The single units of cells have a fusiform shape with a central nucleus, ranging between 300-400μm in length and 5-6μm in diameter (DeLancey et al., 2002; Tasian et al., 2010). Detrusor smooth muscle cells express various types of ion channels, including calcium (Ca^{2+}), potassium (K^+), and chloride (Cl^-) channels (Malysz & Petkov, 2020). The primary integrator of detrusor cell excitability is the population of L-type Ca^{2+} channels, which contribute to the influx of Ca^{2+} in the intracellular space and control smooth muscle contractility. In addition, the detrusor smooth muscle cells express stretch-activated nonselective cation channels, whereby filling of the bladder with urine stretches the cells and activates the channels, contributing to the pacemaking mechanism (Wellner & Isenberg, 1993). Their activation initiates inward depolarisation of smooth muscle cells and enhances the opening of voltage-dependent Ca^{2+} channels that results in the generation of action potentials and subsequent contractions. Coordinated contraction occurs as a result of the presence of gap junctions formed by connexin subunits between muscle cells, which allows action potentials to propagate between neighbouring cells (Brink, 1998; Tasian et al., 2010).

The detrusor muscle functions through the complex coordination of relaxation and contraction to fulfil its vital functions to store urine without leakage for long periods of time and rapidly expel during micturition. During the filling phase, the smooth muscle cells relax and elongate to accommodate large volumes of urine entering the urinary bladder. During the micturition phase, force generation and shortening of muscle cells must be initiated rapidly to allow for synchronous contraction of the bladder wall (Andersson & Arner, 2004). Contractions observed in the detrusor of porcine bladders are biphasic, consisting of an initial phasic

contraction, followed by a slow tonic contraction (Uchida et al., 1994), a mechanism important for ensuring bladder emptying (DeLancey et al., 2002) . The detrusor muscle cells also exhibit spontaneous contractile activity, demonstrated in detrusor strips of human, porcine, guinea pig, and rabbit tissue (McCarthy et al., 2019; Sibley, 1984; Stromberga et al., 2020b). These are brief contractions of variable height and do not represent the maximal force they can generate. However, the presence of spontaneous contractions also suggests that an increased frequency or amplitude of detrusor contractions may result in an enhanced urge to urinate and be an underlying cause of overactive bladder (Drake et al., 2018).

Interstitial cells (IC) are another population of cells present within the detrusor smooth muscle layer, however, they are morphologically different to those found in the lamina propria. The ICs within the bladder have been described as similar in nature to the interstitial cells of Cajal located within the GIT, which are specialised pacemaker cells responsible for gut motility (Foong et al., 2020). In the human detrusor, interstitial cells are found scattered throughout the muscle layers, both intra- and inter-muscular, with a fusiform-shaped cell body containing dendritic processes, large oval nuclei and dispersed chromatin (Rasmussen et al., 2009; Shafik et al., 2004). The urinary bladder ICs may be involved in regulating the activity of the detrusor smooth muscle or nerve terminals in their respective locations (Gillespie et al., 2006; Johnston et al., 2008).

1.3 Function of the urinary bladder

The urinary bladder functions through two key stages: the storage and periodic elimination of urine. The interchange between the periodic filling/storage and voiding phases is referred to as the micturition cycle. For smooth control of the bladder, a complex interplay of nervous system

control, smooth muscle cells, skeletal muscle fibres and the urethra and urethral sphincter is required. These structures are coordinated by the activity of three sets of peripheral nerves: sacral parasympathetic (pelvic nerves); thoracolumbar sympathetic (hypogastric nerves); and somatic nerves (pudendal nerves) (de Groat, 1986). The storage reflexes become active as the bladder fills, and are primarily organised in the spinal cord, whereas the reflex mechanisms for voiding are located in the brain (Fowler et al., 2008). Parasympathetic efferent nerves induce bladder contraction and urethral relaxation, while sympathetic efferent nerves promote bladder relaxation and urethral contraction. Somatic efferent nerves contract the striated muscles of the external urethral sphincter (de Groat & Yoshimura, 2009). The ability to control bladder activity is voluntary in adults but is involuntarily controlled by an entirely spinal reflex in infants, established throughout development as connections to the central nervous system are formed (Ikeda, 2021). Disturbances to storage and voiding function may result in lower urinary tract symptoms, therefore a thorough understanding of the mechanisms involved in normal bladder control is important to target the causes underlying bladder contractile dysfunction.

1.3.1 *Storage*

Normal functional bladder capacity in adults is approximately 300 to 400 ml of urine (Fitzgerald et al., 2002; Latini et al., 2004). As the bladder fills, the tissue maintains a high degree of compliance, which is facilitated by the internal rugae of the urothelium and a dynamic rearrangement of collagen fibres (Cheng et al., 2018). During the storage phase, the urinary bladder maintains a low pressure with no global bladder contraction as it acts as a reservoir to fill. At the same time, the urethra remains closed, noncompliant, and sealed to maintain continence. Sympathetic outflow throughout the hypogastric nerve releases noradrenaline, activating beta-adrenergic receptors that provide inhibitory input to the detrusor tissue to relax the smooth muscle, and alpha-adrenergic excitatory receptors in the urethra and the bladder

neck (de Groat et al., 2011; Fowler et al., 2008). Afferent nerve fibres are activated by increases in bladder wall tension (Downie & Armour, 1992) and intravesical pressure (Häbler et al., 1993), to convey the sense of fullness to the central nervous system. Afferent activity in the lamina propria can also be potentiated by signalling molecules, such as ATP, nitric oxide, and acetylcholine released from the urothelium in response to distension (Birder & Andersson, 2013). Disturbances to the storage functions of the urinary bladder can present with symptoms of increased frequency, nocturia, urgency or urgency incontinence (Gacci et al., 2018).

1.3.2 *Voiding*

The micturition reflex is organised as an on-off circuit that switches between the filling/storage and voiding phases (Fowler et al., 2008). This switch is elicited by slow adapting mechanoreceptors in the bladder wall that respond to increasing intravesical pressure, enhancing nerve activity up to a critical threshold at which the voiding reflex is initiated (Häbler et al., 1993). Sensations of bladder fullness are conveyed to the spinal cord by the hypogastric and pelvic nerves (Fowler et al., 2008). Afferent activity is relayed to the periaqueductal grey area in the pons, which transmits the signal to the pontine micturition centre and provides the initial stimulus to facilitate voiding (Sullivan & Yalla, 2002). Parasympathetic postganglionic axons in the pelvic nerve innervate the bladder and release acetylcholine at the dome region, which acts upon M3 muscarinic receptors to stimulate detrusor contraction and initiates voiding (Andersson & Arner, 2004; Fowler et al., 2008). At the same time as this cholinergic transmission, sympathetic activity is inhibited to relax the urethral smooth muscle and bladder outlet, mediated by nitric oxide released by parasympathetic nerves (Andersson & Arner, 2004). Non-cholinergic excitatory activity is facilitated by the release of ATP acting upon $P2X_1$ purinergic receptors in the detrusor smooth muscle (Burnstock, 1972). The typical bladder function is to facilitate voiding at a frequency

of 4-10 times during the day and 0 times during the night (Wyman et al., 2020). Healthy voiding is characterised by a strong, continuous stream with complete emptying, and when necessary, an individual should be able to defer voiding without leakage (Lukacz et al., 2011). It is important to distinguish between storage and voiding symptoms when assessing lower urinary tract symptoms, and disturbances to the bladder's ability to void may present with symptoms of hesitancy, intermittency, weak stream, or straining (Gacci et al., 2018).

1.4 G protein-coupled receptors

G protein-coupled receptors (GPCRs) are the largest and most versatile family of cell surface receptor proteins (Pierce et al., 2002), with over 800 GPCRs expressed in the human genome (Fredriksson et al., 2003). They are expressed in a wide range of tissues and are responsible for various physiological processes that mediate the majority of cellular responses to external stimuli, including light, odour, hormones, neurotransmitters, and growth factors (Weis & Kobilka, 2018). GPCRs are characterised by a seven-transmembrane configuration, including three extracellular loops and three cytosolic loops, that couple with a G protein in the plasma membrane. The G proteins associated with GPCRs are heterotrimeric in nature, comprising of an α-, β- and γ-subunit (Sanders et al., 2008). The α-subunit is responsible for binding and hydrolysing guanosine triphosphate (GTP), whereas the β- and γ-subunits are associated in a tightly linked complex (Gilman, 1987). Upon ligand binding to the extracellular terminal of the GPCR, conformational changes occur in the receptor which are then transduced and mediated by the G protein complexes through the activation of the G α-subunit promoting displacement of guanosine diphosphate (GDP) with GTP and dissociation of the G protein heterotrimer subunits from the receptor and from each other. The Gα-GTP and Gβγ dimer then transmit the receptor-generated signals to downstream effector molecules and protein binding

partners until the intrinsic GTPase activity of Gα hydrolyses GTP to GDP and the inactive subunits reassociate.

GPCRs are classified based on their function and are generally referred to by their α-subunit, which divides the G proteins into four distinct subfamilies, including G_s, $G_{i/o}$, $G_{q/11}$, and $G_{12/13}$. These subfamilies regulate key effectors and generate second messengers to trigger distinct signalling cascades. The G_s subfamily is the stimulatory activator of adenylyl cyclase leading to increased cyclic adenosine monophosphate, $G_{i/o}$ activate an inhibitory α-subunit that blocks adenylyl cyclase, $G_{q/11}$ proteins activate phospholipase C and its downstream effectors, and $G_{12/13}$ activate small GTPases (Gilman, 1987).

G protein-coupled receptors function through a conserved mechanism for extracellular signal perception, responding to various stimuli, including hormones, neurotransmitters, chemokines, and Ca^{2+} ions. When extracellular signals are received by the GPCR, intracellular signalling cascades are activated via the G proteins which act on various processes, including contraction, secretion, transcription, ion channels, and enzyme activity (Neves et al., 2002). Due to the diverse range of physiological processes controlled by GPCRs, they are considered one of the most successful therapeutic targets for a broad spectrum of diseases, and present as the primary target for >50% of therapeutics currently available (Dhyani et al., 2020; Pierce et al., 2002; Venkatakrishnan et al., 2013; Zhang & Xie, 2012).

1.4.1 *Gq/11 signalling cascade*

Of the four receptor subtypes in the GPCR family, the $G_{q/11}$ subfamily is the most widely studied (Sánchez-Fernández et al., 2014). This subfamily can be further characterised by four distinct members, including $G_{\alpha q}$ and $G_{\alpha 11}$ which are ubiquitously expressed, $G_{\alpha 14}$ found in the

kidney, liver and lungs, and $G_{\alpha 15/16}$ (mouse/human orthologues, respectively) expressed in hematopoietic cells (Hubbard & Hepler, 2006). Whilst $G_{\alpha q}$ and $G_{\alpha 11}$ are distinct gene products, they have an identical number of amino acids and essentially indistinguishable structures and functions, and therefore often referred to as $G_{\alpha q/11}$ ($G_{q/11}$) receptors.

In the most common signalling pathway, $G_{q/11}$ receptors activate phospholipase C (PLC), which catalyse the hydrolysis of phosphatidylinositol biphosphate (PIP_2) to generate inositol trisphosphate (IP_3) and diacylglycerol (DAG). These second messengers serve to propagate and amplify the $G_{q/11}$-mediated signal with Ca^{2+} mobilisation. IP_3 binds to the IP_3-receptor on the surface of the endoplasmic/sarcoplasmic reticulum, leading to the release of Ca^{2+} from these intracellular stores. In addition, DAG and Ca^{2+} work together to activate protein kinase C (PKC) to further phosphorylate other modules, leading to altered cellular activity (Dhyani et al., 2020). In smooth muscle, this increase in free intracellular Ca^{2+} stimulates contraction, with binding of Ca^{2+} to calmodulin (CAM), and the resulting Ca-$^{2+}$-CAM complex activates myosin light chain kinase (MLCK), which phosphorylates MLC and promotes the interaction of myosin with actin and subsequent contraction (Figure 1-4).

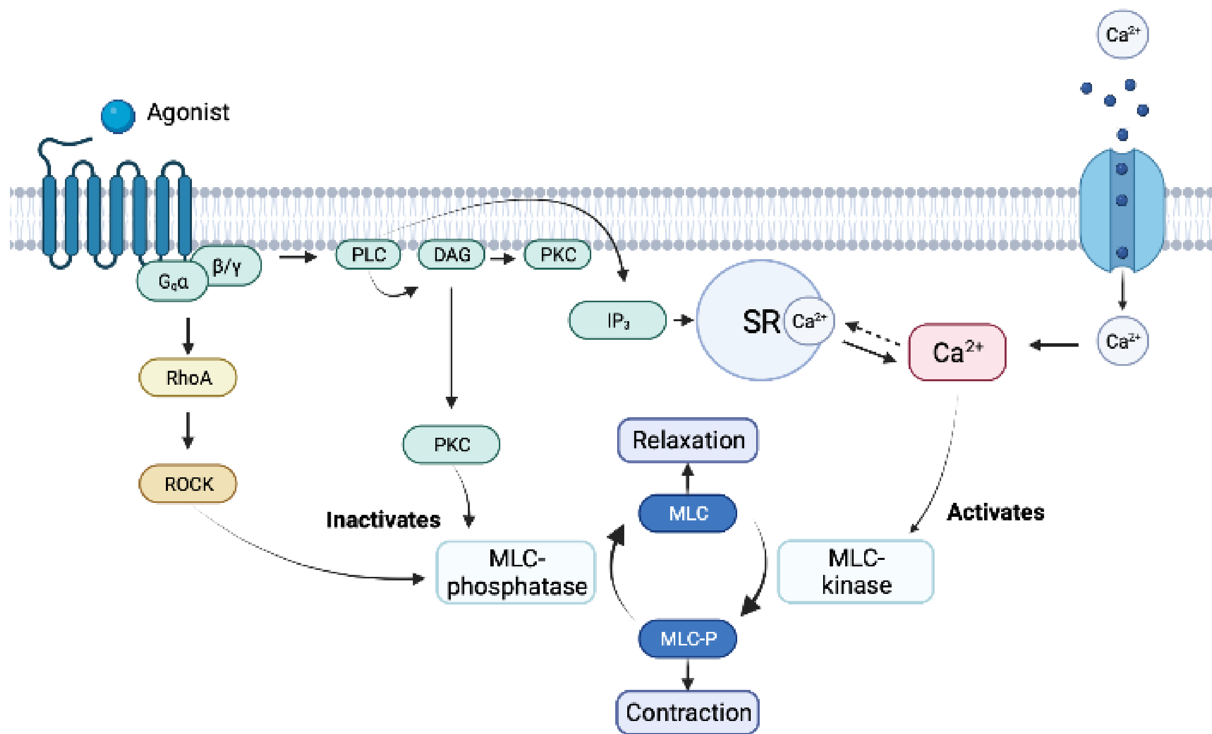

Figure 1-4: Activation of G protein-coupled receptor (Gq/11) signalling cascade. Coupling of agonists to $G_{q/11}$ proteins activate phospholipase C (PLC), with the subsequent formation of inositol trisphosphate (IP_3) and diacylglycerol (DAG) to release Ca^{2+} from intracellular stores and to activate protein kinase C (PKC), respectively. Increases in intracellular cytosolic Ca^{2+} concentration, via both release from intracellular stores and entry through voltage-gated Ca^{2+} channels on the plasma membrane, leads to activation of myosin light chain kinase (MLCK), which phosphorylates MLC and promotes the interaction of myosin with actin and subsequent contraction. $G_{q/11}$ activation can also activate the small G protein RhoA to its active form, which binds and activates Rho kinase to interact with MLC and inhibit its activity by phosphorylating and inactivating MLC phosphatase to cause contraction. Image created with BioRender (biorender.com, Toronto, ON).

1.4.2 *Rho kinase pathway*

Rho kinase (ROCK) has a central role in the regulation of smooth muscle and has been identified in many tissues throughout the body (Rattan et al., 2010; Shimokawa et al., 2016; Wang et al., 2020), including the urinary bladder (Takahashi et al., 2004; Wibberley et al., 2003). There are two isoforms of ROCK that have been identified, including ROCK-1 and ROCK-2, which are downstream targets of the ROCK activator, RhoA (Peters et al., 2006). Coupling of agonists to the GPCRs, particularly the $G_{q/11}$ and $G_{12/13}$ proteins (Mizuno & Itoh, 2009), convert the inactive small G protein RhoA to its active form, which binds and activates Rho kinase. However, it is important to note that whilst considerable evidence now indicates that $G_{q/11}$ and its family members are involved in RhoA activation, whether all members of this G protein family induce Rho activation remains to be elucidated (Mizuno & Itoh, 2009; Sah et al., 2000). Upon activation, ROCK interacts with MLC, and inhibits its activity by phosphorylating and inactivating MLC phosphatase. This, in turn, increases the proportion of MLC in its phosphorylated state and promotes further smooth muscle contraction. Pharmacological inhibitors of Rho kinase, such as fasudil and Y-27632, block its activity by competing with the ATP-binding site on the enzyme (Domokos et al., 2019). The activation of smooth muscle via the Rho kinase pathway occurs in the absence of significant changes in intracellular Ca^{2+} concentrations and, therefore considered a Ca^{2+} sensitisation mechanism (Webb, 2003).

1.4.2.1 Rho kinase pathway in the urinary bladder

ROCK-1, ROCK-2, and Rho-A are expressed in the urinary bladder at the mRNA and protein level (Takahashi et al., 2004; Wibberley et al., 2003). The role of Rho kinase in smooth muscle bladder contraction has been reported consistently across the literature, extending to muscarinic receptors (Denizalti et al., 2018; Schneider et al., 2004; Tatsumiya et al., 2009), neurokinin

receptors (Quinn et al., 2004; Wibberley et al., 2003), bradykinin receptors (Sand & Michel, 2014), prostanoids (Molnár et al., 2021), and purinoceptors (Wibberley et al., 2003). This has been confirmed by attenuation of receptor-mediated contractile responses in the presence of selective Rho-kinase inhibitors in the urinary bladder (Zhang & DiSanto, 2011). Pharmacological inhibitors of Rho kinase, fasudil and Y-28632, inhibit the activity of Rho kinase by competing with the ATP-binding site on the enzyme, which induces relaxation of the smooth muscle by blocking ROCK's inhibitory action on MLC phosphatase.

The Rho kinase pathway plays a role in the contraction of the urinary bladder through Ca^{2+} sensitisation events, achieved by modifying the sensitivity of contractile and regulatory proteins to intracellular Ca^{2+} ion concentrations (Somlyo et al., 1999). This is a Ca^{2+} concentration-independent pathway (Somlyo & Somlyo, 2003), which can maintain force generation. The ROCK pathway predominantly participates in the Ca^{2+} sensitisation induction under pathophysiological conditions. Elevated RhoA/ROCK signalling may play a role in the diabetic bladder (Nobe et al., 2009), hypertension-related overactive bladder (Rajasekaran et al., 2005), aged bladder (Kirschstein et al., 2014), as well as in the development of male urogenital disorders, such as benign prostatic hyperplasia and bladder cancer (Gur et al., 2011). As such, Rho kinase presents a novel target in the treatment of urinary bladder disorders (Peters et al., 2006). Furthermore, ROCK inhibitors could have potential advantages over front-line pharmaceuticals, antimuscarinics or parasympathomimetics, in the treatment of OAB and UAB, respectively, as they act downstream of the muscarinic receptors.

In the human detrusor smooth muscle, muscarinic receptor-mediated contraction increases intracellular Ca^{2+} concentrations, as well as increases Ca^{2+} sensitivity of contractile apparatus in a Rho kinase- and PKC-dependent manner (Takahashi et al., 2004). Itt has also been

suggested that the urothelium and lamina propria may be involved in controlling carbachol-induced contractions of the bladder via the RhoA/ROCK pathway, as demonstrated by stronger inhibitory effects of fasudil on contraction in urothelium-intact porcine tissue preparations compared to urothelium-denuded tissue (Tatsumiya et al., 2009). In addition, real-time PCR studies showed that the concentrations of mRNA and enzyme for RhoA was higher in the urothelium than the detrusor in porcine tissue (Nakanishi et al., 2009). Previous studies investigating the effects of ROCK inhibitors on urinary bladder contractile activity either have not reported the presence of the urothelial layer or utilised urothelium-intact tissues, hence this layer is a likely contributor to the enhancement of ROCK signalling pathways in the urinary bladder and warrants further investigation.

1.5 The role of Ca^{2+} in smooth muscle contraction

It has been established that G protein-coupled receptor-dependent smooth muscle contraction is mediated by activation of three key intracellular signalling pathways: Ca^{2+}-dependent pathway activated by IP3 resulting in activation of PLC; increase of Ca^{2+} sensitivity mediated by activation of PKC by DAG; increase of Ca^{2+} sensitivity mediated by receptor-dependent activation of RhoA/Rho kinase. Smooth muscle regulates intracellular Ca^{2+} concentration by altering Ca^{2+} influx via plasma membrane channels and transporters, as well as across the sarcoplasmic reticulum (SR) membrane. Extracellular Ca^{2+} channels are mobilised by Ca^{2+} concentration gradients (Harraz & Altier, 2014).

The influx of Ca^{2+} in cells requires intricate regulatory mechanisms in place to guarantee an equal balance between activation of Ca^{2+}-dependent processes. Intracellular cytosolic Ca^{2+} concentration levels play a central role in mediating diverse cellular processes, including

smooth muscle contraction and proliferation, neurotransmitter release, and Ca^{2+}-induced cell damage (Hill-Eubanks et al., 2011). In smooth muscle, the majority of Ca^{2+} used for cellular processes is stored in the sarcoplasmic reticulum as intracellular Ca^{2+}, where release is induced by receptor-mediated activation via IP3 pathways. When SR intracellular stores of Ca^{2+} are depleted, plasma membrane Ca^{2+} channels are opened (Rivera & Brading, 2006). The elevation of intracellular Ca^{2+} concentration causes contraction through Ca^{2+}-calmodulin and MLCK. On the other hand, inhibition of voltage-gated Ca^{2+} channels by hyperpolarisation mediated through either the activation of K^+ channels or inhibition of nonselective cation channels results in a decrease in intracellular Ca^{2+} and subsequently relaxes smooth muscle (Brading, 2006).

Calcium channels fall into three major gene families, Cav_1, Cav_2, and Cav_3, each containing multiple members (Zamponi et al., 2015). The Cav_1 family consists of four family members that encode L-type Ca^{2+} channels, whereas $Cav_{2.1}$, $Cav_{2.2}$, and $Cav_{2.3}$ correspond to P/Q-type, N-type, and R-type channels, respectively. Cav_3 channels comprise the family of low voltage-activated T-type Ca^{2+} channels (Zamponi et al., 2015). L-type Ca^{2+} channels are long-lasting, voltage-gated, slowly inactivating channels that first activate at relatively depolarised potentials and exhibit a high single-channel conductance. T-type channels are voltage-activated channels that show a more negative range of activation and inactivation, rapid inactivation, slow deactivation, and smaller single-channel conductance. Greater depolarisation in the cell membrane activates L-type Ca^{2+} channels, allowing for a net influx of Ca^{2+} to initiate smooth muscle contraction. However, T-type Ca^{2+} channels have a lower threshold of activation, shown to be active at the resting membrane potential and can regulate smooth muscle excitability. As more negative potentials activate the T-type channels, they also facilitate L-type Ca^{2+} channels opening (Sui et al., 2003).

1.5.1 *The role of Ca^{2+} in the urinary bladder*

The concentration of intracellular Ca^{2+} is essential for normal contractile activity of the urinary bladder. Levels of Ca^{2+} concentration in the cytosol is dependent on Ca^{2+} influx from the cell membrane via Ca^{2+} plasma membrane channels, as well as Ca^{2+} release from the intracellular stores. A primary role for Ca^{2+} channels in mediating urinary bladder contraction has been identified consistently across the literature, whereby spontaneous contractile activity is inhibited in the presence of voltage-gated Ca^{2+} channel antagonists, such as nifedipine (Katsuragi et al., 1990), as well as nimodipine, verapamil and diltiazem (Badawi et al., 2006). Two types of voltage-gated Ca^{2+} channels, L- and T-type voltage-gated Ca^{2+} channels, have been identified in the human myocyte that contribute to the spontaneous contractile activity of the urinary bladder (Sui et al., 2003), and are expressed at the mRNA and protein level across various other species (Malysz & Petkov, 2020). Wegener et al. (2004) identified an essential role for L-type Ca^{2+} channels for normal bladder function that cannot be compensated by other mechanisms. However, inhibition of the T-type Ca^{2+} channel with mibefradil does not seem to have a significant effect in the urinary bladder, except for at the highest concentration used in Badawi et al. (2006) study on potassium-precontracted muscle strips, and as such is presumed to not have an essential role in the bladder.

Ca^{2+} channels underlie the depolarization phase of the action potential that leads to an increase in global intracellular Ca^{2+} concentrations and subsequent generation of urinary bladder smooth muscle phasic contractions (Hashitani & Brading, 2003a; Heppner et al., 1997). Stretch activates non-selective cation channels, which initiates inward currents and depolarises the smooth muscle cells, subsequently opening voltage-gated Ca^{2+} channels (Wellner & Isenberg, 1993). This triggers the influx of Ca^{2+} from the extracellular space via voltage-gated Ca^{2+} channels into the intracellular cytosol, leading to the release of Ca^{2+} from the sarcoplasmic

reticulum and inducing contraction. In the urinary bladder, the release of Ca^{2+} from the SR is an important step in the activation of the detrusor muscle. Studies using blockers of SR function have demonstrated that both nerve- (McCarthy et al., 2019) and agonist-induced (Rivera & Brading, 2006), as well as stretch-induced contractions (Ji et al., 2002) of the urinary bladder are dependent on Ca^{2+} influx through the SR. Refilling of intracellular Ca^{2+} stores occurs via the influx of Ca^{2+} through L-type Ca^{2+} channels, and the influx is regulated by a feedback mechanism whereby the decrease of intracellular Ca^{2+} concentrations reduces the activity of Ca^{2+}-activated K^{+} channels, causing cell depolarisation and an enhancement of L-type Ca^{2+} channel conductance (Wu et al., 2002).

Muscarinic receptor stimulation of the urinary bladder detrusor muscle can increase the sensitivity of the contractile machinery to Ca^{2+}, leading to an influx of Ca^{2+} through L-type Ca^{2+} channels (Kishii et al., 1992). However, the contribution of extracellular Ca^{2+} influx and intracellular store release to muscarinic receptor stimulation often differs between studies (Batra et al., 1987; Rivera & Brading, 2006) and species (Wuest et al., 2007). Furthermore, other receptors systems, such as histamine (Rueda et al., 2002), neurokinin (Quinn et al., 2004), and purinergic receptors (Wu et al., 1999), have also been identified to depend on Ca^{2+} influences for urinary bladder contraction, however, there are also inconsistencies reported on the contribution of extracellular and intracellular Ca^{2+} for these systems. Therefore, there is a need to identify the influence of Ca^{2+} sources in G protein-coupled receptor-mediated contraction to further the understanding of the mechanisms underlying urinary bladder contractile activity.

Interestingly, in pathological states, the propagation of Ca^{2+} signals across the urinary bladder wall is faster compared with controls, which may be due to faster propagation between cells in

the U&LP, where Cx26 and Cx43 expression is increased, and more effective coupling between detrusor bundles (Ikeda et al., 2007). Across the years, there has been increasing interest towards the use of GPCRs as targets for pharmaceutical treatments, which are responsible for the modulation of Ca^{2+} signalling in various disease states, however additional studies are required to investigate the effect of specific drugs in a particular tissue type (Dhyani et al., 2020). Agonists or antagonists of Ca^{2+}-sensitive pathways may represent alternative targets in the treatment of bladder contractile disorders, such as underactive or overactive bladder, respectively. Therefore, the identification of common mechanisms of action between G protein-coupled receptor-mediated contractions of the urinary bladder is important in the development of future therapeutics for bladder contractile dysfunction.

1.6 G protein-coupled receptors in the urinary bladder

With stimulation of the GPCRs resulting in contractions of the urinary bladder urothelium and lamina propria, there is an importance towards identifying any common mechanisms of action between them. In particular, over the years, an increasing interest has developed into the potential role of M3 muscarinic (An et al., 2002; Moro et al., 2011), H1 histamine (Kühn et al., 1996; Stromberga et al., 2019), $5\text{-}HT_{2A}$ (Mizuta et al., 2008; Moro et al., 2016), neurokinin-2 (Grundy et al., 2018), EP1 prostaglandin E2 (Stromberga et al., 2020c), and AT_1 angiotensin II (Lim et al., 2021) receptors in mediating bladder contraction, all of which belong to the $G_{q/11}$ receptor subfamily and play a role in the contractile activity of the urinary bladder.

1.6.1 *Muscarinic*

Muscarinic receptors are the primary receptor group responsible for mediating urinary bladder contractions and are therefore the main target for the treatment of contractile disorders

(Andersson, 2011; Moro et al., 2021b; Yamada et al., 2018). Activation of the muscarinic receptors was previously understood to be primarily mediated by acetylcholine released from cholinergic nerves that run through the detrusor muscle and into the lamina propria (Dixon et al., 2000). However, it is now widely accepted that acetylcholine is also released from the urothelium, identifying a non-neuronal mechanism of muscarinic receptor activation (Kim et al., 2023; Lips et al., 2007; Yoshida et al., 2006; Yoshida et al., 2008).

The muscarinic receptor family comprises five subtypes, which have been pharmaceutically identified and cloned as M1 to M5 (Caulfield & Birdsall, 1998; van Koppen & Kaiser, 2003). The odd-numbered receptors, M1, M3 and M5, are coupled to $G_{q/11}$ proteins which activate PLC, and the even-numbered receptors, M2, and M4, couple to $G_{i/o}$ to inhibit adenyl cyclase. An et al. (2002) reported that acetylcholine-induced contraction of the bladder detrusor was selectively inhibited by $G_{q/11}$ antibody, but not by other subunits, and concluded that stimulation of the M3 receptors mediates urinary bladder contractility via this receptor pathway. All five muscarinic receptor subtypes have been identified in the urinary bladder by molecular studies, with the M2 followed by the M3 receptors found to be the most predominant (Sigala et al., 2002). The M3 receptors are responsible for the contraction of the detrusor smooth muscle (Moro et al., 2011; Sellers et al., 2000b), as well as spontaneous phasic contractile activity exhibited in the urothelium and lamina propria (Moro et al., 2011). The role of the M2 receptors in urinary bladder contraction is more complex, however, it is understood that they can oppose the sympathetically mediated detrusor relaxation through inhibition of adenyl cyclase (Hegde et al., 1997). Therefore, of particular interest are the M3 receptors present in the urinary bladder, which couple to $G_{q/11}$ proteins to activate signalling cascades for muscle contraction.

Bladder function in response to direct muscarinic receptor stimulation is highly dependent on extracellular Ca^{2+} (Fovaeus et al., 1987) through translocation of extracellular Ca^{2+} via L-type Ca^{2+} channels, as well as the release of Ca^{2+} from the SR through IP_3-sensitive and -insensitive sites. Inhibition of these sites can reduce the bladder's ability to generate and sustain pressure, generate power, and perform work (Damaser et al., 1997). Low concentrations of carbachol increase intracellular Ca^{2+} levels primarily through an influx of extracellular Ca^{2+}, whereas at high concentrations, the release of Ca^{2+} from intracellular sources becomes increasingly important (Masters et al., 1999). Several studies have reported on the requirements for both extracellular and intracellular Ca^{2+} sources for urinary bladder contraction, as demonstrated by Ca^{2+} antagonist studies across multiple species, including rabbit (Damaser et al., 1997), guinea pig (Rivera & Brading, 2006), human, and porcine bladder (Wuest et al., 2007). However, there has been shown to be considerable differences in the relative contributions of Ca^{2+} influx and muscarinic receptor stimulated Ca^{2+} release between species (Wuest et al., 2007), highlighting precaution should be taken when drawing conclusions about human urinary bladder contraction when using different animal models. These signalling pathways can be altered in pathological states, as demonstrated in the diabetic state where increased expression of muscarinic receptors was identified, leading to an increased influx of extracellular Ca^{2+} and subsequent stronger contractions (Kamata et al., 1992). Furthermore, in aged models, the contractile response to muscarinic stimulation of isolated urinary bladder strips is significantly reduced, which is mediated at least in part by a decrease in the rate of Ca^{2+} entry through receptor-operated channels (Yu et al., 1997).

The density of muscarinic receptors has been reported to be 40% greater in the urothelium and lamina propria than the detrusor in the pig bladder (Hawthorn et al., 2000). In the U&LP of the human urinary bladder, all five muscarinic receptor subtypes are expressed at the mRNA level,

with the M2 and M3 subtypes showing the highest expression (Bschleipfer et al., 2007; Mansfield et al., 2005; Ochodnický et al., 2012). In addition, immunohistochemical studies have identified a high prevalence of the M2 and M3 receptors in the human U&LP (Mukerji et al., 2006; Tyagi et al., 2006). However, whilst M2 receptors predominate at the mRNA and protein level in the U&LP, frequency responses to carbachol are significantly reduced by M3 selective antagonists, while M1 and M2 selective antagonists have minimal effects, indicating spontaneous contractions of the U&LP are mediated by the M3 receptor subtype (Moro et al., 2011). The presence of muscarinic receptors in the U&LP has demonstrated responses to stretch and muscarinic agonist stimuli (Moro et al., 2011) by releasing ATP, nitric oxide, and acetylcholine. These signals can then transduce to closely located afferent nerves, myofibroblasts, and smooth muscle cells to modulate overall bladder activity (Birder et al., 2010). Antimuscarinics remain the front-line pharmaceutical treatment for overactive bladder (Robinson & Cardozo, 2019), and the U&LP may present an additional site of action for these drugs (Moro et al., 2011), however further research is required into the mechanism of action of this class of medication due to low adherence rates, and associated physical and cognitive side effects.

1.6.2 *Histamine*

Histamine is an inflammatory mediator that is stored within granules in a range of immune and non-immune cells, and it has a particularly strong presence in mast cells and basophils where it is synthesised upon activation. Histamine is present in all tissues in varying amounts and is particularly rich in the skin, connective tissue, lungs and the gastrointestinal tract (Parsons & Ganellin, 2006). It acts as a neurotransmitter in the nervous system and as a local mediator in the skin, lungs and GIT (Panula et al., 2015). In the target tissue, it causes vasodilation,

contraction of smooth muscles, and increased blood vessel permeability (Lundequist & Pejler, 2011).

Histamine exerts its effects via four G protein-coupled receptor subtypes, where it binds to H1, H2, H3, and H4 receptors (Obara et al., 2020). The H3 and H4 receptors have a higher affinity to histamine (nM range) than the H1 and H2 receptors (µM range) (Obara et al., 2020). H1 receptors are coupled to $G_{q/11}$ receptors, whereby binding of agonists stimulate the downstream signalling cascade to release intracellular Ca^{2+} and mediate extracellular Ca^{2+} entry to initiate smooth muscle contraction (Panula et al., 2015). The H2 receptors couple to G_s and H3 and H4 couple to the $G_{i/o}$ proteins.

Histamine receptors have been identified in the various tissue layers of porcine (Stromberga et al., 2019; Stromberga et al., 2020a), guinea pig (Kondo et al., 1985), and human urinary bladders (Rubinstein et al., 1987). One of the primary functions of the histaminic receptor system is the contraction of smooth muscle, including the modulation of urinary bladder contractions and spontaneous activity (Stromberga et al., 2020a). Administration of histamine has generated a contractile response via activation of H1 receptors in the isolated guinea pig (Khanna et al., 1977; Kondo et al., 1985; Poli et al., 1988), rabbit (Fredericks, 1975), and porcine bladder (Stromberga et al., 2019; Stromberga et al., 2020a). In addition, the H1 and H3 receptors have been found to be involved in inducing Ca^{2+} release through the IP_3 pathway or the modulation of N-type Ca^{2+} channels in detrusor smooth muscle cells (Neuhaus et al., 2006).

The direct mechanism of action of histamine has yet to be fully elucidated. Some authors believe that the contractile response to histamine is mediated by histamine-enhanced acetylcholine release from a site proximal to the muscle (Rubinstein et al., 1987), or histamine-

potentiated release of ATP (Patra & Westfall, 1994), rather than direct action of histamine. Histamine is likely involved in the pathology of OAB (Christensen et al., 1990), where the release of histamine has been found to cause an inflammatory response in guinea pig urinary bladder. However, the specific action of histamine, whether direct or mediated by other chemicals released in the bladder, requires further investigation.

1.6.3 *5-hydroxytryptamine*

5-hydroxytryptamine (5-HT), also known as serotonin, is a monoamine neurotransmitter that functions across various body systems, including the central nervous system, cardiovascular system, gastrointestinal tract, and genitourinary system. In non-neuronal tissues, 5-HT also has many roles, including platelet aggregation, and smooth muscle growth and contraction (Berger et al., 2009). A total of fifteen 5-HT receptors have been identified, which are divided into seven main families, from $5\text{-HT}_{1\text{-}7}$, all of which are GPCRs except 5-HT_3 which are ion channels (Kroeze et al., 2002). Several of the families also include multiple members encoded by distinct gene products (Roth et al., 2000), for example, the 5-HT_2 family currently comprises of three subtypes: 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors (Leysen, 2004). 5-HT_1 and 5-HT_5 receptors couple to the $G_{i/o}$ protein, and 5-HT_4 and 5-HT_7 couple to G_s (Alexander et al., 2011). The 5-HT_2 subtypes couple to the $G_{q/11}$ protein, leading to excitatory effects via regulation of PLC to increase IP_3 and cytosolic Ca^{2+} (Hannon & Hoyer, 2008).

In the urinary bladder, a range of 5-HT receptors have been identified to play a role in the micturition reflex and urinary continence at both central and peripheral levels (Andersson & Pehrson, 2003; Ramage, 2006). 5-HT has been shown to produce a variety of pharmacological effects on the urinary bladder, such as contraction and/or relaxation through either a direct effect on smooth muscle or an indirect action on the autonomic innervation of the bladder. The

5-HT receptor subtypes mediating these pharmacological effects appear to differ between species. For example, direct stimulation of 5-HT_2 receptors has been shown to produce contractions of detrusor smooth muscle cells in human (Klarskov & Hørby-Petersen, 1986), rat (Kodama & Takimoto, 2000), and canine (Cohen, 1990), as well as in the U&LP of the porcine bladder (Moro et al., 2016). In the cat bladder, a biphasic response has been reported, mediated by 5-HT_2 and 5-HT_3 receptors (Saxena et al., 1985), and 5-HT_3 receptors mediate contractions in the rabbit (Chen, 1990). However, in electrical field studies, Hattori et al. (2017) found 5-HT_2 does not participate in neural modulation of human detrusor motor function, and instead, 5-HT-induced potentiation of cholinergic responses is mediated by the 5-HT_4 receptor in the isolated detrusor of human (Corsi et al., 1991) and porcine bladders (Sellers et al., 2000a).

5-HT has been identified to play a role in the urothelium and lamina propria of the urinary bladder, with the 5-HT_{2A} receptor subtype found to be the predominant receptor influencing contractile activity (Moro et al., 2016). An upregulation of 5-HT_{2A} receptor mRNA in the urothelium was induced by partial bladder outlet obstruction, which may be related to enhanced contractile responses to 5-HT (Michishita et al., 2015). Furthermore, in the underactive bladder, ageing is accompanied by a decrease in the number of 5-HT-positive cells, suggesting decreased 5-HT availability can lead to urethral sensory fibre excitation and decrease the number of effective voiding contractions (Coelho et al., 2019). However, whilst ageing is associated with decreased micturition frequency, the maximal contractile response to 5-HT is not altered between young and aged healthy rat models (Takanashi et al., 2019).

1.6.4 *Neurokinin*

Neurokinin (NK) is a member of the tachykinin family, which are closely related peptides that are actively involved in the central and peripheral nervous systems, as well as in the

cardiovascular and immune systems. In mammals, tachykinins act as neurotransmitters, paracrine or endocrine factors, and neuroimmunomodulators that have roles in the nervous system, gastrointestinal tract, and cardiovascular system (Liu & Burcher, 2005). They are also integral to the urinary bladder, known to influence contraction and trigger micturition via activation of the G protein-coupled receptors NK1, NK2, and NK3, which show selectivity for substance P, neurokinin-A (NKA), and neurokinin-B (NKB), respectively (Alexander et al., 2011). NKA-induced contractions are directly mediated via NK2 receptors in both the detrusor (Templeman et al., 2003) and U&LP (Grundy et al., 2018; Ishizuka et al., 1995b; Sadananda et al., 2008). This response has shown effectiveness on pig (Sadananda et al., 2008), human (Templeman et al., 2003), rat (Quinn et al., 2004), mouse (Grundy et al., 2018), and dog urinary bladder (Rizzo & Hey, 2000). Templeman et al. (2003) identified an almost identical response to the NK2 receptor in human and pig detrusor, highlighting the pig bladder to be an effective species to study neurokinin-induced contractions in the human bladder.

Contractile activity of detrusor muscle via neurokinin activation involves extracellular Ca^{2+} from voltage-operated Ca^{2+} channels and RhoA-Rho kinase activation, however, a recent study has identified little involvement of sarcoplasmic Ca^{2+} release, as indicated by the limited response to PLC-inhibitors (Dér et al., 2019). This is a property unique to the common $G_{q/11}$-mediated downstream signalling, which involves PLC and sarcoplasmic reticulum Ca^{2+} release for contraction. Furthermore, activation of the NK2 receptors determines prostanoid synthesis in the hamster urinary bladder, which can amplify the direct contractile effect of NK2 receptor agonists, suggested to be likely due to L type Ca^{2+} channel activation (Tramontana et al., 2000).

Expression profiling of human urothelium has revealed expression of all three neurokinin receptors (Ochodnický et al., 2012), however, it is the activation of NK2 receptors by NKA

that are responsible for mediating urinary bladder contraction (Grundy et al., 2018). It has been suggested that the NK2-induced contractile response in the U&LP is mediated by suburothelial myofibroblasts (Sadananda et al., 2008). Under pathological conditions, for example, injury and inflammation, neurokinin has been reported to alter their expression pattern in neurons, which could amplify NK2 receptor signalling in bladder contractile dysfunction, such as overactive bladder (Arms & Vizzard, 2011; Callsen-Cencic & Mense, 1997). Furthermore, NK2 receptor inhibition has been shown to reduce the amplitude of detrusor muscle contractions in an experimental cystitis model, further indicating the importance of the NK2 receptor in bladder overactivity (Ishizuka et al., 1994; Maggi et al., 1991). Due to its abundance, there is high potential for the NK2 receptor to be an effective target for therapies of various disease states (Jung & Priefer, 2021). For example, NK2 receptor agonists have been recently considered as a potential therapy to induce on-demand voiding in individuals with or without spinal cord injury (Marson et al., 2018) in both young and aged populations (Marson et al., 2021).

1.6.5 *Prostaglandin*

Prostaglandins (PGE) are inflammatory mediators released from mast cells that can potentially affect the urinary bladder micturition reflex (Andersson et al., 1977; Bultitude et al., 1976). In addition, prostaglandins can be produced locally within the bladder in response to mechanical damage or inflammation (Funk, 2001). The role of prostaglandins in bladder physiology was first documented when the release of prostaglandins was observed immediately after urinary bladder distention (Gilmore & Vane, 1971). Stretch-induced release of PGE_2 from the urothelium has been suggested to exert a direct effect on detrusor smooth muscle cells to evoke contraction, or to enhance the release of local ATP via EP_1 receptor activation resulting in increased afferent activation (Wang et al., 2008). Complex interactions between ATP and nitric

oxide has been reported to modulate PGE_2 release, subsequently initiating a positive feedback process between ATP and PGE_2 (Nile et al., 2010). Prostaglandin release from the bladder wall has been found to originate from both the urothelium and detrusor muscle layers (Abrams et al., 1979; Jeremy et al., 1987; Masunaga et al., 2006; Park et al., 1999; Stromberga et al., 2020c). Overproduction of the release of prostaglandins has been observed in various pathological conditions, such as bladder overactivity due to spinal cord injury (Masunaga et al., 2006), bladder outlet obstruction (Masick et al., 2001), and inflammation (Wheeler et al., 2001).

Prostaglandins are synthesised from arachidonic acid, which is metabolised into PGH_2 by two cyclooxygenase isoforms, COX-1 and COX-2, and subsequently converted into five primary prostaglandins via their respective synthases: PGE_2, PGD_2, $PGF_{2\alpha}$, prostacyclin (PGI_2) and thromboxane (TXA_2) (Stromberga et al., 2020b). These signalling molecules exert their function through the stimulation of nine specific G protein-coupled receptors: prostaglandin E2 receptor 1–4 (EP_1–EP_4), prostaglandin D2 receptor (DP_1-DP_2), prostaglandin F receptor (FP), prostaglandin I2 receptor (IP) and thromboxane receptor (TP), respectively. The urinary bladder is capable of responding to all five major prostaglandins (Stromberga et al., 2020b), however, the production of prostaglandins and the subsequent bladder contractile responses for the different prostaglandin receptor subtypes differs between species (Root et al., 2015). For example, in the rat bladder, PGI_2 is the major prostaglandin produced (Jeremy et al., 1984), whereas in the rabbit it is PGE_2 (Leslie et al., 1984). In the porcine bladder, PGE_2 and $PGF_{2\alpha}$ have the most significant impact on contraction and spontaneous contractile frequency in the urothelium and lamina propria (Stromberga et al., 2020b; Stromberga et al., 2020c). In the human bladder, the primary prostaglandins synthesised are PGI_2, followed by PGE_2, $PGF_{2\alpha}$ and TXA_2 (Jeremy et al., 1984; Masunaga et al., 2006). Prostanoid synthesis, as demonstrated

in vitro in the rat urinary bladder, can be stimulated by postganglionic muscarinic receptors and involved a muscarine receptor-linked Ca^{2+} influx system (Jeremy et al., 1986).

Of the five prostaglandins produced endogenously within the bladder and released from bladder-infiltrated mast cells, PGE_2 is believed to be the most likely contributor to bladder dysfunction. It is involved in the control of bladder function via afferent signalling (Andersson & Wein, 2004), and has been proposed to be involved in detrusor overactivity by sensitising capsaicin-sensitive afferent nerve endings (Maggi, 1988; Park et al., 1999). Administration of intravesical PGE_2 has been found to cause a strong sense of urgency, bladder instability, and reduced bladder capacity (Ishizuka et al., 1995a; Rahnama'i et al., 2012; Schussler, 1990), and have been considered in the treatment of underactive bladder, although little clinical benefit has been observed (Hindley et al., 2004; Kim, 2017; Wagner et al., 1985). Of the four PGE_2 receptor subtypes ($EP_{1\text{-}4}$), the EP1 receptors are the only subtype to act via the $G_{q/11}$ proteins, and therefore induce excitatory effects due to their involvement with the intracellular signalling pathway that increases intracellular Ca^{2+} for smooth muscle contraction (Katoh et al., 1995). The expression of EP1 receptors have been located within the urothelium (Wang et al., 2008) and lamina propria in the myofibroblasts, as well as in the smooth muscle cells of the detrusor (Rahnama'i, 2010). Activation of EP_1 receptors have been demonstrated to trigger the release of Ca^{2+} from the sarcoplasmic reticulum via the transient receptor potential cation channel (TRPV1) (Moriyama et al., 2005), which are channels that also open voltage-gated Ca^{2+} channels on the plasma membrane. Furthermore, in isolated detrusor smooth muscle strips, prostaglandins augment Ca^{2+} permeability through acting at the Ca^{2+} channel or as a carrier for Ca^{2+} across smooth muscle cell membranes to produce a marked increase in spontaneous motility (Anderson & Kohn, 1978).

1.6.6 *Angiotensin*

Angiotensin (AT) is an active peptide hormone of the renin-angiotensin system (RAS) with a primary function in cardiovascular and renal regulation. However, when produced locally by RAS, angiotensin can influence tissue functions in an autocrine or paracrine manner (Patel & Mehta, 2012; Weaver-Osterholtz et al., 1996). In the urinary bladder, angiotensin I (ATI) is converted into angiotensin II (ATII) by angiotensin converting enzyme and a serine protease similar to human chymase (Andersson et al., 1992; Waldeck et al., 1997). ATII acts on two subtypes of receptors, classified as angiotensin II type-1 receptors (AT_1) and angiotensin II type-2 receptors (AT_2), and exhibit equal affinity in binding to both receptors (Gasparo et al., 1995). The AT_1 receptors couple to $G_{q/11}$ receptors, activating PLC, L-type Ca^{2+} channels, and inhibiting adenyl cyclase to reduce intracellular cAMP (McKinley & Oldfield, 2009). When isolated human detrusor strips are exposed to an extracellular Ca^{2+}-free environment, contractions induced by angiotensin are completely abolished, however, L-type Ca^{2+} channel antagonists only partially inhibit contractions, suggesting alternative mechanisms of Ca^{2+} entry (Andersson et al., 1992).

In the human detrusor smooth muscle, angiotensin I and angiotensin II produces concentration-dependent contractions, and ATI receptors can be blocked by the competitive ATII receptor antagonist, saralasin, which indicates the actions of both peptides are mediated through stimulation of ATII receptors (Andersson et al., 1992). However, in the dog bladder, the responses to both ATI and ATII were minor or lacking, highlighting variation in responses between species (Steidle et al., 1990). A number of studies have reported angiotensin II as a potential and effective contractile agent (Andersson et al., 1992; Erspamer et al., 1981; Saito et al., 1993b; Waldeck et al., 1997). The response of isolated detrusor smooth muscle is primarily mediated by the AT_1 receptors, as indicated in rat urinary bladder (Tanabe et al.,

1993). However, Hadzhibozheva et al. (2021) reported increased strength and velocity of the contractile response of isolated rat detrusor tissue strips to ATII in response to pre-treatment with an AT_2 receptor blocker, indicating the possibility for counteraction between AT_1 and AT_2 in modulating the contractile process in the detrusor. Moreover, Lim et al. (2021) was the first to study the effects of ATII on the muscularis mucosae within the lamina propria of the bladder wall and identified the responses of porcine tissue to be mediated primarily via the AT_1 receptors. A consistent observation across stimulation of the urinary bladder with ATII is tachyphylaxis, as indicated by failure to initiate contractions after repeat administration of the peptide, which is a phenomenon identified in both detrusor (Andersson et al., 1992; Lam et al., 2000) and the U&LP (Lim et al., 2021).

Angiotensin II AT_1 receptors have been identified in the human urothelium at the mRNA (Ochodnický et al., 2012) and protein level (Cho et al., 2012). Angiotensin II sensitivity in the muscularis mucosae of the lamina propria appears to be higher than the detrusor smooth muscle, however, the presence of urothelium appears to inhibit muscularis mucosae contractility (Lim et al., 2021). Angiotensin II has been studied in pathological states, whereby expression of AT_1 receptors is significantly increased in the urothelium and detrusor of rats with bladder outlet obstruction (BOO). However, the authors reported a downregulation of the expression of ATII receptors after administration with an AT_1 receptor antagonist, telmisartan, in both layers, suggesting this could improve bladder function in rats with BOO, as well as decrease detrusor overactivity symptoms (Cho et al., 2012).

1.7 Contractile dysfunction of the urinary bladder

Lower urinary tract symptoms (LUTS) can be categorised into symptom syndromes based on which symptoms are most prevalent (Mancini et al., 2020). For example, overactive bladder (OAB) syndrome occurs as a result of storage dysfunction that may be caused by detrusor overactivity (DO), while underactive bladder (UAB) syndrome is voiding dysfunction frequently caused by detrusor underactivity (DU). However, as these syndromes occur in the storage and voiding phases, there is significant overlap in the presenting symptoms, and it is possible to be affected by both overactive and underactive symptoms concurrently. There has been a particular focus on the detrusor in the pharmaceutical management of bladder contractile disorders. Despite this, the urothelium has the potential to influence whole intact bladder activity, and it has been recently ascertained that dysfunction of the physiologically active receptor systems within the urothelium could be the underlying cause in some bladder disorders (Drake et al., 2018).

1.7.1 *Overactive bladder*

Overactive bladder is a lower urinary tract disorder associated with a set of chronic symptoms that have significant impacts on the quality of life of those affected. OAB is a highly prevalent condition, affecting over 12% of the adult population, with an increasing burden predicted over time (Irwin et al., 2011). The overall prevalence is similar between men and women of all ages, with a marked increase after 40 years of age (Stewart et al., 2003). Overactive bladder is defined as urinary urgency, usually with urinary frequency and nocturia, with or without urgency urinary incontinence (Abrams et al., 2002). It is important to note that there is overlap between the definitions of OAB and detrusor overactivity DO (Guralnick et al., 2010). OAB is a symptom syndrome, defined by urinary urgency in the absence of identifiable pathology,

whereas DO is defined as a urodynamic observation characterised by involuntary detrusor contractions during filling that may be spontaneous (Abrams, 2003). Approximately 50% of patients with OAB present without urodynamically diagnosed detrusor overactivity, which becomes more prevalent with age, resulting in a reduced quality of life and increasing the chance of developing incontinence (Ptashnyk et al., 2021). DO is often myogenic (urgency initiated from autonomous contraction of the detrusor smooth muscle) or neurogenic (urgency signalled from the central nervous system initiating detrusor contractions) in nature. However, the pathophysiology of OAB is complex, and idiopathic causes represent the largest population of patients. There is an increasing interest on the role of the urothelium and lamina propria in initiating urinary urgency, as well as the urethra, which has been regarded as a possible afferent origin of OAB (de Groat, 2004; Fry et al., 2020; Peyronnet et al., 2019). In addition, OAB can result from various other potential mechanisms, including gastrointestinal functional disorders, subclinical autonomic nervous system dysfunctions, metabolic syndrome, and affective disorders (Peyronnet et al., 2019).

1.7.1.1 Pharmacological treatment options for OAB

Due to the poorly understood nature of the pathophysiology of OAB, it is common to label the diagnosis as idiopathic, however, it should be seen as a complex, multifactorial symptom syndrome, resulting from multiple pathophysiological mechanisms. Identification of the mechanisms causing a patient to experience OAB symptoms may help tailor treatment to individual patients and improve outcomes (Peyronnet et al., 2019). OAB symptoms that affect the overall quality of life (QOL) require therapeutic interventions, whereby the goals of OAB management are to improve symptoms and urinary continence, protect the kidney, decrease the risk complications second to impaired bladder emptying, restore LUT function, and improve QOL (Gormley et al., 2015). Initial management of OAB typically involves behavioural

changes, such as diet and lifestyle modifications, however, the rate of improvement is typically low (Monteiro et al., 2018). Antimuscarinic treatment has been used as the first-line pharmaco-therapeutic intervention for OAB for many years, which exerts its effects by competitively inhibiting the binding of acetylcholine to M3 receptors on the urothelium and detrusor smooth muscle cells (Veer et al., 2023). However, there is a need for alternative treatment options, due to the large number of side effects associated with antimuscarinic use (Chapple et al., 2008), and the low rates of patient adherence (Basra et al., 2008).

1.7.2 *Underactive bladder*

Underactive bladder is a complex clinical condition with a limited amount of research focused on identifying the mechanisms underlying its presentation. In elderly men and women with lower urinary tract symptoms, over 45% exhibit an underactive bladder, presenting this as an increasingly important and clinically relevant syndrome (Jeong et al., 2012). UAB is an increasingly prevalent problem that commonly results in urinary retention, incomplete bladder emptying, and other bothersome urinary symptoms (Stoffel et al., 2017). Although a range of definitions have been provided in past literature (Chapple et al., 2015; Dewulf et al., 2017; Li & Liao, 2016; Vale et al., 2019), the currently accepted definition comes from a recent International Continence Society (ICS) terminology report, that underactive bladder is "characterised by a slow urinary stream, hesitancy, and straining to void, with or without a feeling of incomplete bladder emptying sometimes with storage symptoms" (Chapple et al., 2018). This ICS report also proposes underactive bladder as the clinical syndrome that includes urodynamic diagnosis of detrusor underactivity, defined as a contraction of reduced strength and/or duration, resulting in prolonged bladder emptying and/or a failure to achieve complete bladder emptying within a normal time span (Taylor & Kuchel, 2006).

Underactive bladder is a multifactorial condition that can result from a variety of pathological processes, including idiopathic, neurogenic, myogenic, or functional (Chang et al., 2018). However, misdiagnosis of UAB is common, as the symptoms often overlap with the clinical features of other disorders, such as overactive bladder and bladder outlet obstruction (Jeong et al., 2012). It is possible that bladder urothelial dysfunction, along with sensory nerve dysfunction, and detrusor myogenic dysfunction all play a part, whether partial or complete, in the development of underactive bladder. In a study comparing urothelial dysfunction in patients with detrusor underactivity to healthy controls, the junction protein E-cadherin was significantly lower, and mast cell count and urothelial apoptosis significantly higher in DU, indicating that chronic inflammation and urothelial dysfunction are present in underactive bladder (Jhang et al., 2015). However, there remains a paucity of research into the mechanisms underlying the disorder in general. This is of particular importance as Australia's older population (aged 65 and over) has been growing rapidly. As the incidence of bladder dysfunction increases with ageing, this trend is likely to exacerbate the number of people seeking help and assistance nationwide (Wilson et al., 2020).

1.7.2.1 Pharmacological treatment options for UAB

There are currently no outcome-validated effective therapeutics for the management, treatment, or prevention of underactive bladder. Prior studies have investigated the use of parasympathomimetics (muscarinic agonists) as a treatment, which act to directly stimulate muscarinic receptors or inhibit anticholinesterase to increase acetylcholine concentration between synapses. However, much of the evidence may not have the academic or clinical rigour required to provide evidence-based support for the use of this class of medications in treating underactive bladder (Barendrecht et al., 2007; Chancellor et al., 2020; Osman & Chapple, 2018; Osman et al., 2018). In particular, there are a range of adverse effects associated

with parasympathomimetic use, such as nausea, vomiting, diarrhea, sweating, salivation and headache, which also need to be taken into account (Osman & Chapple, 2018). A recent systematic review with meta-analysis investigating the effectiveness of parasympathomimetic drugs for the treatment of underactive bladder identified a paucity of quality evidence suggesting benefits for this class of medicine, and highlights the need for future well-controlled clinical trials (Moro et al., 2021b). Intravesical administration of prostanoids has been studied as a treatment option for UAB, as PGE2 can increase detrusor contraction and promote urethral relaxation (Rahnama'i et al., 2012), although little clinical benefit has been observed compared to controls (Hindley et al., 2004; Kim, 2017; Wagner et al., 1985). Alpha-adrenoceptor antagonists have also been investigated in the treatment of UAB, which work to reduce outlet resistance through urethral smooth muscle relaxation. The treatment can improve lower urinary tract symptoms and voiding function but does not improve detrusor underactivity itself. Combination therapy has shown effectiveness in the treatment of underactive bladder, with the combination of muscarinic agonists and alpha-adrenoceptor agonists improving underactive bladder symptoms when compared to monotherapy (Yamanishi et al., 2004). The recent interest into the development of pharmacological treatments for underactive bladder is of particular importance as it increases with prevalence (Bayrak & Dmochowski, 2019). Therefore, a greater understanding into mediators of contraction will help identify future therapeutic targets that can be employed in the pharmaceutical management of this disorder and provide insights into the mechanisms of action underlying the various presentations.

1.7.3 *Coexistent overactive-underactive bladder*

The association between the symptoms of both overactive and underactive bladder was first described by Resnick and Yalla (1987) as detrusor hyperreflexia and impaired contractility (DHIC) in the elderly. DHIC, also referred to as detrusor overactivity with impaired

contractility (DOIC), comprises detrusor overactivity during storage but poor detrusor contraction in the emptying phase. However, this term may be unsuitable as it refers only to detrusor contractility and does not take into consideration other causative factors such as innervation. Until recently, there has been little research into the coexistent nature of overactive and underactive bladder. During the International Consultation on Incontinence-Research Society (ICI-RS) meeting in 2019, it was considered whether coexistent overactive-underactive bladder (COUB) is the combination of the two separate OAB and UAB syndromes, or if it is a real unique clinical syndrome associated with symptoms of both in the same patient (Mancini et al., 2020). A consensus for the definition of COUB was described as *coexisting storage and emptying symptoms in the same patient, without implying any specific urodynamic/functional findings or causative physiology;* and that these *symptoms are suggestive of urodynamically demonstrable coexistent detrusor overactivity/underactivity, but can be caused by other forms of urethro-vesical dysfunctions.*

The coexistent nature of overactive with underactive bladder has been found to have the most significant impact on patient quality of life among the lower urinary tract symptoms (Murukesu et al., 2019). The prevalence rate of COUB has been reported to be 18.8% and 5.5% in community-dwelling elderly men and women, respectively, and increases with age (Jeong et al., 2021). In a retrospective study, Gammie et al. (2018) identified major differences in the signs and symptoms of patients having detrusor underactivity and detrusor overactivity compared to patients with DU without DO. For example, both male and female patients with DU and DO were older, had lower bladder volumes at first desire to void, lower voided volumes, lower post-void residual volumes, and more urgency than patients with DU without DO (Gammie et al., 2018). However, the diagnostic criteria of COUB have not yet been established, and the urodynamic correlates of this syndrome are poorly understood.

1.7.3.1 Pharmacological treatment options for COUB

There are currently no therapeutics available targeted specifically towards coexistent overactive-underactive bladder. Current studies examining the pharmaceutical treatment of COUB have been directed towards treating the OAB symptoms, which could be due to the lack of adequate medical treatment options for UAB (Moro et al., 2021b). However, combination therapies would be the most obvious strategy in the treatment of COUB (Mancini et al., 2020). For example, the combination of bladder relaxants, such as antimuscarinics, with alpha-blockers to enhance bladder emptying could be suitable. One retrospective study of five males with DOIC treated with antimuscarinics and alpha-blockers identified this combination to be effective in improving symptoms in four of the five patients (Liu et al., 2014). Despite this, as there are currently no prospective studies investigating the effectiveness of this combination therapy, the long-term effectiveness remains to be elucidated. With only a recent consensus arising on the existence of coexistent overactive-underactive bladder, and a lack of understanding on the underlying mechanisms of OAB and UAB, this presents a significant issue for patients suffering with symptoms of both syndromes and could result in a reduced quality of life. In addition, with a paucity of effective treatment options currently available, this presents a need to identify novel targets for the pharmaceutical treatment of COUB.

1.8 General aims and hypotheses

The overall aim of this research is to identify variations in the mechanisms of action between G protein-coupled receptor system pathways in mediating urinary bladder contractions on porcine urothelium, lamina propria and detrusor smooth muscle. Specifically, this project aims to:

1. Measure and ascertain the functional responses to the G protein-coupled receptors, namely the muscarinic, histamine, serotonin (5-HT), neurokinin, prostaglandin, and angiotensin receptor systems, as they have the potential to stimulate contractile activity of the urinary bladder.
2. Characterise and identify the mechanisms of action between extracellular and intracellular receptor pathways. This includes Ca^{2+} channels and second messenger systems, including Rho kinase, involved in responses to these contractile-mediating receptor systems.
3. Determine the effects of ageing on G protein-coupled receptor-mediated contractile activity in the detrusor smooth muscle.

It is hypothesised that there are common mechanisms of action between receptor-mediated contractions within the various tissue layers of the urinary bladder. Activation of selective receptors may be responsible for inhibiting contractile activity, and as such, dysfunction could result in disorders such as overactive or underactive bladder. Therefore, uncovering insights into the mechanisms underlying these particular receptor systems may present novel therapeutic targets for future pharmacological therapies that can be used in bladder management. In addition, as the prevalence of contractile disorders increases with age, it is

anticipated that ageing may also introduce a variable that can influence the receptor-mediated contractile activity within the urinary bladder.

Chapter 2

General materials and methods

2.1 Materials

Specific materials and methods for each experiment are contained within the relevant methods section of each results chapter. This section provides an overview of the general techniques and resources applied throughout the study.

2.1.1 ***Tissue source and collection***

Due to the amount of tissue required for experimentation throughout this report, and the difficulty in obtaining human bladder tissue as a result of availability and ethical constraints, porcine tissue was the chosen tissue for experimentation. Porcine tissue has been used widely for bladder contractile studies and has shown effectiveness in modelling the physiology and pathophysiology of the lower urinary tract based on anatomical and functional similarities to humans (Steiner et al., 2018; Yamanishi et al., 2002). In addition, the porcine urinary bladder can be regarded as a reliable model for pharmacological studies (Leonhäuser et al., 2019).

The urinary bladders from female Large White-Landrace-Duroc cross-bred pigs (*Suf scrofa domestica*) at six months old and weighing between 80 and 100 kg were used in this study. For the alterations in age studies, the bladders from two- to three-year-old sow pigs weighing approximately 200 kg were used to depict an adult model, as at two years they will have undergone puberty and reproduced. Only female bladders were used in this study due to local availability at the abattoir, and there are no differences between sexes in resting frequency, baseline tension or amplitude of spontaneous contractile activity (Moro, 2013). Fresh tissues were obtained on the morning of experimentation from Highchester Meats abattoir (Gleneagle, QLD) after slaughter for the routine commercial provision of food. The abattoir confirmed they did not supply any porcine tissues where the pigs were found to be infected, and prior to initial

dissection of the porcine tissue, each bladder used in this study was visually assessed for infection, blood, or pus.

2.1.2 *Chemicals and pharmacological agents*

Krebs-Henseleit bicarbonate solution (Krebs) was used as the physiological buffer for functional tissue bath experiments. When equilibrated with carbogen (95% oxygen and 5% carbon dioxide), the solution maintained a pH of 7.4. The solution was made fresh each day with distilled water and the chemical components as outlined in Table 2-1. Krebs was used within 24 hours from the initial dilution of salts. For studies using a nominally zero Ca^{2+}-free Krebs solution, calcium chloride was omitted when preparing the Krebs solution from the chemical components.

Table 2-1: Components of Krebs-Henseleit bicarbonate solution including chemical name and formula, concentration used and supplier.

Chemical name	Molecular formula	Concentration (mM)	Supplier
Sodium chloride	$NaCl$	118	Sigma-Aldrich
Glucose	$C_6H_{12}O_6$	11.7	Sigma-Aldrich
Sodium bicarbonate	$NaHCO_3$	24.9	Sigma-Aldrich
Potassium chloride	KCl	4.7	Sigma-Aldrich
Magnesium sulphate	$MgSO_4$	2.4	Sigma-Aldrich
Potassium phosphate monobasic	KH_2PO_4	1.15	Sigma-Aldrich
Calcium chloride	$CaCl_2$	1.9	Sigma-Aldrich

The chemical name, concentration used, mechanism of action and source of drugs used throughout this research project are outlined in Table 2-2. 2-aminoethyl diphenylborinate, carbamylcholine chloride, ethylenediamine-tetraacetic acid, glycol ether diamine tetraacetic acidhistamine dihydrochloride, and procaine hydrochloride were obtained from Sigma-Aldrich (Missouri, U.S.), cyclopiazonic acid, fasudil hydrochloride, neurokinin-A and nifedipine were from Tocris Bioscience (Bristol, U.K.), 5-hydroxytryptamine was from Toronto Research Centre (Toronto, CA), angiotensin II and prostaglandin E2 were obtained from Cayman Chemicals (Michigan, U.S.), and ruthenium red was obtained from Sapphire Bioscience (NSW, AUS).

Table 2-2: Chemical name, concentration used, mechanism of action, and supplier of pharmacological agents used in this research.

Chemical name	Abbreviated	Conc.	Action	Supplier
2-aminoethyl diphenylborinate	2-APB	300μM	Modulates intracellular IP3-induced Ca^{2+} release	Sigma-Aldrich
5-hydroxytryptamine (serotonin)	5-HT	100μM	5-HT receptor agonist	Toronto Research Centre
Angiotensin II	ATII	100nM	Angiotensin receptor agonist	Cayman Chemicals
Carbamylcholine chloride	Carbachol	1μM	Muscarinic receptor agonist	Sigma-Aldrich
Cyclopiazonic acid	CPA	10μM	Sarcoplasmic reticulum Ca^{2+}-ATPase inhibitor	Tocris
Ethylenediamine-tetraacetic acid	EDTA	1mM	Hydrophilic metal chelating agent	Sigma-Aldrich
Glycol ether diamine tetraacetic acid	EGTA	0.5mM	Ca^{2+} chelating agent	Sigma-Aldrich
Fasudil hydrochloride	Fasudil	30μM	Rho-kinase inhibitor	Tocris
Histamine dihydrochloride	Histamine	100μM	Histamine receptor agonist	Sigma-Aldrich
GSK269962: (N-[3-[[2-(4-amino-1,2,5-oxadiazol-3-yl)-1-ethyl-1H-imidazo	GSK	1μM	Selective Rho-associated protein kinase inhibitor	Tocris Bioscience

[4,5-c]pyridin-6-yl]oxy]phenyl]-4-[2-(4-morpholinyl)ethoxy] benzamide)				
Neurokinin-A	NKA	300nM	NK2 receptor agonist	Tocris
Nifedipine	Nifedipine	1μM	L-type Ca^{2+} channel blocker	Tocris
Prostaglandin E2	PGE2	10μM	EP receptor agonist	Cayman Chemicals
Ruthenium red	RuR	10μM	Inhibits sarcoplasmic reticulum Ca^{2+} release	Sapphire Bioscience
Y-27632 hydrochloride	Y-27632	1μM	Selective Rho-associated protein kinase inhibitor	AdooQ BioScience

Nifedipine and PGE2 were dissolved in 100% ethanol. 2-APB and CPA were dissolved in 100% dimethyl sulfoxide (DMSO). The volume of drug added to the tissue baths was kept to a minimum, and the volume of ethanol and DMSO used as vehicle controls did not affect the tension, frequency, or amplitude of control experiments. All other pharmaceutical agents were soluble in distilled water (dH_2O). The concentrations of agonists and antagonists were unique to each experiment and are outlined in each chapter. The agonist concentrations were chosen as the dose that induced ~80% of this contraction, allowing for a strong, sub-maximal contraction of the tissue.

2.1.3 *Ethics*

All tissues were obtained from the local abattoir after slaughter for the routine commercial provision of food with no animals bred, harmed, culled, interfered, or interacted with as part of this research project. All methods were carried out in accordance with relevant Australian guidelines and regulations, and all experimental protocols were in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (Queensland Government, 2015), and the Bond University institutional ethics policy. As such, no ethics was required for this project.

2.2 Methods

2.2.1 *Tissue preparation*

After collection from the abattoir, tissues were transported in a portable cooler in cold Krebs-bicarbonate solution (NaCl 118.4 mM, $NaHCO_3$ 24.9 mM, D-glucose 11.7 mM, KCl 4.6 mM, $MgSO_4$ 2.41 mM, $CaCl_2$ 1.9 mM, and KH_2PO_4 1.18 mM) maintained at 4°C to the Bond University laboratory facilities and used within three hours of the animal's slaughter. Upon return, tissues were prepared for experimentation by separating the urinary bladder dome from the ureters, urethra, serosa, and blood vessels. Bladders were opened longitudinally and a single transverse strip (4cm x 0.5cm) of full thickness was taken from the bladder wall at the midpoint between the trigone and the bladder apex. To dissect the detrusor smooth muscle, the urothelial and lamina propria layers were removed as well as the serosal connective tissue. For U&LP tissue strips, the white-coloured detrusor smooth muscle was dissected away from the pink-coloured urothelium and lamina propria using fine scissors (Figure 2-1). Histological assessment is known to effectively separate the two layers (Moro et al., 2012; Moro & Phelps, 2022b). After dissection and preparation, this single strip was cut in the middle, resulting in 2 x 2cm paired strips. Tissue strips were continually washed with a cold Krebs-Henseleit bicarbonate solution during the preparation and dissection stage. A maximum of two 4cm strips were taken from each unique porcine bladder across the experiments. Throughout this manuscript, n values quoted are from paired tissue strips, and as such, the number of animals (N) used can be calculated using $n \div 2$. The remaining tissue was placed in containers containing Krebs solution and stored in a fridge maintained at 4°C for no longer than 10 hours.

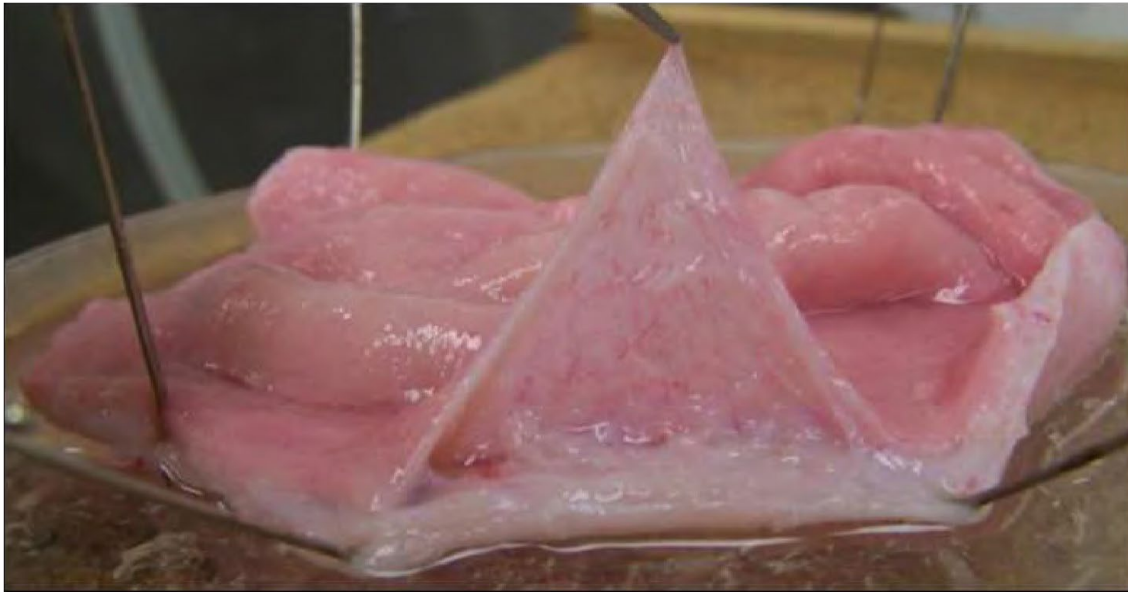

Figure 2-1: Porcine urinary bladder opened longitudinally and showing the separation of the pink-coloured urothelium with lamina propria dissected away from the underlying, white-coloured detrusor smooth muscle. Reproduced with permission by C Moro.

2.2.2 *Functional organ bath studies*

Functional organ bath studies were utilised in this project, consistent with previous research (Moro & Chess-Williams, 2012; Moro et al., 2011; Stromberga et al., 2019; Stromberga et al., 2020c). Adjacent tissue strips of urothelium and lamina propria or detrusor smooth muscle were immersed in 10mL isolated organ baths (Labglass, Brisbane, Australia, Figure 2-2) containing Krebs-bicarbonate solution, maintained at 37°C and continually perfused with carbogen gas (95% oxygen and 5% carbon dioxide). Tissues were suspended in the baths and attached to isometric force transducers (MCT050/D, ADInstruments, Castle Hill, Australia) that were manually adjusted to approximately 2.0g resting tension during equilibration. Each bath was washed through with warmed Krebs a total of three times prior to experimentation. The contractile tension, frequency, and amplitude of spontaneous contractions were recorded through force transducers and transmitted on a Powerlab computer system using LabChart v7 software (ADInstruments). At the conclusion of each experiment, tissue weight was measured on a scale to an accuracy of 0.001 g.

Eight functional organ baths can run simultaneously during one experiment, where the tissue response to agonists and antagonists can be recorded. Studies utilised paired preparations (four paired baths), where the ‘control’ bath contained tissue that was not treated with antagonists, and the adjacent ‘experimental’ bath contained tissue from the same bladder strip incubated with selective antagonists for 30 minutes before the addition of agonists.

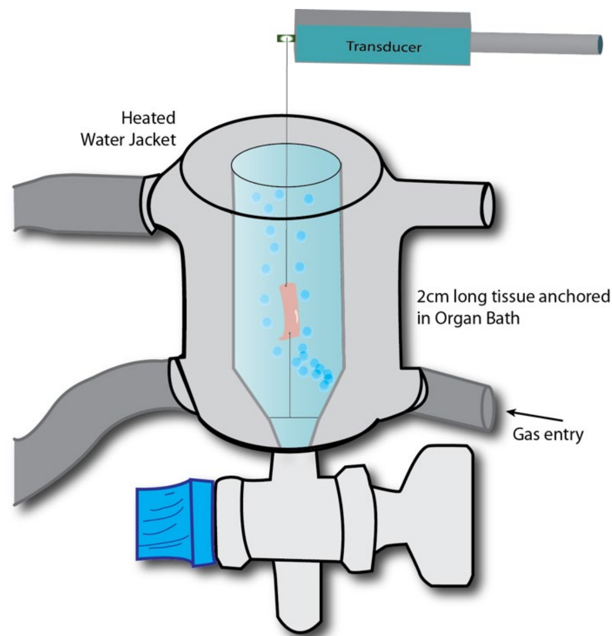

Figure 2-2: Depiction of a single custom-made functional organ bath set-up. Isolated tissue strips of U&LP are suspended in each bath containing Krebs-bicarbonate solution maintained at 37°C and continually perfused with carbogen gas. Contractile activity of the tissue was recorded using isolated force transducers. Reproduced with permission by C Moro.

2.2.3 *Measurements*

The frequency of contractile activity was recorded across a two- to five-minute time period and expressed as the number of spontaneous phasic contractions per minute. Baseline tension was taken as the lowest point of the spontaneous phasic contraction. Amplitude was measured from the lowest point of spontaneous phasic contraction to the peak (Figure 2-3). The three parameters (frequency, tension, and amplitude) were recorded before the agonist was added and immediately after the addition of agonist at the peak response (Figure 2-4).

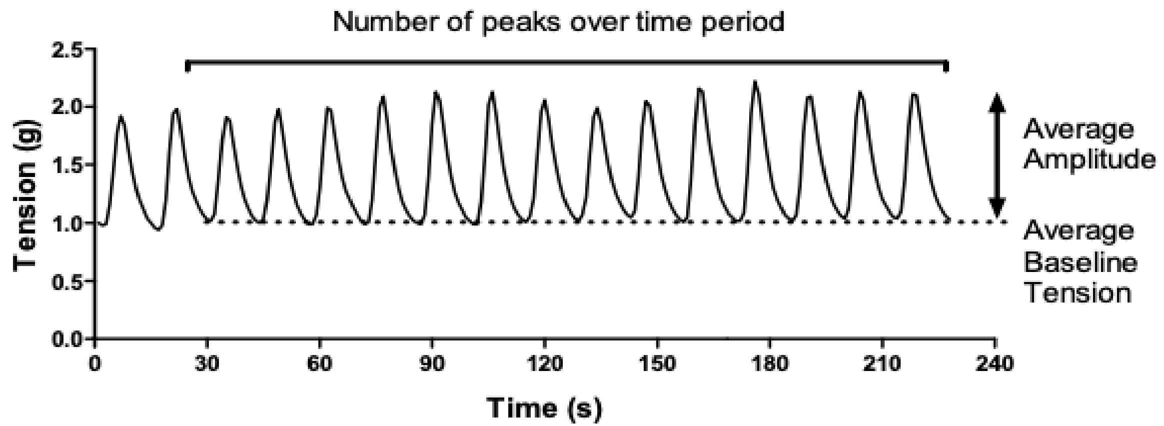

Figure 2-3: Calculation of tension, frequency, and amplitude of spontaneous phasic contractions.

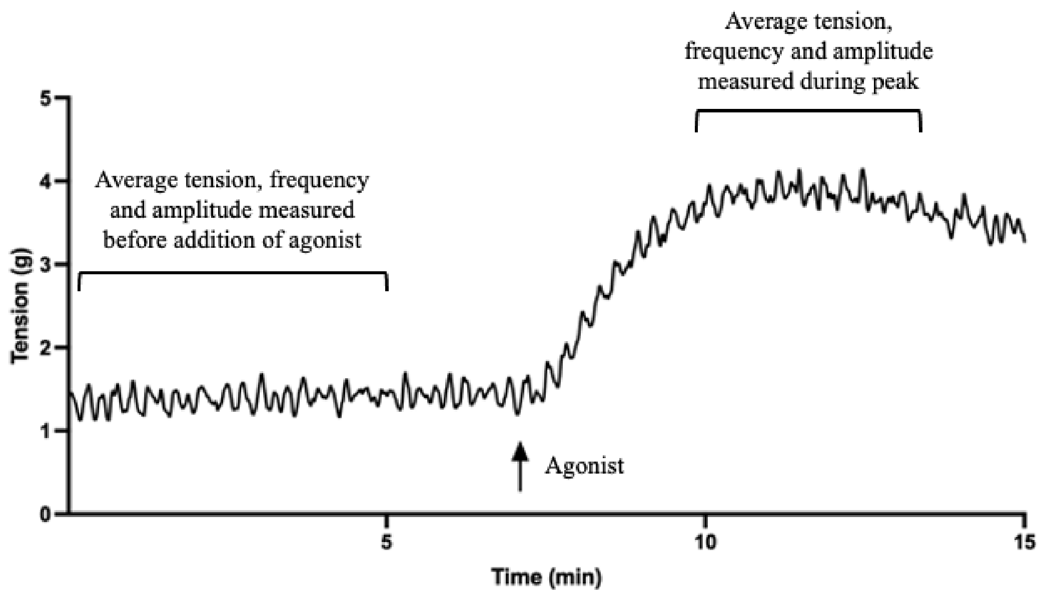

Figure 2-4: Sample trace of spontaneous phasic contractile activity. Average tension (g), frequency (cpm), and amplitude (g) recorded before and immediately after the addition of agonist.

2.2.4 *Analysis of data*

Raw data was initially collected and analysed using LabChart v8 software. Tension, frequency, and amplitude measurements of spontaneous contractions were taken from a single trace at two time points (before and after the addition of agonist). Changes in tension and amplitude were expressed in grams (g), and the frequency of spontaneous phasic contractions was expressed as the number of contractions per minute (cpm). The raw data from each experiment was tabulated in Microsoft Excel 2023 (Microsoft, Redmond, Washington, US). All data was analysed using GraphPad Prism version 10 (San Diego, CA), and results were presented as mean ± standard error of the mean (SEM) or standard deviation (SD). A paired Student's *t*-test was used to analyse tissue responses before and after the addition of agonist. A paired Student's two-tailed *t*-test was used to analyse the significance of results to compare control and experimental tissues, as per previous studies (Stromberga et al., 2020a). To compare two different groups, such as responses across different time points, an unpaired Student's two-tailed *t*-test was used. A one-way ANOVA with Tukey post-test was also undertaken to compare the means where more than two variables were assessed. For all statistical analysis, $p < 0.05$ was considered statistically significant.

Chapter 3

The dependence of urinary bladder responses on extracellular calcium varies between muscarinic, histamine, 5-HT (serotonin), neurokinin, prostaglandin, and angiotensin receptor activation.

PUBLISHED CHAPTER

Phelps, C., Chess-Williams, R., & Moro, C. (2022). The dependence of urinary bladder responses on extracellular calcium varies between muscarinic, histamine, 5-HT (serotonin), neurokinin, prostaglandin, and angiotensin receptor activation. *Frontiers in Physiology, 13*, 1-9. https://doi.org/10.3389/fphys.2022.841181

Additional published abstracts and conference presentations arising from this chapter:

Phelps, C., Chess-Williams, R., & Moro, C. (2022, November 23). Urinary bladder contractions and the influence of extracellular calcium. *AuPS Scientific Meeting.* Hobart, Tasmania.

Phelps, C., Chess-Williams, R., & Moro, C. (2021, November). Identifying novel mediators of contraction within the urinary bladder urothelium and lamina propria tissue layers. *Proceedings of the Australian Physiological Society 60th Diamond Jubilee Conference.* Gold Coast, Australia.

Phelps, C., Chess-Williams, R., & Moro, C. (2021, October). Extracellular ion channels for the induction of bladder urothelium and lamina propria contractions. *Bond University Pitching Research Competition.* Gold Coast, Australia.

Phelps, C., Chess-Williams, R., & Moro, C. (2021, July). Potential targets for underactive bladder treatments: Receptor-mediated contractions of the urinary bladder urothelium. *Physiology 2021.* United Kingdom, virtual conference.

Preface

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Formatting and terminology changes have been made to maintain consistency throughout this thesis. The final version of this manuscript can be accessed at: https://www.frontiersin.org/articles/10.3389/fphys.2022.841181/full.

All data presented in this manuscript are the sole work of Charlotte Phelps.

3.1 Abstract

With many common bladder diseases arising due to abnormal contractions, a greater understanding of the receptor systems involved may aid the development of future treatments. The aim of this study was to identify any difference in the involvement of extracellular calcium (Ca^{2+}) across prominent contractile-mediating receptors within cells lining the bladder. Strips of porcine urothelium and lamina propria were isolated from the urinary bladder dome and mounted in isolated tissue baths containing Krebs-bicarbonate solution, perfused with carbogen gas at 37°C. Tissue contractions, as well as changes to the frequency and amplitude of spontaneous activity were recorded after the addition of muscarinic, histamine, 5-hydroxytryptamine, neurokinin-A, prostaglandin E2, and angiotensin II receptor agonists in the absence and presence of 1μM nifedipine or nominally zero Ca^{2+} solution. The absence of extracellular Ca^{2+} influx after immersion into nominally zero Ca^{2+} solution, or the addition of nifedipine, significantly inhibited the contractile responses ($p < 0.05$ for all) after stimulation with carbachol (1μM), histamine (100μM), 5-hydroxytryptamine (100μM), neurokinin-A (300nM), prostaglandin E2 (10μM), and angiotensin II (100nM). On average, Ca^{2+} influx from extracellular sources was responsible for between 20-50% of receptor-mediated contractions. This suggests that although the specific requirement of Ca^{2+} on contractile responses varies depending on the receptor, extracellular Ca^{2+} plays a key role in mediating G protein-coupled receptor contractions of the urothelium and lamina propria.

3.2 Introduction

While strong and sustained bladder contractions are vital for urinary voiding, during the filling stage abnormal and spontaneous contractions can result in bladder dysfunction. One common presentation is underactive bladder, characterized by a slow urinary stream, hesitancy and straining to void, with or without a feeling of incomplete bladder emptying, and sometimes with storage symptoms (Chapple et al., 2018). Normally, voiding commences when M3 muscarinic receptors in the smooth muscle of the bladder wall are stimulated by neuronally derived acetylcholine. However, recent research has identified a number of other receptor systems on cells within the bladder wall which may also modulate bladder contractions (Stromberga et al., 2019; Stromberga et al., 2020c). Of particular interest are those linked to $G_{q/11}$ proteins, with their stimulation often resulting in strong tissue contractions. With many common bladder diseases arising as a result of abnormal contractions, an understanding of the potential receptor systems involved in this response is vital for the development of new and upcoming treatments for those affiliated with bladder dysfunctions.

A continuing interest has developed into the potential role of histamine (Stromberga et al., 2019), prostaglandins (Stromberga et al., 2020c), angiotensin (Lim et al., 2021), adrenergic (Moro et al., 2013), neurokinin (Grundy et al., 2018), muscarinic (Moro et al., 2011), and 5-hydroxytryptamine (Moro et al., 2016) receptors in influencing contractions of the urothelium and lamina propria (U&LP) layers. This layer has been observed to modulate overall bladder activity through the release of chemical mediators (Moro et al., 2011), as well as other pacemaking functions via the presence of the muscularis mucosae in the underlying connective tissue layer (Fry et al., 2007). The U&LP (also referred to as the bladder mucosa) may also play a key role in the mechanisms of action for current therapeutics (Moro et al., 2011), such

as the parasympathomimetic bethanechol, which is one of the more commonly prescribed first-line treatments in the pharmaceutical management of underactive bladder (Kim, 2017). However, aside from this focus on the M3 muscarinic receptor, there is also a growing interest in combination therapies where, for example, administering both muscarinic agonists and alpha-adrenoceptor antagonists concurrently demonstrated greater success when compared to monotherapy in the management of underactive bladder (Yamanishi et al., 2004). This potential for alternative therapeutic options is important, as many patients who are administered muscarinic-acting pharmaceuticals in the management of bladder contractile disorders cease the regimen due to adverse side effects or lower than expected treatment outcomes (Yeowell et al., 2018). This is a growing area of research, with more work to be done regarding the potential benefits or harms from using parasympathomimetics in the management of underactive bladder (Moro et al., 2021b).

Although the detrusor smooth muscle layer of the bladder has been the traditional target for research and therapeutic development, in recent years the importance of the U&LP in the maintenance of normal bladder function has been highlighted. The bladder generates spontaneous contractions, which can be altered in cases such as outlet obstruction or nerve injury (Kushida & Fry, 2016). The role of this spontaneous activity in the intact bladder is unclear (Fry & McCloskey, 2019), but may be mediated by signals originating in the U&LP, suggesting mechanisms where this tissue might underlie bladder contractile disorders (Fry & Vahabi, 2016). Calcium (Ca^{2+}) and membrane potential transients commence in the lamina propria and spread towards the detrusor. In addition, the U&LP can release mediators, such as acetylcholine during stretching (Moro et al., 2011), which can induce increased spontaneous contractions in the detrusor (Kanai, 2007). A number of cells within the tissue may induce this spontaneous activity, as well as generate spontaneous Ca^{2+} transients, such as myofibroblasts

(Sui et al., 2008), pericytes (Hashitani et al., 2018; Lee et al., 2016), interstitial-like cells (Fry & McCloskey, 2019) or muscularis mucosae (Heppner et al., 2011; Kushida & Fry, 2016). These cells remain highly sensitive to Ca^{2+}, and there is value in identifying sources of Ca^{2+} influx into the tissue from extracellular fluids. Of note, however, is the potential for species differences in the generation and activity of phasic contractions. For example, the spontaneous activity of the bladder urothelium and lamina propria in the pig appears to predominantly arise from the muscularis mucosae, although there is evidence that this is not the case across all species (Mitsui et al., 2020).

With stimulation of the G protein-coupled receptors (GPCRs) resulting in contractions of the urinary bladder U&LP, identifying any common mechanisms of action between them is important. One primary function of the GPCRs in the urinary bladder may be the modulation of Ca^{2+} channels in the cell membranes, accommodating an influx of Ca^{2+} from extracellular fluids, and mediating a variety of physiological responses, from bladder contractions to increased pacemaker activity (Wuest et al., 2007). This study aims to identify similarities in extracellular Ca^{2+} requirements between muscarinic, histamine, 5-hydroxytryptamine (5-HT), neurokinin-A (NKA), prostaglandin E2 (PGE2), and angiotensin II (ATII) receptors for mediating contractile activity of the urinary bladder U&LP.

3.3 Materials and methods

3.3.1 *Tissue collection*

Urinary bladders from Large White-Landrace-Duroc pigs (6 months old, 80kg live weight) were used as the tissue in this study. All bladders were obtained from the local abattoir after slaughter for the routine commercial provision of food with no animals bred, harmed, culled, interfered, or interacted with as part of this research project. As such, animal ethics approval was not required (Queensland Government, 2015). Only female bladders were used in this study due to local availability at the abattoir. After collection from the abattoir, tissues were transported in a portable cooler in cold Krebs-Henseleit bicarbonate solution (NaCl 118.4 mM, $NaHCO_3$ 24.9 mM, D-glucose 11.7 mM, KCl 4.6 mM, $MgSO_4$ 2.41 mM, $CaCl_2$ 1.9 mM, and KH_2PO_4 1.18 mM) maintained at 4°C to the University research facilities and used within three hours of the animal's slaughter.

3.3.2 *Tissue preparation*

A single 4cm strip was taken longitudinally from the bladder wall at the midpoint between the trigone and the bladder apex. Tissue strips were continually washed with a cold Krebs-Henseleit bicarbonate solution during the preparation and dissection stage. The white-coloured detrusor smooth muscle was dissected away from the pink-coloured urothelium and lamina propria using fine scissors. Histological assessment is known to effectively separate the two layers (Moro et al., 2012). After dissection and preparation, this single strip was cut in the middle, resulting in 2 x 2cm strips which were mounted and suspended in 10mL isolated tissue baths (Labglass, Brisbane, Australia) containing warmed Krebs solution at 37°C and perfused with carbogen gas (95% oxygen and 5% carbon dioxide). A maximum of two 4cm strips were

taken from each unique porcine bladder across the experiments. Throughout this manuscript, *n* values quoted are from paired tissue strips, and as such, the number of animals (N) used can be calculated using $n \div 2$. After mounting, tissue tension was manually adjusted to 2g using each transducer positioner's fine adjustment knob. Each bath was washed with warmed Krebs a total of three times prior to the start of experimentation. At the conclusion of each experiment, tissue weight was measured on a scale to an accuracy of 0.001g. The mean weight of porcine U&LP tissues was 0.22 ± 0.01g (n = 208).

3.3.3 *Pharmaceutical agents*

Krebs-Henseleit bicarbonate solution ingredients were obtained from Sigma-Aldrich (Missouri, U.S.). Carbamylcholine chloride (carbachol) and histamine dihydrochloride (histamine) were obtained from Sigma-Aldrich (Missouri, U.S.), nifedipine and NKA were from Tocris Bioscience (Bristol, U.K.), 5-HT was from Toronto Research Centre (Toronto, CA), and ATII and PGE2 were obtained from Cayman Chemicals (Michigan, U.S.). Nifedipine and PGE2 were dissolved in 100% ethanol, while all other pharmaceutical agents were soluble in distilled water. Concentrations chosen for the agonists and antagonists were selected based on their selectivity at each receptor and consistent with concentrations used in previous studies. In each case, the agonist concentration used induced a submaximal contraction (with an aim to achieve around 80% of peak receptor-induced contraction).

3.3.4 *Measurements and data collection*

In the nifedipine studies, a vehicle control of 100% ethanol was added to control tissues or nifedipine (1µM) was added to the experimental tissues and left to equilibrate for 30 minutes. Nifedipine was kept in darkness until the final application in the organ bath and experiments

were concluded within 30 minutes to ensure no adverse light impacts. In the Ca^{2+}-free studies, control tissues were washed with Krebs solution as normal, whereas the experimental tissues were washed three times with a nominally Ca^{2+}-free Krebs solution. This also ensured that any excess Ca^{2+} on the tissue, or Ca^{2+} leaking out from intracellular sources, was cleared from the bath. Tissues were then left to equilibrate for three minutes in nominally zero Ca^{2+} solution before agonists were added. A single dose of a select GPCR agonist was added to both the control and experimental tissues after equilibration. Tension, frequency, and amplitude of spontaneous contractions were measured with an isometric force transducer (MCT050/D, ADInstruments, Castle Hill, Australia) and recorded on a Powerlab system using Labchart v7 software (ADInstruments). Although a threshold was not applied, in all cases each spontaneous contraction exceeded 0.4g.

3.3.5 *Statistical analysis*

Changes in tension and amplitude were measured in grams (g), where amplitude was measured from the lowest point of spontaneous phasic contraction to peak. Frequency was expressed as the number of spontaneous phasic contractions per minute (cpm). All data was analysed using GraphPad Prism version 9 (San Diego, CA), and results were presented as mean ± standard error of the mean (SEM). A paired *t*-test was used to analyse tissue responses before and after the addition of agonist. A paired Student's two-tailed *t*-test was used to analyse the significance of results when comparing tissues with direct controls, as per previous studies (Stromberga et al., 2020a). A one-way ANOVA with Tukey post-test was also undertaken to compare the means where more than two variables were assessed. For all statistical analysis, $p < 0.05$ was considered statistically significant.

3.3.6 *Preliminary results related to the omission of extracellular Ca^{2+}*

There is variation in the literature surrounding methods to remove extracellular Ca^{2+} from an isolated tissue bath. Firstly, this may involve the omission of calcium chloride (CaCl) from Krebs-Henseleit bicarbonate solution (Poyser, 1984). Secondly, the addition of Ca^{2+} chelating agents ethylenediaminetetraacetic acid (EDTA, 1-5mM) (Maggi et al., 1989) or glycoletherdiaminetetraacetic acid (EGTA, 0.5mM) solution (Yoshimura & Yamaguchi, 1997) have been proposed. However, EGTA may have impacts on other ions (Wheeler and Weiss, 1979) or other mechanisms within the tissue (Poyser, 1984), as well as potentially destabilise cell membranes, leading to increased Ca^{2+} permeability (Guan et al., 1988). To assess this feasibility of using Ca^{2+} chelators, we observed that compared to the simple omission of CaCl, the addition of 1mM EDTA or 0.5mM EGTA had no effect on contractile responses to our agonists (e.g., carbachol 1µM). Thirdly, in some studies, the removal of Ca^{2+} appeared to result in a hypovolemic solution, and in order to rectify this, the concentration of other ions was altered. In our pilot studies, when incorporating additional Mg^{2+} to substitute for omitted Ca^{2+}, there was no significant effect on contractions (n = 8). As such, no additional magnesium was added to replace the omission of the Ca2+ divalent cation. Fourthly, there is the potential for Ca^{2+} to enter the Ca^{2+}-free bath solution from the cytoplasm, or via the activation of other cellular pumps/exchangers. To minimise the impact of this, after mounting in the bath, tissues were washed three times over five minutes in warmed Ca^{2+}-free Krebs. As a final check, before and after contractions, the extracellular buffer was collected and checked with spectrophotometric assessments (Pacific Laboratory Products, Victoria, Australia), with no Ca^{2+} detected on this assay. This provided confidence in a nominally zero Ca^{2+} solution. As there were no observable or significant impacts to any contractions from adjusting for the methods listed above, the decision was made to henceforth solely remove Ca^{2+} from the Krebs solution and not alter anything else, as this was the path of least manipulation.

3.4 Results

3.4.1 *Influence of GPCR agonists in U&LP*

In the absence of stimulation from any agonist, U&LP tissue strips developed spontaneous phasic contractions at a frequency of 3.65 ± 0.08 cpm with an amplitude of 0.73 ± 0.04 g (n = 104). When receptor agonists carbachol (1µM), histamine (100µM), 5-HT (100µM), NKA (300nM), PGE2 (10µM), and ATII (100nM) were added to the tissues, U&LP baseline tension increased significantly for all activated receptors ($p < 0.001$, Table 3-1). In addition, the frequency of spontaneous phasic contractions increased for carbachol ($p < 0.001$), histamine ($p < 0.05$), 5-HT ($p < 0.01$), and ATII ($p < 0.05$), but not NKA or PGE2. Amplitude was reduced by all the agonists, but the changes were statistically significant for only carbachol ($p < 0.05$), 5-HT ($p < 0.001$), and ATII ($p < 0.01$).

Table 3-1: Summary of U&LP tissue spontaneous phasic activity. Tension, frequency, and amplitude recorded in response to receptor agonists. Data presented as mean ± SEM.

Agonist	Conc.	Δ Tension (g)	Δ Frequency (cpm)	Δ Amplitude (g)	*n*
Carbachol	1μM	3.77 ± 0.31***	1.15 ± 0.20***	-0.27 ± 0.10*	22
Histamine	100μM	1.54 ± 0.20***	0.64 ± 0.24*	-0.08 ± 0.07	16
5-HT	100μM	6.23 ± 0.64***	2.15 ± 0.64**	-0.37 ± 0.09***	17
NKA	300nM	2.50 ± 0.25***	0.11 ± 0.24	-0.12 ± 0.06	17
PGE2	10μM	2.43 ± 0.22***	-0.05 ± 0.34	-0.03 ± 0.04	16
ATII	100nM	1.50 ± 0.18***	0.20 ± 0.08*	-0.22 ± 0.06**	16

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (paired *t*-test).

3.4.2 *Influence of nifedipine on U&LP contractions*

When nifedipine (1µM) or vehicle control (totalling 0.035% ethanol) were added to tissues at the start of the 30-minute equilibration period, there were no immediate changes to baseline tension, frequency, or amplitude for any of the agonists.

3.4.2.1 Effect of nifedipine on baseline tensions

After activation of the muscarinic, histamine, 5-HT, NKA, PGE2 and ATII receptors, the baseline tension of the U&LP tissue increased, and the increases were similar for all the agonists. In the presence of nifedipine (1µM), the contractions were inhibited as follows (paired Student's two-tailed *t*-tests): carbachol by 54% (1µM, $n = 11$, $p < 0.01$, Figure 3-1); histamine by 45% (100µM, $n = 8$, $p < 0.05$, Figure 3-2); 5-HT by 28% (100µM, $n = 8$, $p < 0.001$, Figure 3-3); neurokinin-A by 49% (300nM, $n = 8$, $p < 0.001$, Figure 3-4); prostaglandin-E2 by 29% (10µM, $n = 8$, $p < 0.05$, Figure 3-5); and angiotensin-II by 47% (100nM, $n = 8$, $p < 0.05$, Figure 3-6). The impact of nifedipine was relatively consistent after the activation of each agonist, with no significant differences found between any of the responses (p = NSD, ANOVA with Tukey's post-test for this final statistical assessment only).

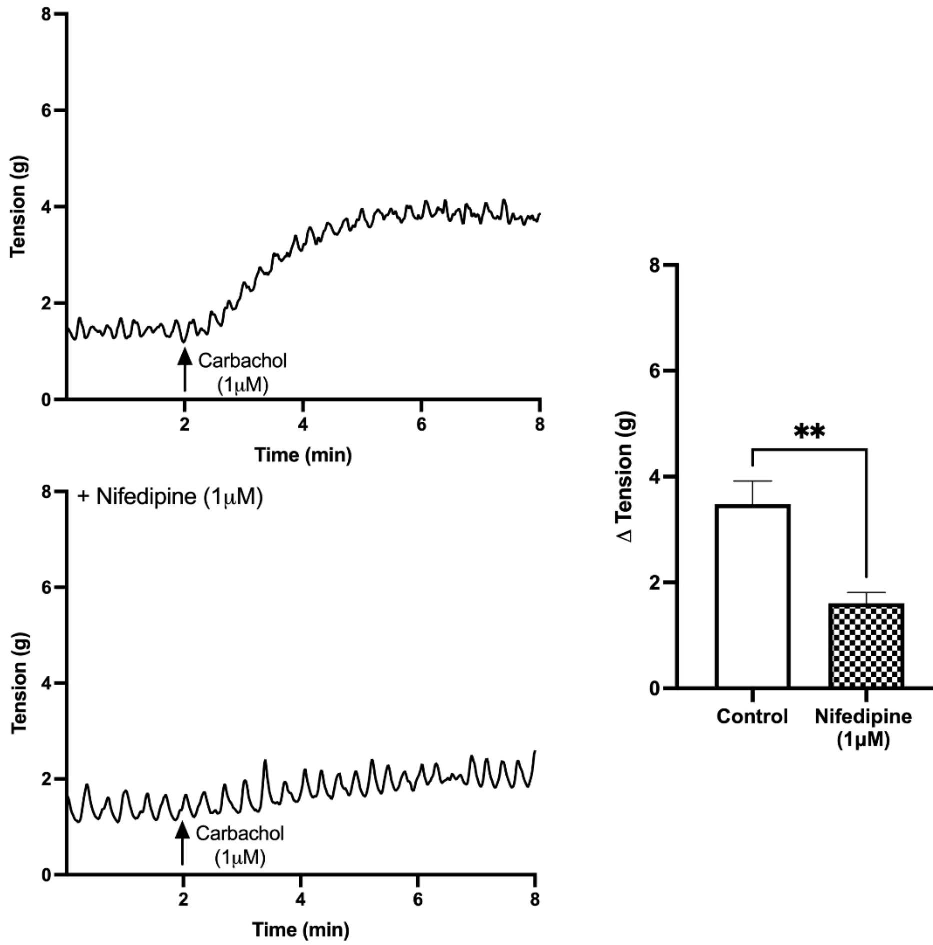

Figure 3-1: U&LP baseline tension responses to receptor agonist carbachol (1μM) in the absence of (*upper left trace*) and in the presence of (*lower left trace*) L-type Ca^{2+} inhibitor, nifedipine (1μM). Changes to baseline tension (*right*) presented as mean ± SEM. $^{**}p < 0.01$ (paired Student's two-tailed *t*-test).

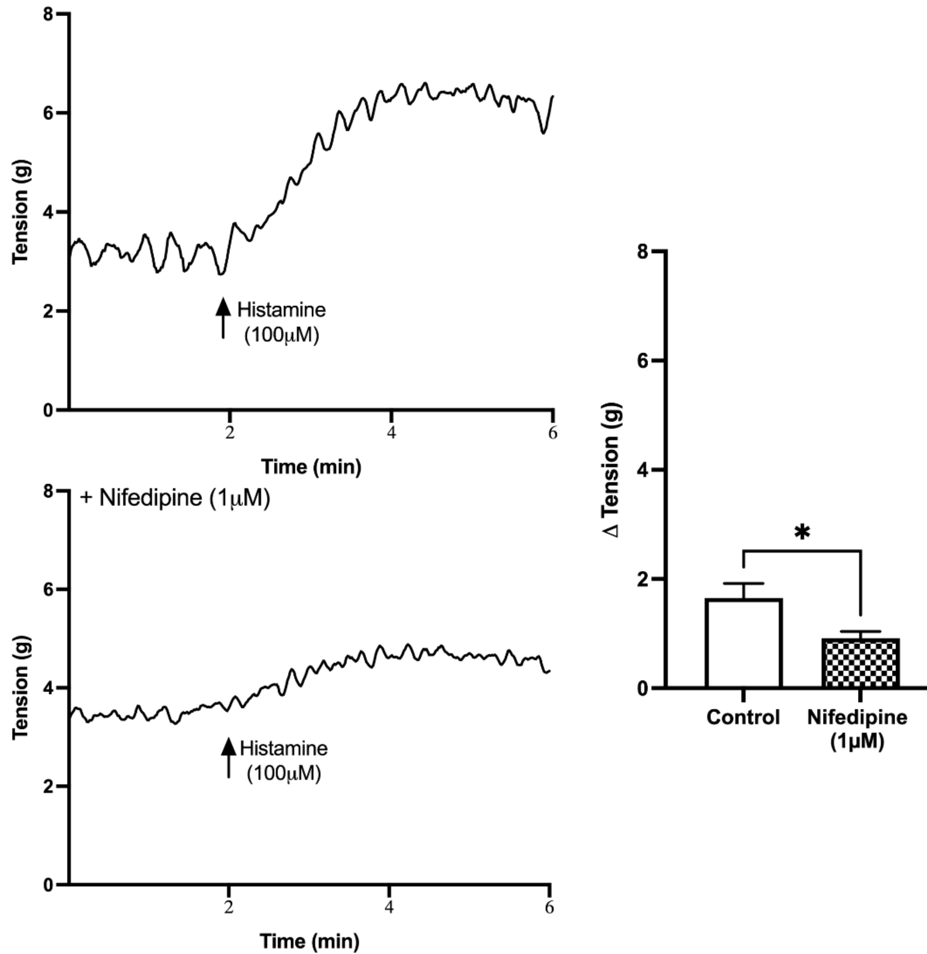

Figure 3-2: U&LP baseline tension responses to receptor agonist histamine (100µM) in the absence of (*upper left trace*) and in the presence of (*lower left trace*) L-type Ca^{2+} inhibitor, nifedipine (1µM). Changes to baseline tension (*right*) presented as mean ± SEM. *$p < 0.05$ (paired Student's two-tailed *t*-test).

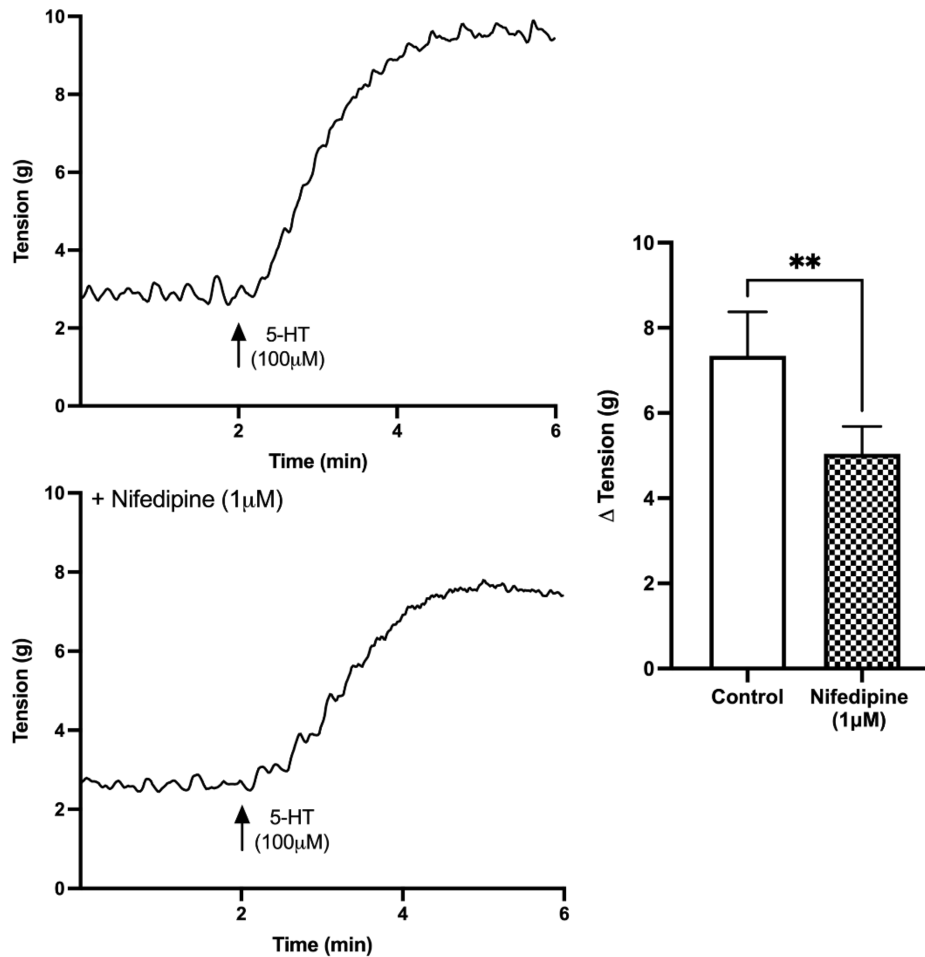

Figure 3-3: U&LP baseline tension responses to receptor agonist 5-HT (100µM) in the absence of (*upper left trace*) and in the presence of (*lower left trace*) L-type Ca^{2+} inhibitor, nifedipine (1µM). Changes to baseline tension (*right*) presented as mean ± SEM. ***p* < 0.01 (paired Student's two-tailed *t*-test).

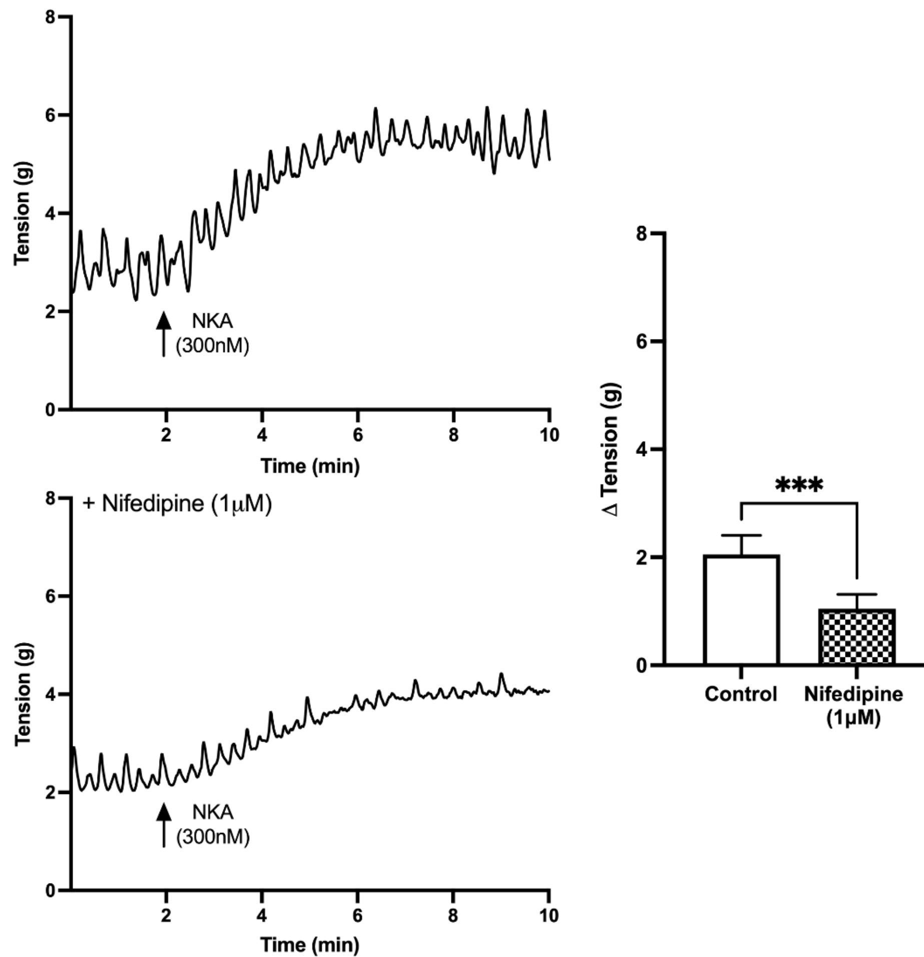

Figure 3-4: U&LP baseline tension responses to receptor agonist NKA (300nM) in the absence of (*upper left trace*) and in the presence of (*lower left trace*) L-type Ca^{2+} inhibitor, nifedipine (1µM). Changes to baseline tension (*right*) presented as mean ± SEM. ***$p < 0.001$ (paired Student's two-tailed *t*-test).

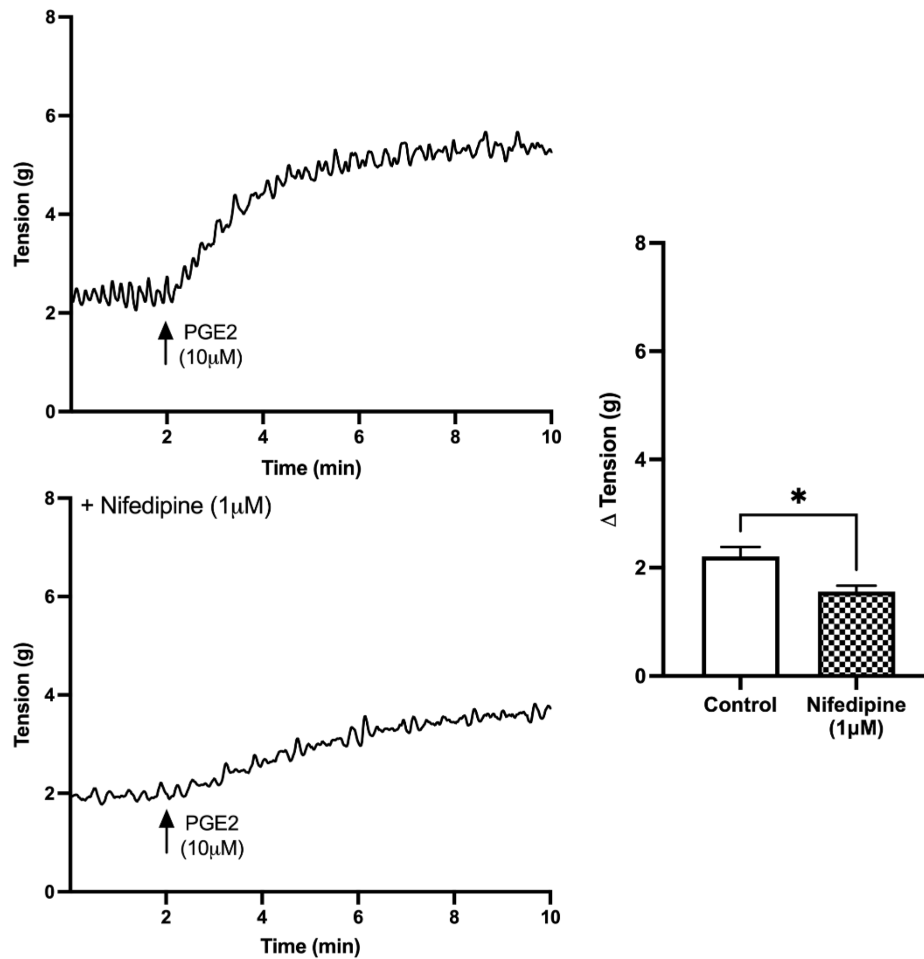

Figure 3-5: U&LP baseline tension responses to receptor agonist PGE2 (10µM) in the absence of (*upper left trace*) and in the presence of (*lower left trace*) L-type Ca^{2+} inhibitor, nifedipine (1µM). Changes to baseline tension (*right*) presented as mean ± SEM. *$p < 0.05$ (paired Student's two-tailed *t*-test).

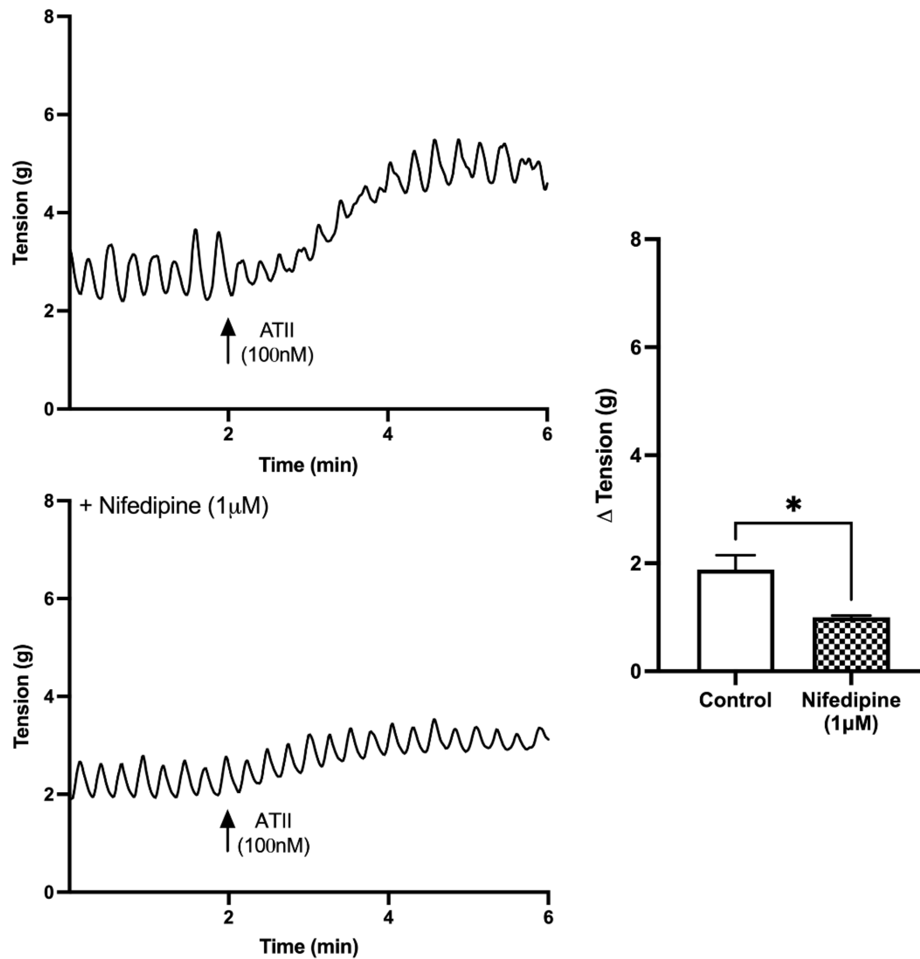

Figure 3-6: U&LP baseline tension responses to receptor agonist ATII (100nM) in the absence of (*upper left trace*) and in the presence of (*lower left trace*) L-type Ca^{2+} inhibitor, nifedipine (1µM). Changes to baseline tension (*right*) presented as mean ± SEM. *$p < 0.05$ (paired Student's two-tailed *t*-test).

3.4.2.2 Effect of nifedipine on frequency and amplitude of phasic contractions

The frequencies and amplitudes of spontaneous phasic contractions produced by the U&LP tissues were investigated in the absence and presence of nifedipine for each of the receptor agonists. No significant differences in the contractile frequency (Table 3-2) or amplitude (Table 3-3) were observed between the presence and absence of nifedipine (1µM) for responses to carbachol, histamine, 5-HT, or NKA. Contractions for PGE2 (10µM, $n = 8$, $p < 0.05$) and ATII (100nM, $n = 8$, $p < 0.05$) exhibited a reduction in the amplitude of spontaneous phasic contractions in the presence of nifedipine, and when compared to the response in the absence of nifedipine, was a statistically significant difference (Table 3-3).

Table 3-2: U&LP change in frequency responses to receptor agonists in the absence (control) and presence of nifedipine (1μM). There were no significant differences between the average frequency changes between the absence and presence of nifedipine for any of the agonists. Data presented as mean ± SEM.

		Δ Frequency (cpm)			
Agonist	Conc.	Control	+ nifedipine	*p*-value	*n*
Carbachol	1μM	0.92 ± 0.17***	1.20 ± 0.44*	0.58	11
Histamine	100μM	0.76 ± 0.28*	0.83 ± 0.33*	0.88	8
5-HT	100μM	2.58 ± 0.95*	3.67± 1.66	0.26	8
NKA	300nM	0.44 ± 0.40	0.50 ± 0.59	0.91	8
PGE2	10μM	-0.23 ± 0.38	-0.41 ± 0.41	0.78	8
ATII	100nM	0.06 ± 0.17	0.24 ± 0.18	0.45	8

*$p < 0.05$, ***$p < 0.001$ (paired *t*-test) after the addition of each agonist (listed to the left) in the absence and presence of nifedipine (1μM). *p*-values (paired Student's two-tailed *t*-test) in the right column denote the differences between the responses to agonist in the absence of and the responses to agonist in the presence of nifedipine.

Table 3-3: U&LP change in amplitude responses to receptor agonists in the absence (control) and presence of nifedipine (1µM). Data presented as mean ± SEM.

		Δ Amplitude (g)			
Agonist	Conc.	Control	+ nifedipine	*p*-value	*n*
Carbachol	1µM	-0.51 ± 0.17*	-0.18 ± 0.06*	0.07	11
Histamine	100µM	-0.03 ± 0.05	-0.07 ± 0.04	0.54	8
5-HT	100µM	-0.25 ± 0.10*	-0.28 ± 0.08**	0.70	9
NKA	300nM	-0.23 ± 0.08*	-0.18 ± 0.07*	0.48	8
PGE2	10µM	-0.04 ± 0.04	-0.16 ± 0.03***	0.01**	8
ATII	100nM	-0.32 ± 0.08**	-0.16 ± 0.06**	0.05*	8

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (paired *t*-test) after the addition of each agonist (listed to the left) in the absence and presence of nifedipine (1µM). *p*-values (paired Student's two-tailed *t*-test) in the right column denote the differences between the responses to agonist in the absence of and the responses to agonist in the presence of nifedipine.

3.4.3 *Influence of nominally zero Ca^{2+}-free solution on U&LP contractions*

3.4.3.1 Effect of nominally zero Ca^{2+}-free solution on baseline tensions

U&LP contractions for all receptor agonists in the presence of nominally zero Ca^{2+} solution were significantly inhibited compared to the control group in normal Krebs solution. In the absence of any extracellular Ca^{2+} sources, the contractions of the U&LP were impaired (paired two-tailed Student's *t*-test). Contractions were inhibited as follows: carbachol by 39% (1µM, $n = 11$, $p < 0.01$, Figure 3-7); histamine by 46% (100µM, $n = 8$, $p < 0.05$, Figure 3-8); 5-HT by 28% (100µM, $n = 8$, $p < 0.05$, Figure 3-9); NKA by 22% (300nM, $n = 9$, $p < 0.05$, Figure 3-10); PGE2 by 32% (10µM, $n = 8$, $p < 0.05$, Figure 3-11); and ATII by 43% (100nM, $n = 8$, $p < 0.01$, Figure 3-12). Across all receptors, there were no significant differences between the averaged responses (*p* = NSD, ANOVA with Tukey post-test).

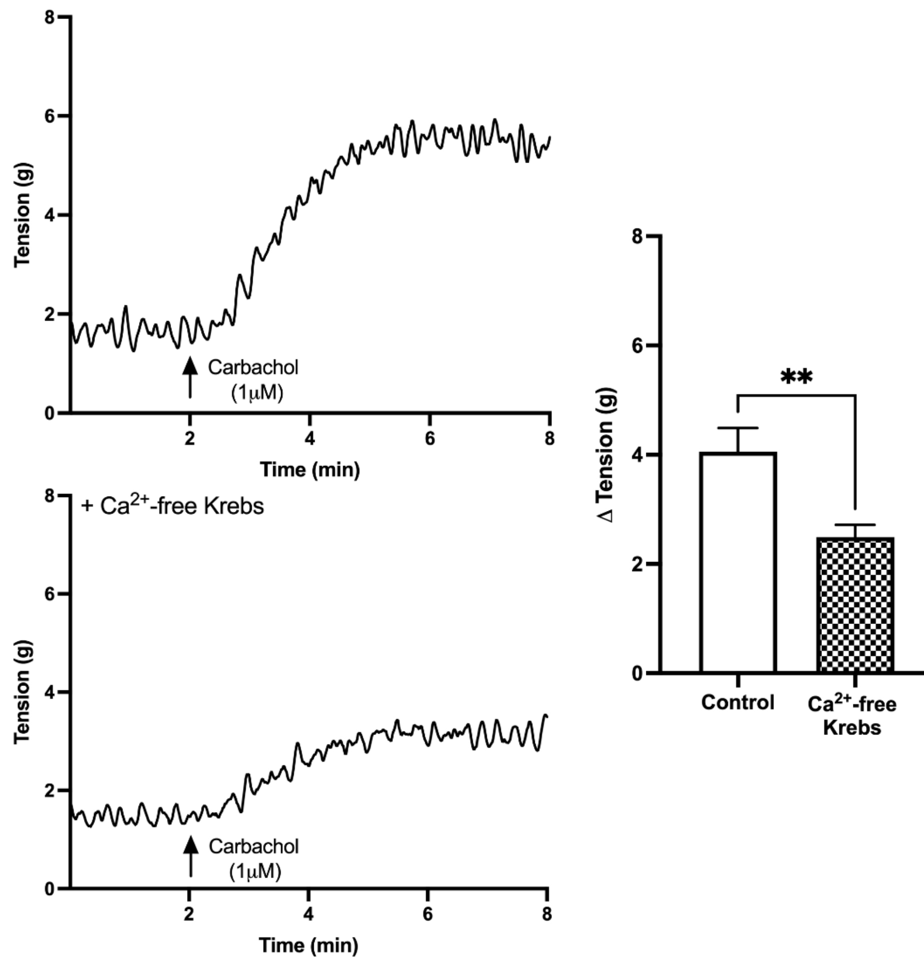

Figure 3-7: U&LP baseline tension responses to receptor agonist carbachol (1μM) as controls in the normal Krebs (*upper left trace*) and in nominally zero Ca^{2+} solution (*lower left trace*). Changes to baseline tension (*right*) presented as mean ± SEM. **$p < 0.01$ (paired Student's two-tailed *t*-test).

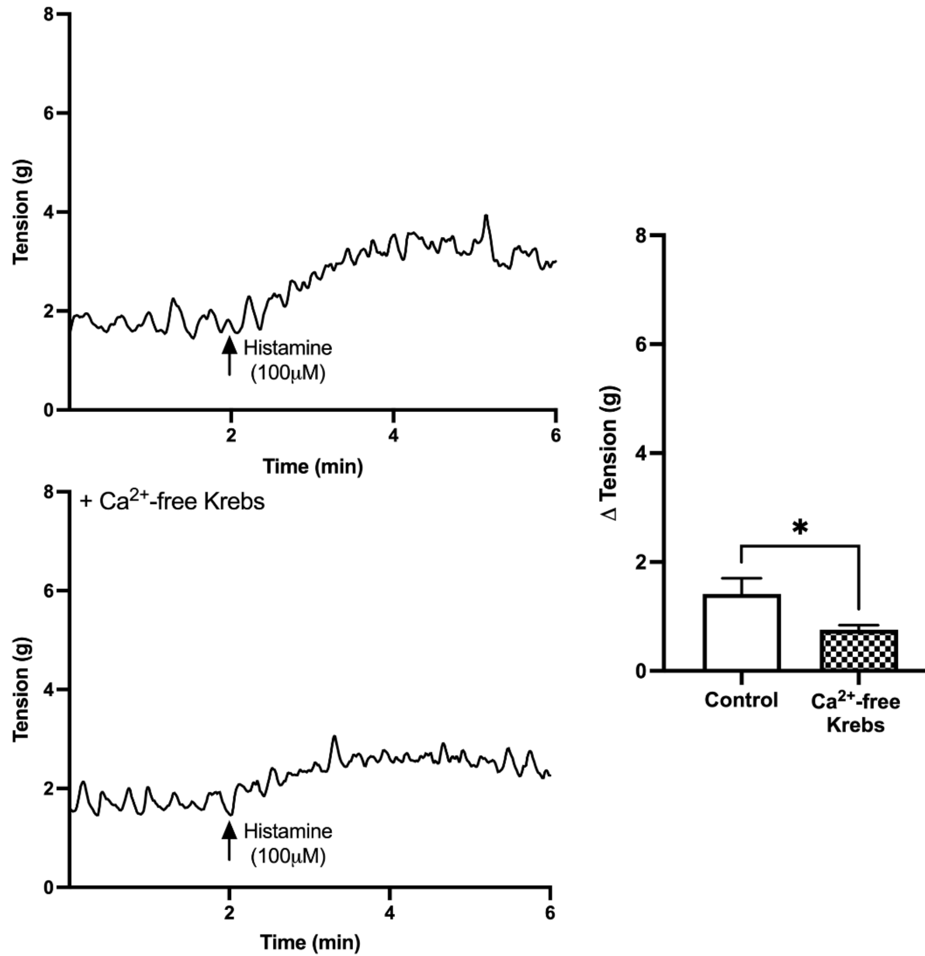

Figure 3-8: U&LP baseline tension responses to receptor agonist histamine (100µM) as controls in the normal Krebs (*upper left trace*) and in nominally zero Ca^{2+} solution (*lower left trace*). Changes to baseline tension (*right*) presented as mean ± SEM. *p < 0.05 (paired Student's two-tailed *t*-test).

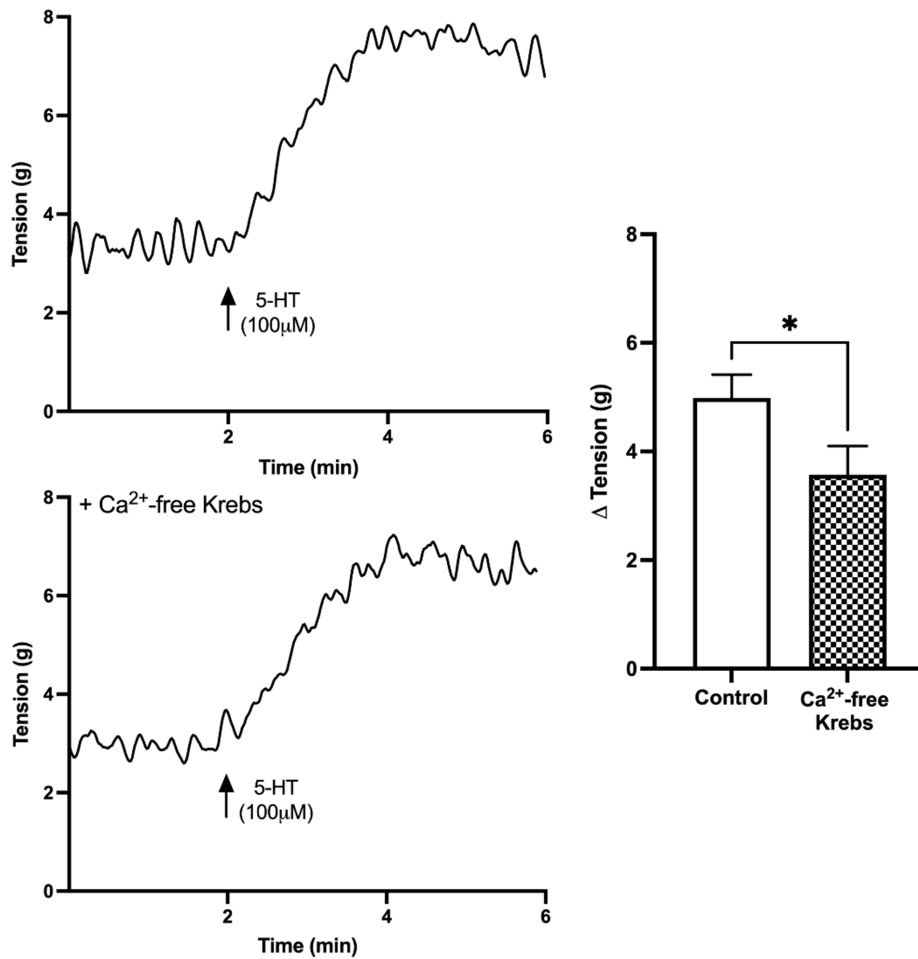

Figure 3-9: U&LP baseline tension responses to receptor agonist 5-HT (100μM) as controls in the normal Krebs (*upper left trace*) and in nominally zero Ca^{2+} solution (*lower left trace*). Changes to baseline tension (*right*) presented as mean ± SEM. *$p < 0.05$ (paired Student's two-tailed *t*-test).

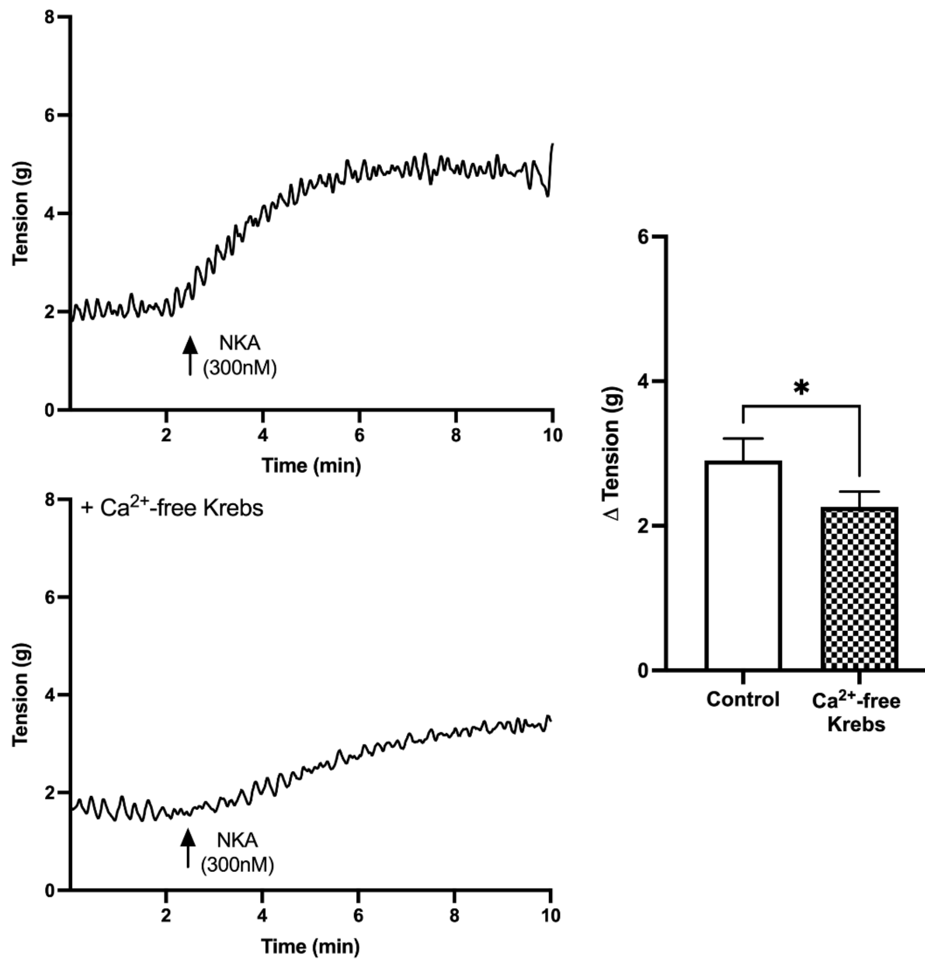

Figure 3-10: U&LP baseline tension responses to receptor agonist NKA (300nM) as controls in the normal Krebs (*upper left trace*) and in nominally zero Ca^{2+} solution (*lower left trace*). Changes to baseline tension (*right*) presented as mean ± SEM. *$p < 0.05$ (paired Student's two-tailed t-test).

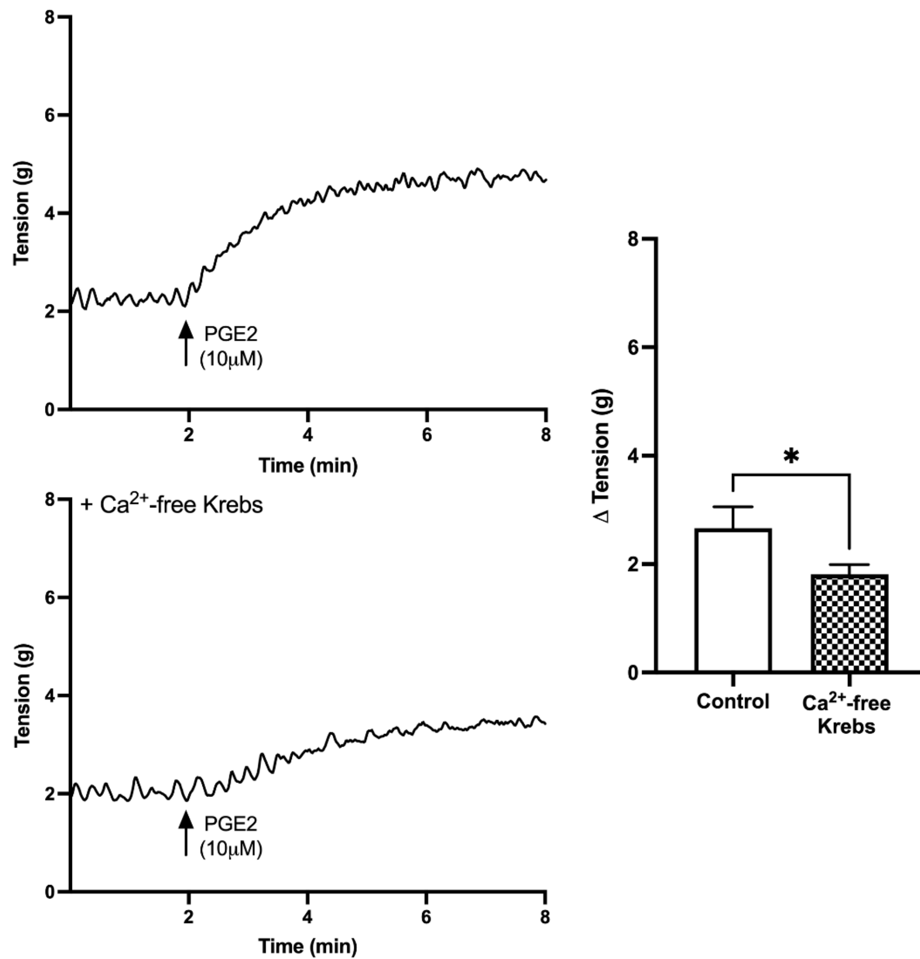

Figure 3-11: U&LP baseline tension responses to receptor agonist PGE2 (10µM) as controls in the normal Krebs (*upper left trace*) and in nominally zero Ca^{2+} solution (*lower left trace*). Changes to baseline tension (*right*) presented as mean ± SEM. *$p < 0.05$ (paired Student's two-tailed *t*-test).

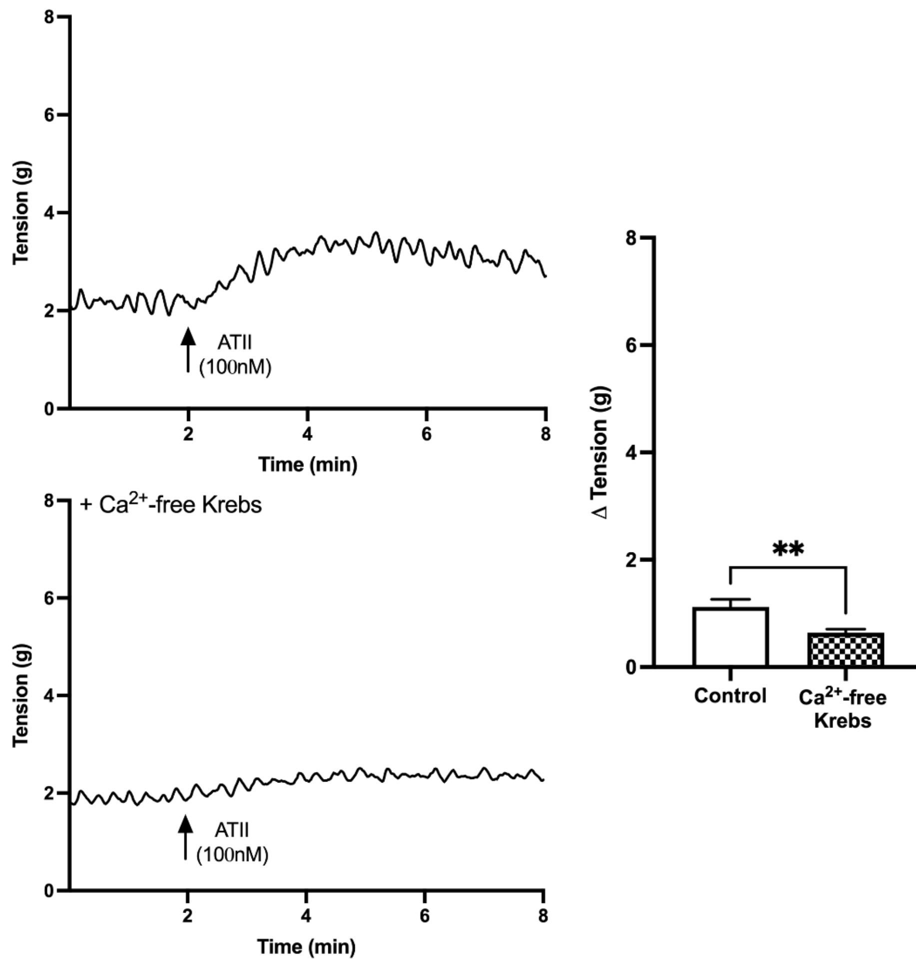

Figure 3-12: U&LP baseline tension responses to receptor agonist ATII (100nM) as controls in the normal Krebs (*upper left trace*) and in nominally zero Ca^{2+} solution (*lower left trace*). Changes to baseline tension (*right*) presented as mean ± SEM. $^{**}p < 0.01$ (paired Student's two-tailed *t*-test).

3.4.3.2 Effect of nominally zero Ca^{2+}-free solution on frequency and amplitude of spontaneous contractions

Tissues exposed to a Ca^{2+}-free extracellular environment had no alterations to their frequency (Table 3 4) or amplitude (Table 3-5) of spontaneous phasic contraction during responses to receptor agonists carbachol (1µM), histamine (100µM), 5-HT (100µM), NKA (300nM) or PGE2 (10µM). Although the activation of all receptors induced increases to baseline tensions, only ATII (100nM) also resulted in the significant decrease of frequency of spontaneous phasic contractions in the presence of nominally zero Ca^{2+} solution (Table 3-4).

Table 3-4: U&LP change in frequency responses to receptor agonists in the absence (control) and presence of nominally zero Ca^{2+} solution. Data presented as mean ± SEM.

		Δ Frequency (cpm)			
Agonist	Conc.	Control	+ Ca^{2+}-free Krebs	*p*-value	*n*
Carbachol	1μM	1.38 ± 0.36**	1.88 ± 0.84*	0.48	11
Histamine	100μM	0.74 ± 0.27*	0.93 ± 0.70	0.52	6
5-HT	100μM	2.44 ± 0.77*	2.68 ± 1.31	0.83	8
NKA	300nM	0.01 ± 0.24	1.93 ± 0.47	0.09	9
PGE2	10μM	0.14 ± 0.59	0.63 ± 0.45	0.49	8
ATII	100nM	0.22 ± 0.09*	-0.32 ± 0.16	0.02*	8

*$p < 0.05$, **$p < 0.01$ (paired *t*-test) after the addition of each agonist (listed to the left) in the absence and presence of Ca^{2+}-free Krebs. *p*-values (paired Student's two-tailed *t*-test) in the right column denote the differences between the responses to agonist in the absence of and the responses to agonist in the presence of nifedipine.

Table 3-5: U&LP change in amplitude responses to receptor agonists in the absence (control) and presence of nominally zero Ca^{2+} solution. No significant differences between the average amplitude changes between the absence and presence of nominally zero Ca^{2+} solution for any of the agonists. Data presented as mean ± SEM.

		Δ Amplitude (g)			
Agonist	Conc.	Control	+ Ca^{2+}-free Krebs	*p*-value	*n*
Carbachol	1µM	-0.02 ± 0.05	0.02 ± 0.05	0.58	11
Histamine	100µM	-0.13 ± 0.12	-0.19 ± 0.07*	0.71	8
5-HT	100µM	-0.51 ± 0.14**	-0.22 ± 0.22	0.17	8
NKA	300nM	-0.01 ± 0.07	-0.05 ± 0.05	0.63	9
PGE2	10µM	-0.02 ± 0.08	-0.08 ± 0.08	0.66	8
ATII	100nM	-0.12 ± 0.08	0.04 ± 0.11	0.32	8

*$p < 0.05$, **$p < 0.01$ (paired *t*-test) after the addition of each agonist (listed to the left) in the absence and presence of Ca^{2+}-free Krebs. *p*-values (paired Student's two-tailed *t*-test) in the right column denote the differences between the responses to agonist in the absence of and the responses to agonist in the presence of nifedipine.

3.4.4 *Overall impact of extracellular Ca^{2+} on baseline tension contractions*

In this study, two different methods were applied to assess the impact of extracellular Ca^{2+}: the immersion of the tissue in nominally zero Ca^{2+} solution; or through Ca^{2+} channel antagonism with nifedipine (1µM). Both methods significantly inhibited contractile activity changes in response to the assessed agonists. Overall, when looking at the impact of either nifedipine or nominally zero Ca^{2+} solution, there were no significant differences (Student's two-tailed unpaired *t*-tests for each) between the effectiveness of inhibitions of tension, frequency, or amplitude after the addition of carbachol (1µM), histamine (100µM), 5-HT (100µM), NKA (300nM), PGE2 (10µM), and ATII (100nM, p = NSD for all).

3.5 Discussion

The extent of influence that either extracellular or intracellular Ca^{2+} has on smooth muscle contraction varies throughout the body, promoting a specific interest towards identifying the prominent sources of Ca^{2+} influx across different organs. Ca^{2+} has a clear role in mediating the contractile activity of the bladder (Ikeda & Kanai, 2008), but the specific source of the Ca^{2+} in the urothelium and lamina propria tissue layer has never been investigated. Extracellular Ca^{2+} is of particular interest as it plays an essential role in many physiologic functions throughout the human body, and stimulates not only contraction, but also key underlying Ca^{2+} -dependent systems, which could be altered in bladder disorders (de Groat, 2004). As such, identifying the potential mechanisms involved in receptor-mediated contractions can support the development of new and novel pharmaceuticals, as well as develop a greater understanding of potential mechanisms underlying bladder dysfunction.

This study has identified prominent extracellular Ca^{2+} influences on muscarinic, histamine, 5-HT, neurokinin-A, prostaglandin E2 and angiotensin-II receptor mechanisms of action. Each of these receptor systems are G protein-coupled receptors of the G_{q11} class. Particular subtypes of interest have been previously identified as the M3 muscarinic (Moro et al., 2011), H1 histamine (Stromberga et al., 2019), 5-HT_{2A} serotonergic (Moro et al., 2016), neurokinin-A (Grundy et al., 2018), EP1 prostaglandin (Stromberga et al., 2020c), and AT_1 angiotensin II (Lim et al., 2021) receptors. This broad presence of GPCRs in the U&LP constitutes the majority of surface receptors in the bladder, and many can be activated through neurotransmitters, hormones, and external stimuli that elicit a variety of cellular responses to stimulate downstream signalling activities (Mizuno & Itoh, 2009).

Both inhibition of the L-type Ca^{2+} channels with nifedipine or inhibiting Ca^{2+} influx from extracellular fluids showed similar influences towards impairing GPCR contractions. This suggests that Ca^{2+} influx from extracellular sources via the L-type Ca^{2+} channels is responsible for around 20-50% of the receptor-mediated contractions. These findings are consistent with the extracellular Ca^{2+} influences in the detrusor smooth muscle layer of the urinary bladder. Heppner et al. (2011) demonstrated the activity of L-type voltage-gated Ca^{2+} channels by inhibiting their activity, which reduced carbachol-induced contractions of human tissue by 74%, 18% in pig, and 27% in mouse tissue.

In this study, the response of each of the GPCRs to nifedipine has supported the presence of L-type voltage-gated Ca^{2+} channels in the U&LP of the urinary bladder. Lining the bladder lumen, the urothelium not only has an integral role in acting as the highly resistant physical barrier between urine and the underlying tissues, but also responds to stimuli and can transfer information to underlying cells (Birder & Andersson, 2013). Heppner et al. (2011) also

observed the bladder U&LP of guinea pig tissue to exhibit dependence on Ca^{2+} influx through L-type Ca^{2+} channels, with spontaneous contractions significantly inhibited by nifedipine. However, clear evidence is lacking on the location of the L-type Ca^{2+} channels within these tissue layers. The lamina propria, a layer of highly innervated connective tissue located between the basement membrane of the urothelium and luminal surface of the detrusor, has demonstrated an essential role in signalling functions (Andersson & McCloskey, 2014; Heppner et al., 2017). In particular, its role in Ca^{2+} signalling may be of importance to the maintenance of normal bladder function and may present as a core site of extracellular Ca^{2+} influence. This could be through myofibroblasts, which have been identified to contribute to spontaneous activity through extracellular sources, and also express muscarinic and purinergic receptors that could assist in propagating signals to the urothelium (Heppner et al., 2011). Moreover, it has been suggested that the muscularis mucosae found within the underlying lamina propria of some species may also be impacted by the entry of extracellular Ca^{2+} (Heppner et al., 2011). In addition, interstitial cells of Cajal-like cells, located within the lamina propria layers, have demonstrated firing activity of Ca^{2+} transients (Hashitani et al., 2004; Johnston et al., 2008), and there is some influence on the activity of pericytes surrounding blood vessels within the tissue (Mitsui & Hashitani, 2020).

The partial reduction (20-50%) in GPCR-mediated contractions of the U&LP by nifedipine may indicate additional sources of Ca^{2+} entry. This could include other voltage-gated channels such as T-type or P-type Ca^{2+} channels, previously identified in the urinary bladder (Deng et al., 2012). In addition, this partial reduction which was maintained in a Ca^{2+}-free environment suggests internal stores of Ca^{2+}, such as from the sarcoplasmic reticulum, or other signalling pathways activated by $G_{q/11}$ receptor proteins, may play a role in mediating contractile activity of the U&LP.

3.5.1 *Clinical relevance*

Antimuscarinics and parasympathomimetics have shown success for the management of bladder contractile disorders and currently sit as first-line pharmaceutical treatment for overactive bladder and underactive bladder, respectively. However, most patients cease treatment regimens due to lower than expected benefits and adverse side effects. Recent success has been found with combination therapies and there is increasing interest in the identification of alternative receptors systems that may be involved in contraction, and hence future targets for therapeutic treatments. This study's identification of similarities between receptors mediating and modulating contraction in the urinary bladder may present future therapeutic targets or provide insights into mechanisms that may be dysfunctional in overactive or underactive bladder. Of particular interest was this study's finding that histamine, neurokinin-A and angiotensin II are highly dependent on extracellular Ca^{2+}, warranting further investigation into their clinical use for the management of bladder dysfunction.

3.5.2 *Limitations and future direction*

A limitation of this study was the use of single-dose applications of receptor agonists to examine changes in frequencies and amplitudes of phasic contractions over 30-minute time periods. It should be noted that in detrusor studies of carbachol-induced bladder contractions, human tissue responded to inhibited extracellular Ca^{2+} to a lesser extent than pig tissue (Wuest et al., 2007), however, this has not been demonstrated in urothelial and lamina propria studies. Future studies could investigate the potential role of ageing in influencing receptor responses to extracellular contractions, such as histamine (Stromberga et al., 2020a), as well as explore the influence of other GPCRs and the influence of Ca^{2+} on their responses.

3.6 Conclusions

This study identified a potential role of extracellular Ca^{2+} for urinary bladder contractile activity, which may warrant further investigation regarding its impact on bladder dysfunction. A plausible explanation for these findings may be that an imbalance of Ca^{2+}, or an abnormal influx of extracellular Ca^{2+}, via subsequent opening of Ca^{2+} extracellular channels after activation of muscarinic, histamine, 5-HT, neurokinin-A, prostaglandin E2 and angiotensin II receptors could be a causative factor in contractile disorders such as underactive bladder.

Chapter 4

The role of intracellular calcium and Rho kinase pathways in G protein-coupled receptor-mediated contractions of urinary bladder urothelium and lamina propria

PUBLISHED CHAPTER

Phelps, C., Chess-Williams, R., & Moro, C. (2023). The role of intracellular calcium and Rho kinase pathways in G protein-coupled receptor-mediated contractions of urinary bladder urothelium and lamina propria. *American Journal of Physiology – Cell Physiology, 324*(3), C787-C797. https://doi.org/10.1152/ajpcell.00441.2022

Additional published abstracts and conference presentations arising from this chapter:

Phelps, C., Chess-Williams, R., & Moro, C. (2022, October). Future therapies for the treatment of bladder contractile disorders? *ASCEPT Special Interest Group Virtual National Symposium – Advances in Urogenital and Gastrointestinal Research.* Virtual conference.

Phelps, C., Chess-Williams, R., & Moro, C. (2021, October). Novel targets for the pharmaceutical management of bladder contractile disorders: Identifying mediators of contraction in the urinary bladder urothelium. *ASCEPT Special Interest Group Virtual National Symposium on Advances in Urogenital and Gut Research Symposium.* Virtual conference.

Preface

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Formatting and terminology changes have been made to maintain consistency throughout this thesis. The final version of this manuscript can be accessed at: https://journals.physiology.org/doi/full/10.1152/ajpcell.00441.2022.

All data presented in this manuscript are the sole work of Charlotte Phelps.

4.1 Abstract

The influence of extracellular and intracellular calcium on smooth muscle contractile activity varies between organs. In response to G protein-coupled receptor (GPCR) stimulation, the urinary bladder detrusor muscle has shown a 70% dependence on extracellular calcium, while the urothelium and lamina propria (U&LP) has a 20-50% dependence. However, as this only accounts for partial contractile activity, the contribution of intracellular calcium and calcium sensitization pathways remains unclear. This study assessed the role of intracellular signalling pathways on GPCR-mediated urinary bladder U&LP contraction. Porcine U&LP responses to activation of the $G_{q/11}$-coupled muscarinic, histamine, 5-hydroxytryptamine (serotonin), neurokinin, prostaglandin, and angiotensin II receptors were assessed with three selective inhibitors of store-released intracellular calcium, 2-APB, CPA, and ruthenium red, and three Rho kinase inhibitors, fasudil, Y-27632, and GSK269962. There was no discernible impact on receptor agonist-induced contractions of the U&LP after blocking intracellular calcium pathways, suggesting this tissue is more sensitive to alterations in the availability of extracellular calcium. However, an alternative mechanism of action for GPCR-mediated contraction was identified to be the activation of Rho kinase, such as when Y-27632 significantly reduced the GPCR-mediated contractile activity of the U&LP by 50% ($p < 0.05$, $n = 8$). This suggests that contractile responses of the bladder U&LP do not involve a significant release of calcium from intracellular stores, but that $G_{q/11}$-coupled receptor activation causes calcium sensitization via Rho kinase. This study presents a key role for Rho kinase in the urinary bladder, which may provide a novel target in the future pharmaceutical management of bladder contractile disorders.

4.2 Introduction

The most common front-line target in the pharmaceutical management of bladder contractile disorders is the $G_{q/11}$-coupled M3 muscarinic receptor (Moro et al., 2021b). However, in many cases, the prescription of muscarinic receptor antagonists for overactive bladder and muscarinic receptor agonists for underactive bladder, is ineffective, with most patients ceasing their pharmaceutical regimens (Basra et al., 2008) due to lower-than-expected treatment outcomes or recurring side effects (Chapple et al., 2008; Moro et al., 2021b). This presents a need to further the current understanding of receptor systems involved in urinary bladder contractions. Investigating the influence of receptor-mediated contractions on bladder activity may also identify potential therapeutic targets and novel treatments for underactive and overactive bladder (Moro et al., 2021b). There has been a particular focus on the detrusor smooth muscle in the pharmaceutical management of bladder contractile disorders. However, the luminal urothelial layer and underlying lamina propria, also termed the ‘bladder mucosa’, has the potential to influence whole bladder activity (Moro & Chess-Williams, 2012). It has been hypothesized that dysfunction of receptor systems within the urothelium and lamina propria (U&LP) may be an underlying cause in a variety of bladder disorders (Drake et al., 2018).

The contraction of the urinary bladder to stimulate emptying is primarily initiated by the release of acetylcholine from cholinergic nerves, which stimulates muscarinic receptors and triggers intracellular signalling cascades. In contrast to the human detrusor, where over 70% of GPCR-activated contraction of the smooth muscle is known to be dependent on extracellular sources of calcium (Ca^{2+}) (Wuest et al., 2007), extracellular Ca^{2+} only accounts for 20-50% of GPCR-mediated contractions in the porcine U&LP (Phelps et al., 2022). This lowered dependence on extracellular sources presents the potential for intracellular signalling pathways to play an

important functional role within the U&LP. Activation of $G_{q/11}$-coupled receptors activates phospholipase C and catalyses the hydrolysis of phosphatidylinositol biphosphate to generate inositol trisphosphate (IP_3) and diacylglycerol (DAG) (Sánchez-Fernández et al., 2014). These second messengers propagate and amplify the $G_{q/11}$-mediated signal with Ca^{2+} mobilization by Ca^{2+} release from intracellular stores and DAG-dependent protein kinase C activation (Mizuno & Itoh, 2009). An increase in the intracellular Ca^{2+} concentration is typically the primary stimulus for smooth muscle contraction, where Ca^{2+} binds to calmodulin, and the resulting complex activates myosin light chain kinase (MLCK), which phosphorylates MLC and promotes the interaction of myosin with actin for subsequent contraction. An elevated concentration of intracellular Ca^{2+} is essential for the normal contractile activity of the urinary bladder. Levels of Ca^{2+} in the cytosol are dependent on the influx of extracellular Ca^{2+} via plasma membrane Ca^{2+} channels, as well as Ca^{2+} release from intracellular stores. However, there remains uncertainty about whether this intracellular Ca^{2+} concentration is due to Ca^{2+} influx entering the membrane from extracellular sources or the release of Ca^{2+} from the sarcoplasmic reticulum (SR) (Batra et al., 1987; Rivera & Brading, 2006; Wuest et al., 2007).

The Rho kinase pathway also plays a role in urinary bladder contraction by modifying the sensitivity of contractile and regulatory proteins to intracellular Ca^{2+} concentrations (Somlyo et al., 1999). Rho kinase is the major Ca^{2+}-dependent pathway in contraction and has been shown to play an important role in the spontaneous contractile activity of the intact urinary bladder (Wang et al., 2018). The Ca^{2+} sensitization events inhibit myosin light chain phosphatase (MLCP) and cause a leftward shift of the Ca^{2+}-force response curve at consistent Ca^{2+} and MLCK levels. Agonists acting on $G_{q/11}$-coupled receptors induce Ca^{2+} sensitization via the activation of Rho kinase. This occurs when the small monomeric G protein, RhoA, activated from GTP binding, phosphorylates the regulatory subunit of MLCP, which inhibits

phosphatase activity and leads to sustained contractions (Somlyo & Somlyo, 2000). In the human detrusor muscle, it has been observed that muscarinic receptor-mediated contraction involves increases in intracellular Ca^{2+} concentrations, and also increases the Ca^{2+} sensitivity of contractile apparatus in a Rho kinase- and protein kinase C-dependent manner (Takahashi et al., 2004).

To investigate the contribution of intracellular Ca^{2+}-dependent and Ca^{2+}-independent sources for receptor-mediated U&LP contractions, selective pharmacological inhibitors for these pathways were chosen. 2-aminoethyl diphenylborinate (2-APB) is a membrane-penetrable inhibitor of IP_3-induced Ca^{2+} release (Luptak et al., 2018). Cyclopiazonic acid (CPA) is an inhibitor of the Ca^{2+}-ATPase pump of the SR (Munro & Wendt, 1994). Ruthenium red has a primary effect on the mitochondrial Ca^{2+} uniporter (Bae et al., 2003), and a secondary effect on impacting Ca^{2+} efflux from the SR via IP_3-gated and ryanodine receptors (Xu et al., 1998). Fasudil (Tatsumiya et al., 2009), Y-27632 (Wibberley et al., 2003), and GSK269962 (N-[3-[[2-(4-Amino-1,2,5-oxadiazol-3-yl)-1-ethyl-1H-imidazo[4,5-c]pyridin-6-yl]oxy]phenyl]-4-[2-(4-morpholinyl)ethoxy]benzamide)) (Wróbel et al., 2018) are selective Rho-associated protein kinase inhibitors.

There is increasing evidence to support the prominent role of the U&LP in overall bladder contractile activity (Birder et al., 2010; Stromberga et al., 2020a). In particular, this tissue layer may release mediators such as acetylcholine, ATP (Sui et al., 2014), or prostaglandins (Stromberga et al., 2020b) to stimulate spontaneous contractions, or impact the activity of the underlying detrusor smooth muscle (Sellers et al., 2018). However, most past research into bladder contraction has focused on the activity of the detrusor (Dalghi et al., 2020), leaving a limited understanding of mechanisms taking place within the U&LP. The aim of this study is

to identify intracellular pathways responsible for mediating contractions across a range of $G_{q/11}$-coupled receptor systems in the bladder U&LP. This includes identifying whether intracellular mechanisms of action are different between muscarinic, histamine, 5-hydroxytryptamine (5-HT), neurokinin, prostaglandin, and angiotensin receptors. The influence that both Ca^{2+} release from intracellular sources, as well as Ca^{2+}-independent pathways through the Rho kinase system, plays in urinary bladder U&LP contractions will be assessed.

4.3 Materials and methods

4.3.1 *Tissue collection and preparation*

Urinary bladders were obtained from Large White-Landrace-Duroc pigs (6 months old, 80 kg live weight) from the local abattoir. As no animals were bred, harmed, culled, interfered, or interacted with as part of this research project, animal ethics approval was not required for this use of offal (Queensland Government, 2015). After collection, tissues were transported in a portable cooler in cold Krebs-Henseleit bicarbonate solution ('Krebs', composition: NaCl 118.4 mM, $NaHCO_3$ 24.9 mM, D-glucose 11.7 mM, KCl 4.6 mM, $MgSO_4$ 2.41 mM, $CaCl_2$ 1.9 mM, and KH_2PO_4 1.18 mM), maintained at 4 °C, to the University research facilities and used within five hours of the organ's retrieval.

Adjacent paired strips (2 cm x 0.5 cm) of isolated U&LP were separated from the underlying detrusor smooth muscle of whole urinary bladders (Moro & Phelps, 2022b). Strips of U&LP were mounted and suspended in 10 mL organ baths (Labglass, Brisbane, Australia) containing Krebs solution maintained at 37 °C and constantly perfused with carbogen gas (95% oxygen and 5% carbon dioxide). Each bath was washed with warmed fresh Krebs solution a total of

three times prior to experimentation. The tension placed on the tissues was manually adjusted to 20 mN using a moveable transducer positioner with a fine adjustment level. The mean ± standard deviation weight of isolated U&LP tissues used in this study was 160 ± 20 mg (average taken from 578 tissue samples).

4.3.2 *Pharmaceutical agents*

Carbamylcholine chloride (carbachol), histamine dihydrochloride (histamine), 2-aminoethyl diphenylborinate (2-APB), and fasudil hydrochloride (fasudil) were obtained from Sigma Aldrich (St. Louis, MO, USA). Neurokinin-A (NKA), cyclopiazonic acid (CPA), and GSK269962 (GSK; N-[3-[[2-(4-Amino-1,2,5-oxadiazol-3-yl)-1-ethyl-1H-imidazo[4,5-c]pyridin-6-yl]oxy]phenyl]-4-[2-(4-morpholinyl)ethoxy]benzamide) were obtained from Tocris Bioscience (Bristol, UK). 5-hydroxytryptamine (5-HT) was obtained from Toronto Research Chemicals (Toronto, ON, Canada), and Y-27632 hydrochloride (Y-27632) from AdooQ BioScience (Irvine, CA, USA). Prostaglandin-E2 (PGE2), angiotensin-II (ATII), and ruthenium red were obtained from Cayman Chemicals (Ann Arbor, MI, USA). All ingredients for the Krebs solution were from Sigma Aldrich (St. Louis, MO, USA). PGE2 was dissolved in 100% ethanol, and CPA and fasudil were dissolved in 100% dimethyl sulfoxide (DMSO). All other pharmaceutical agents were soluble in distilled water. Concentrations selected for the agonists and antagonists were chosen based on their selectivity at each receptor and consistent with concentrations used in previous studies utilizing porcine tissue. In all cases, the receptor agonist concentration used induced submaximal contraction. For this assessment, preliminary studies using concentration-response curves were performed to identify maximal contractions. The agonist concentrations were then chosen as the dose that induced ~80% of this contraction, allowing for a strong, sub-maximal contraction of the tissue.

4.3.3 *Measurements of contractile activity*

A single dose of carbachol (1 µM, muscarinic receptor agonist), histamine (100 µM), 5-hydroxytryptamine (100 µM), neurokinin-A (300 nM), prostaglandin-E2 (10 µM), or angiotensin-II (100 nM) was applied to the U&LP tissue strips following a 30-minute equilibration period. Baseline tension (millinewtons, mN), frequency (cycles per minute, cpm), and amplitude (mN) of spontaneous phasic contractions was measured before and after the application of the GPCR agonist with an isometric force transducer (MCT050/D, ADInstruments, Castle Hill, Australia) and recorded on a Powerlab system using Labchart v7 software (ADInstruments). Throughout this manuscript, baseline change in tension (ΔmN) is related specifically to baseline force.

2-APB (300 µM), ruthenium red (10 µM), fasudil (30 µM), Y-27632 (1 µM), and GSK269962 (1 µM) were applied separately to tissues for a 30-minute incubation period before a single dose of GPCR agonist was added. In separate experiments using the intracellular Ca^{2+}-ATPase pump inhibitor, CPA (10 µM), a wash-out method was employed in order to exhaust the intracellular Ca^{2+} stores from the SR to avoid reuptake by Ca^{2+}-ATPase. Initially, tissues were washed with warm Krebs containing a DMSO vehicle control (totalling 0.03% DMSO) for the control tissues, or CPA (10 µM) for experimental tissues and equilibrated for 30 minutes. After equilibration, a single dose of carbachol (1 µM) was added and when contraction reached its peak, tissues were washed with either control or CPA Krebs three times to remove any excess agonist. This process was repeated a total of three times for all tissues, and after the third wash, a single dose of a select GPCR agonist was added. During the wash-out procedure, spontaneous phasic activity was disrupted, and as such, only baseline tension (mN) was recorded and analysed for CPA studies.

Data was graphed and analysed using Prism 9.4.1 for Windows (GraphPad Software, La Jolla, California, USA). All values were reported as mean change ± standard deviation (SD). Statistical analysis was conducted using paired Student's two-tailed *t*-tests when comparing tissues with their paired, direct control tissues. On one occasion (Section 3.1), an unpaired Student's *t*-test was applied when overall contractile responses between all receptor agonists were assessed. For all statistical analyses, $p < 0.05$ was considered statistically significant. Throughout this manuscript, *n* (number of tissues) values quoted are from paired tissue strips, and as such, the number of animals (N) used can be calculated using $n \div 2$. This study followed an exploratory nature (Vollert et al., 2022; Vollert et al., 2020) with *p* values presented used for descriptive analysis, rather than the formal testing of a pre-specified statistical null hypothesis. Effect sizes (Erdogan et al., 2020) were calculated using an unbiased estimate of Cohen's *d* (Hedges & Olkin, 1985) and values determined small ($d = 0.2$), medium ($d = 0.5$), and large ($d = 0.8$).

4.4 Results

4.4.1 *Spontaneous phasic activity of the U&LP in response to GPCR agonists*

Spontaneous phasic activity was observed in strips of U&LP in the absence of any stimulation at a mean ± SD baseline tension of 19.32 ± 4.09 mN ($n = 241$), frequency of 4.03 ± 0.91 cpm ($n = 241$), and amplitude of 6.29 ± 0.24 mN ($n = 241$). In all cases, responses to GPCR agonists increased baseline tension ($p < 0.001$) and the frequency of spontaneous phasic contractions ($p < 0.05$). A decrease in the amplitude of contractions was observed with all agonists except histamine and NKA (Table 4-1).

Table 4-1: Summary of the changes in U&LP tension, frequency, and amplitude of contraction after the addition of a single dose of GPCR agonist (mean change ± SD)

Agonist	Concentration	Δ Tension (mN)	Δ Frequency (cpm)	Δ Amplitude (mN)	*n*
Carbachol	1 μM	35.39 ± 15.43***	0.90 ± 1.38***	-2.75 ± 4.30***	38
Histamine	100 μM	12.63 ± 7.67***	0.40 ± 0.87**	-0.18 ± 2.74	42
5-HT	100 μM	53.33 ± 16.22***	2.33 ± 3.27***	-4.36 ± 2.64***	39
NKA	300 nM	21.18 ± 10.97***	0.33 ± 0.98*	-0.99 ± 3.40	40
PGE2	10 μM	18.88 ± 5.93***	0.97 ± 1.41***	-1.29 ± 2.23***	42
ATII	100 nM	12.04 ± 4.62***	0.73 ± 1.19***	-0.88 ± 1.25***	40

Data presented as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (unpaired Student's two-tailed *t*-test).

4.4.2 *Influence of agents that reduce intracellular Ca^{2+} responses on spontaneous phasic contractions*

4.4.2.1 Influence of 2-APB on baseline tension, frequency, and amplitude

The responses to six GPCR agonists in the presence of an inhibitor of IP3-induced Ca^{2+} release, 2-APB, were observed. In the presence of 2-APB, increases in the baseline tension of spontaneous phasic contractions in response to histamine (100 µM) was significantly inhibited by 62% (from 16.35 ± 10.21 mN to 6.18 ± 4.52 mN, $n = 12$, $p < 0.001$). There was no difference to the change in baseline tension for responses to carbachol, 5-HT, NKA, PGE2, or ATII in the presence of 2-APB (Table 4-2). PGE2 (10 µM) in the presence of 2-APB caused a greater increase in the frequency of spontaneous phasic contractions ($n = 8$, $p < 0.01$, Table 4-3) and a decrease in the amplitude of contraction peaks ($n = 8$, $p < 0.01$, Table 4-4) compared to controls. In addition, ATII (100 nM) in the presence of 2-APB, was observed to decrease the frequency of spontaneous phasic contractions ($n = 8$, $p < 0.01$) with no change to amplitude.

Table 4-2: The effect of 2-APB (300 µM) on U&LP baseline tension in response to GPCR agonists. Data presented as mean change ± SD.

Agonist (*n*)	Conc.	Δ Tension (mN)		*p*-value
		Control	+ 2-APB	
Carbachol (8)	1 µM	28.99 ± 9.89	32.62 ± 9.88	0.27
Histamine (12)	**100 µM**	**16.35 ± 10.21**	**6.18 ± 4.52**	**0.001*******
5-HT (8)	100 µM	53.43 ± 18.16	48.42 ± 20.68	0.47
NKA (8)	300 nM	16.88 ± 12.43	14.64 ± 9.08	0.70
PGE2 (8)	10 µM	21.15 ± 4.39	27.99 ± 10.84	0.13
ATII (8)	100 nM	8.59 ± 2.96	10.19 ± 4.63	0.43

***$p < 0.001$ (paired Student's two-tailed *t*-test). Bold font designates significant difference.

Table 4-3: The effect of 2-APB (300 μM) on U&LP frequency of phasic contractions in response to GPCR agonists. Data presented as mean change ± SD.

Agonist (*n*)	Conc.	Δ Frequency (cpm)		*p*-value
		Control	+ 2-APB	
Carbachol (8)	1 μM	0.58 ± 1.18	1.09 ± 1.69	0.22
Histamine (12)	100 μM	0.14 ± 0.90	1.40 ± 2.80	0.12
5-HT (8)	100 μM	0.78 ± 2.26	2.15 ± 1.81	0.23
NKA (8)	300 nM	-0.49 ± 0.98	-0.84 ± 3.78	0.78
PGE2 (8)	**10 μM**	**0.30 ± 1.11**	**1.45 ± 0.89**	**0.006****
ATII (8)	**100 nM**	**0.72 ± 0.96**	**-0.58 ± 0.97**	**0.004****

**$p < 0.01$ (paired Student's two-tailed *t*-test). Bold font designates significant difference.

Table 4-4: The effect of 2-APB (300 µM) on U&LP amplitude of phasic contractions in response to GPCR agonists. Data presented as mean change ± SD.

Agonist (*n*)	Conc.	Δ Amplitude (mN)		*p*-value
		Control	+ 2-APB	
Carbachol (8)	1 µM	-1.04 ± 2.62	-1.86 ± 1.54	0.42
Histamine (12)	100 µM	-1.28 ± 2.18	-1.00 ± 1.56	0.71
5-HT (8)	100 µM	-3.41 ± 2.22	-4.76 ± 2.38	0.28
NKA (8)	300 nM	-0.60 ± 2.07	-1.01 ± 1.06	0.53
PGE2 (8)	**10 µM**	**-1.19 ± 0.88**	**-2.95 ± 1.02**	**0.006****
ATII (8)	100 nM	-0.36 ± 0.89	0.82 ± 1.17	0.08

**$p < 0.01$ (paired Student's two-tailed *t*-test). Bold font designates significant difference.

4.4.2.2 Influence of CPA on spontaneous phasic contractions in U&LP

After equilibration with CPA (10 μM), a wash-out method was undertaken to deplete SR intracellular Ca^{2+} stores from tissues. Baseline tension responses to receptor activation were observed to decrease after each tissue wash-out, both in the absence (control) and presence of CPA, indicating an effective depletion of Ca^{2+} stores. Following the wash-out method, in the presence of CPA (10 μM, n = 8 for all), there was no difference between responses to 1 μM carbachol, 100 μM histamine, 100 μM 5-HT, 10 μM PGE2 or 100 nM ATII compared to control contractions (all contractions $p < 0.001$ in both the presence and absence of CPA). However, tension responses to 300 nM NKA were enhanced in the presence of CPA (NKA: Δ26.11 ± 7.93 mN; NKA + CPA: Δ47.80 ± 13.00 mN; $p < 0.001$, n = 8).

4.4.2.3 Influence of ruthenium red on spontaneous phasic contractions in U&LP

In the presence of the non-specific inhibitor of intracellular Ca^{2+} signalling, ruthenium red (10 μM), the baseline tension responses to histamine (100 μM) were inhibited by 61% (histamine alone: Δ 5.34 ± 2.52 mN; histamine + ruthenium red: Δ 2.08 ± 4.32 mN; $p < 0.05$, n = 8). However, there were no significant differences between the absence and presence of ruthenium red for frequency and amplitude of spontaneous phasic contractions in response to histamine. In all other cases, there were no differences between observed responses in the absence and presence of ruthenium red (10 μM, n = 8 for all) to carbachol (1 μM), 5-HT (100 μM), NKA (300 nM), PGE2 (10 μM) or ATII (100 nM).

4.4.3 *Influence of Rho kinase inhibitors on spontaneous phasic contractions*

4.4.3.1 Influence of fasudil on spontaneous phasic contractions in U&LP

In the presence of the Rho kinase inhibitor, fasudil (30 µM), the baseline tension for all tissues decreased during the 30-minute equilibration period by ~8.63 mN (n = 50, $p < 0.001$). No change was observed from the addition of a DMSO vehicle control during equilibration.

Baseline tension responses to agonists

After activation of the six GPCRs with agonists, the baseline tension of the U&LP increased. In the presence of the Rho kinase inhibitor fasudil (30 µM), increases in the baseline tension of contractions in response to receptor agonists were inhibited for carbachol (1 µM, Figure 4-1), histamine (100 µM, Figure 4-2), 5-HT (100 µM, Figure 4-3), and ATII (100 nM, Figure 4-6). However, there was no difference to baseline tension in response to receptor agonists NKA (300 nM, Figure 4-4) and PGE2 (10 µM, Figure 4-5) in the presence of fasudil. Upon observation, the effect on the responses to histamine (100 µM, n = 8) appeared significantly greater than its effect on 5-HT (100 µM, n = 8), where fasudil inhibited contraction by 67% for histamine (histamine alone: Δ 12.61 ± 3.67 mN; histamine + fasudil: Δ 4.12 ± 1.25 mN, $p <$ 0.001) and 17% for 5-HT (5-HT alone: Δ 50.79 ± 15.65 mN; 5-HT + fasudil: Δ 42.06 ± 13.00 mN, $p < 0.01$).

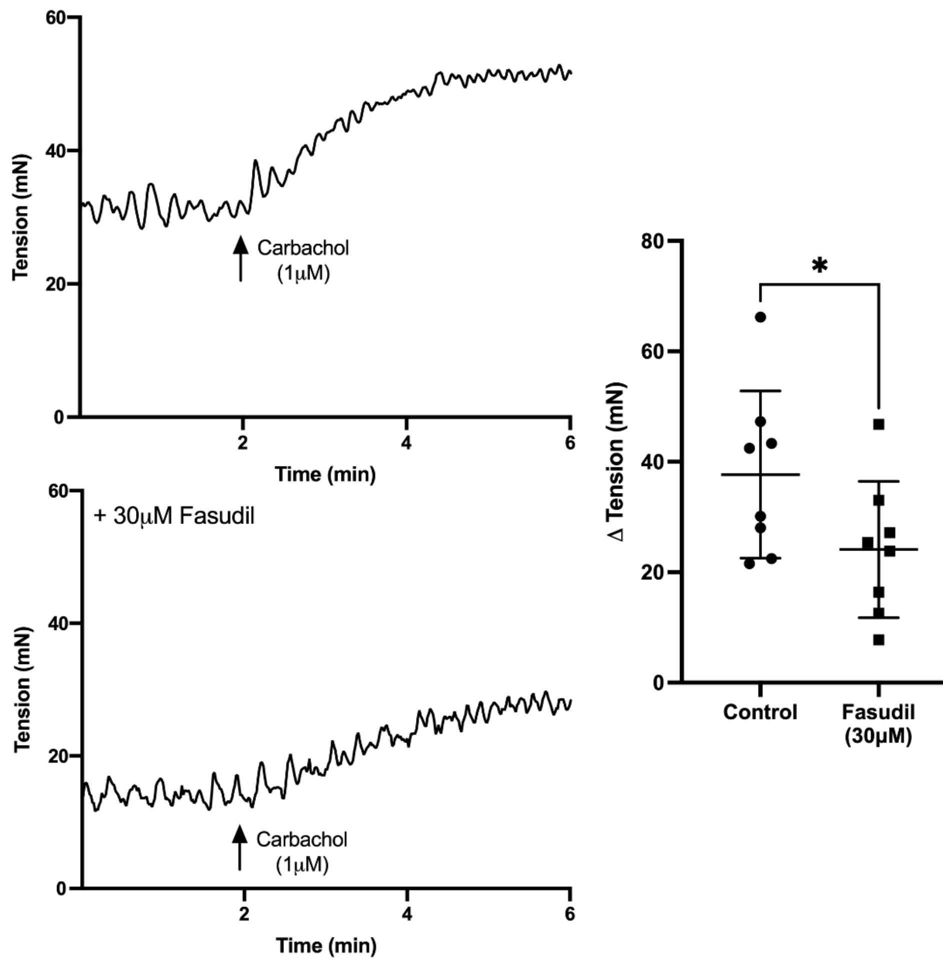

Figure 4-1: U&LP baseline tension responses to receptor agonist carbachol (1 μM, n = 8) as controls in the absence of (*upper left trace*) and in the presence of (*lower left trace*) fasudil (30 μM). Changes to baseline tension (*right*) presented as mean ± SD. *$p < 0.05$ (paired Student's two-tailed t-test).

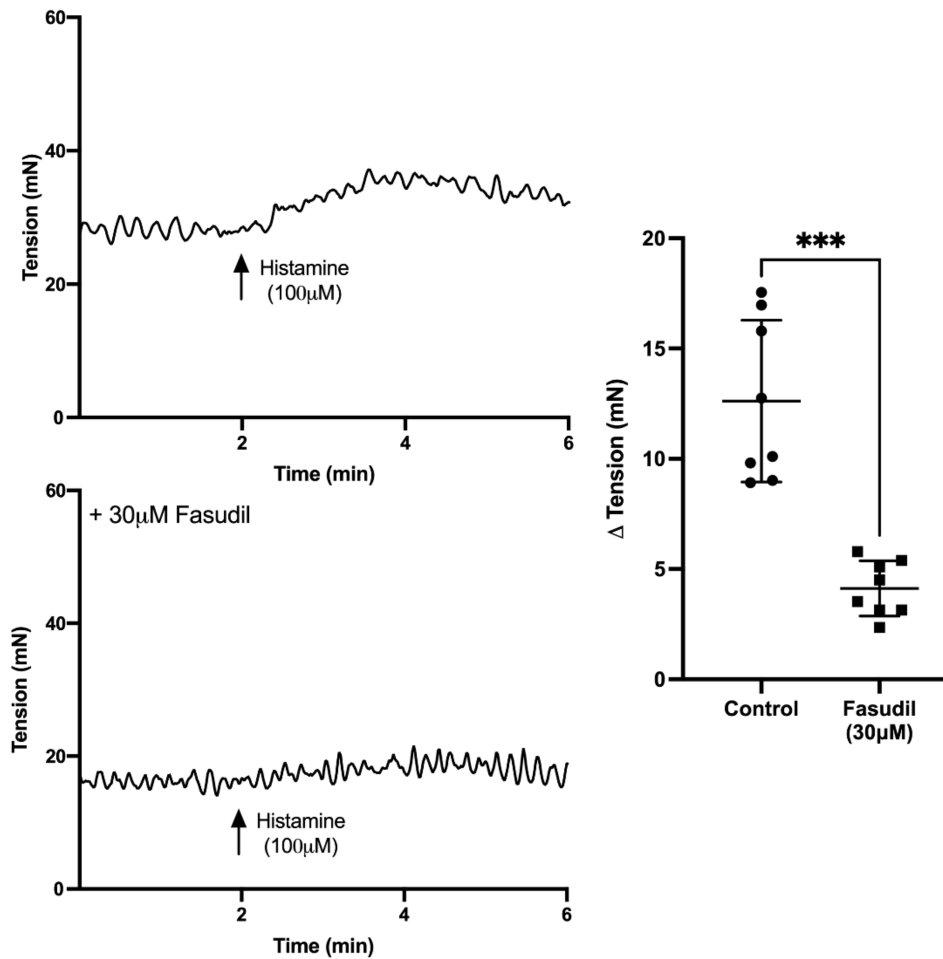

Figure 4-2: U&LP baseline tension responses to receptor agonist histamine (100 µM, $n = 8$) as controls in the absence of (*upper left trace*) and in the presence of (*lower left trace*) fasudil (30 µM). Changes to baseline tension (*right*) presented as mean ± SD. ***$p < 0.001$ (paired Student's two-tailed *t*-test).

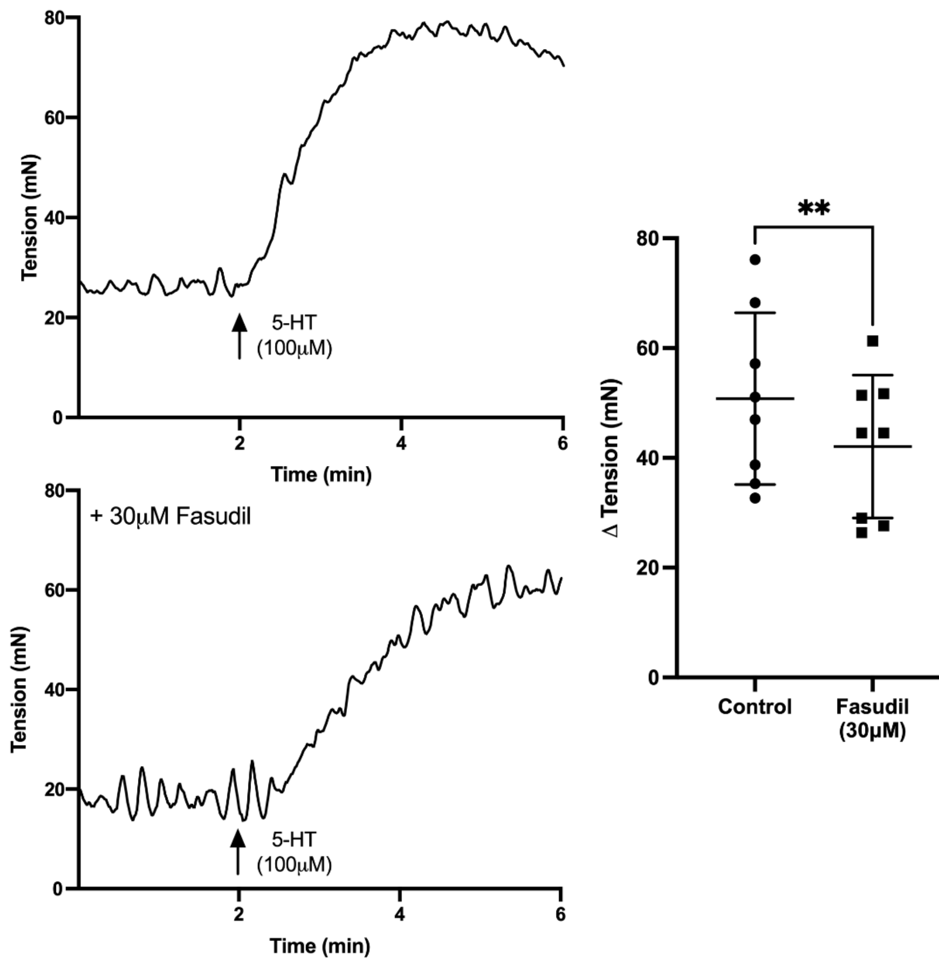

Figure 4-3: U&LP baseline tension responses to receptor agonist 5-HT (100 μM, n = 8) as controls in the absence of (*upper left trace*) and in the presence of (*lower left trace*) fasudil (30 μM). Changes to baseline tension (*right*) presented as mean ± SD. **p < 0.01 (paired Student's two-tailed t-test).

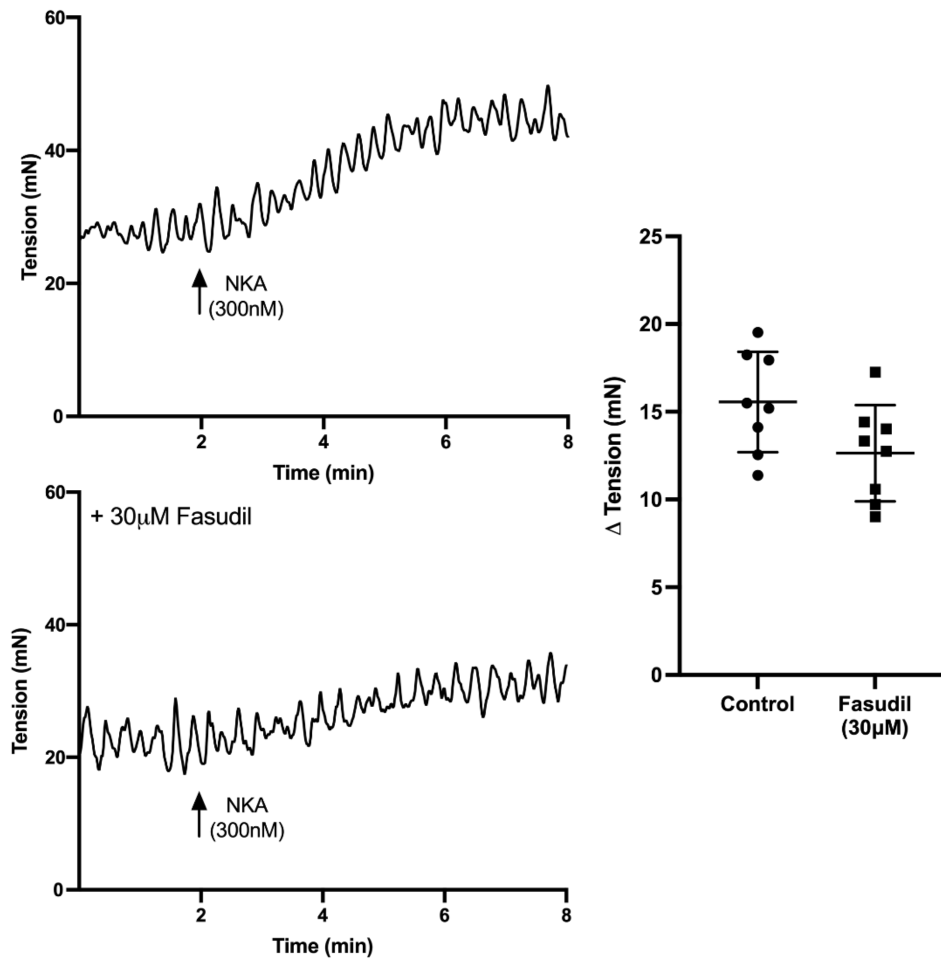

Figure 4-4: U&LP baseline tension responses to receptor agonist NKA (300 nM, *n* = 8) as controls in the absence of (*upper left trace*) and in the presence of (*lower left trace*) fasudil (30 μM). Changes to baseline tension (*right*) presented as mean ± SD.

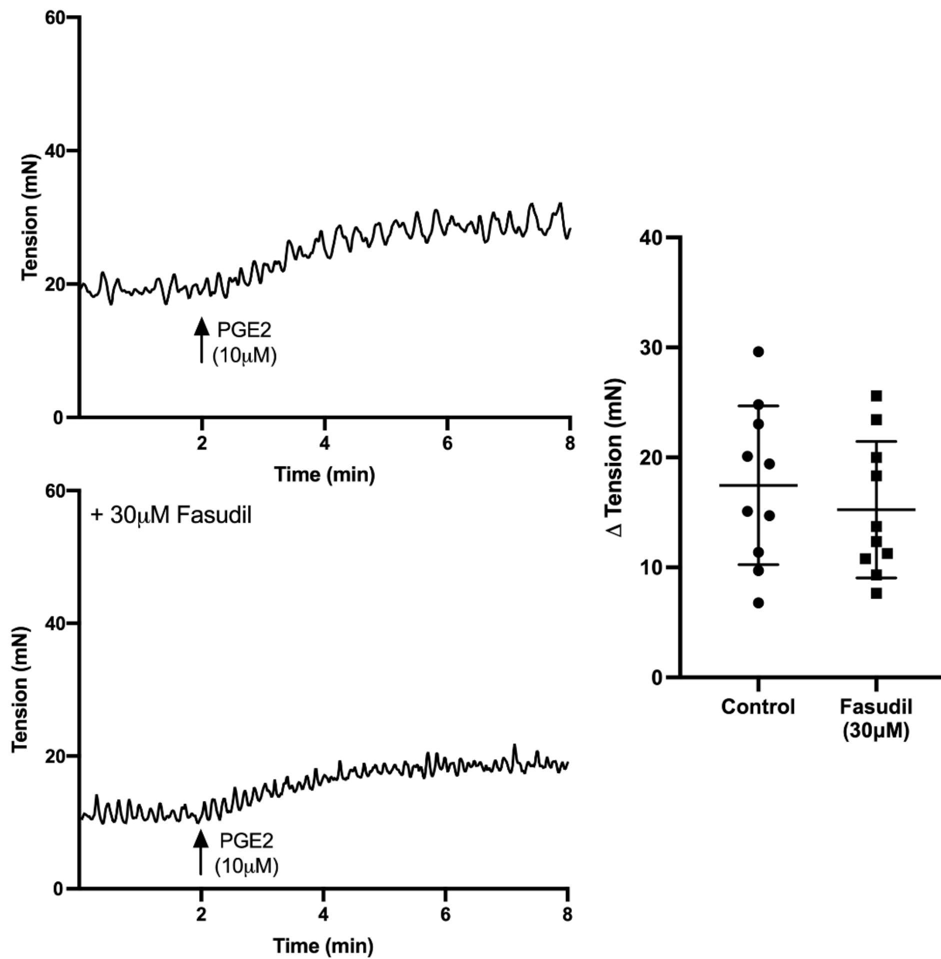

Figure 4-5: U&LP baseline tension responses to receptor agonist PGE2 (10 μM, n = 10) as controls in the absence of (*upper left trace*) and in the presence of (*lower left trace*) fasudil (30 μM). Changes to baseline tension (*right*) presented as mean ± SD.

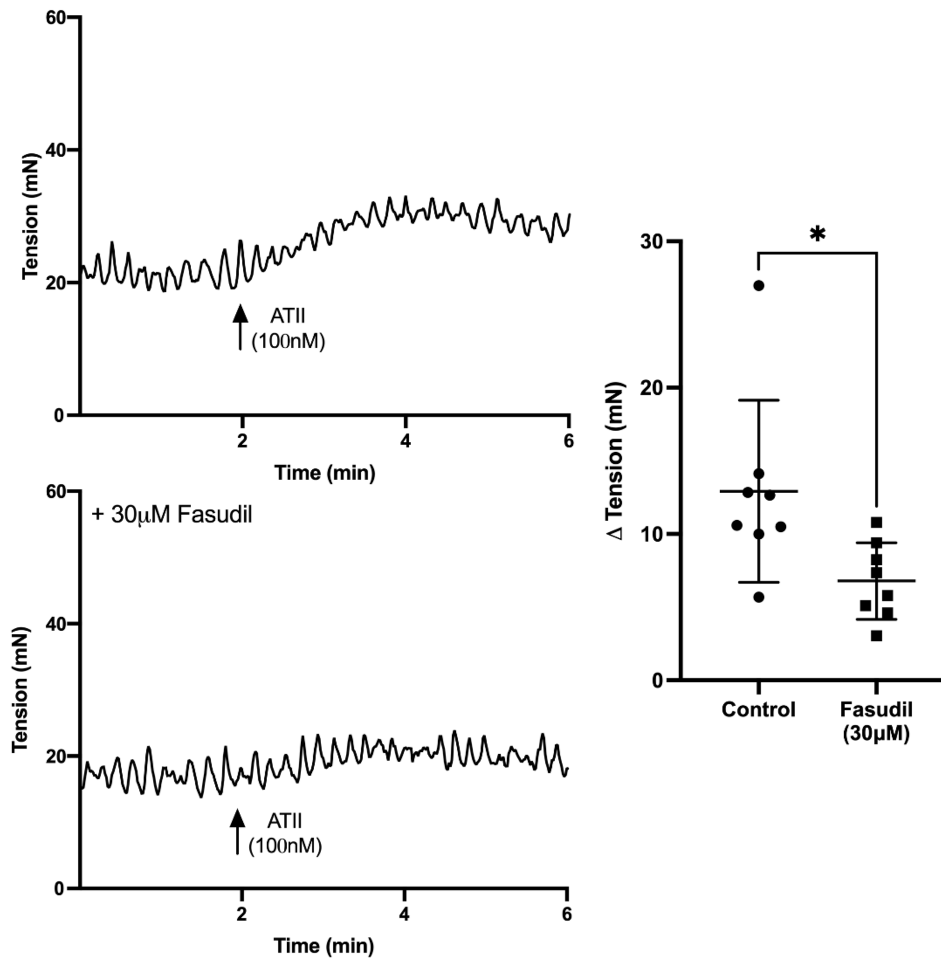

Figure 4-6: U&LP baseline tension responses to receptor agonist ATII (100 nM, n = 8) as controls in the absence of (*upper left trace*) and in the presence of (*lower left trace*) fasudil (30 µM). Changes to baseline tension (*right*) presented as mean ± SD. *$p < 0.05$ (paired Student's two-tailed t-test).

Frequency and amplitude of phasic contractions

The frequency and amplitude of spontaneous phasic contractions produced by the U&LP tissues in response to each of the receptor agonists were investigated in the absence and presence of 30 µM fasudil. No change to frequency was observed in response to carbachol, 5-HT, NKA, PGE2, or ATII in the presence of fasudil (Table 4-5). In addition, fasudil did not have any effect on the amplitude of contractile activity in response to histamine, NKA, PGE2, or ATII (Table 4-6). However, fasudil (30 µM) reduced the frequency of spontaneous phasic contractions in response to histamine (100 µM, $n = 8$, $p < 0.01$) compared to the control tissues. Furthermore, in response to carbachol (1 µM, $n = 8$, $p < 0.01$) and 5-HT (100 µM, $n = 8$, $p < 0.05$), the amplitude of spontaneous phasic contractions was altered in the presence of fasudil compared to in the absence of the Rho kinase inhibitor.

Table 4-5: The effect of fasudil (30 μM) on U&LP frequency of phasic contractions in response to receptor agonists carbachol, histamine, 5-HT, NKA, PGE2, and ATII. Data presented as mean ± SD.

Agonist (*n*)	Conc.	Δ Frequency (cpm)		*p*-value
		Control	+ fasudil	
Carbachol (8)	1 μM	1.51 ± 2.15	0.46 ± 1.29	0.17
Histamine (8)	**100 μM**	**0.64 ± 0.66**	**0.06 ± 0.46**	**0.009****
5-HT (8)	100 μM	2.30 ± 1.60	2.27 ± 2.98	0.97
NKA (8)	300 nM	0.52 ± 0.88	0.29 ± 1.49	0.75
PGE2 (10)	10 μM	0.59 ± 1.43	-0.01 ± 1.75	0.38
ATII (8)	100 nM	-0.11 ± 0.61	0.26 ± 0.66	0.32

**$p < 0.01$ (paired Student's two-tailed *t*-test). Bold font designates significant difference.

Table 4-6: The effect of fasudil (30 µM) on U&LP amplitude of phasic contractions in response to receptor agonists carbachol, histamine, 5-HT, NKA, PGE2, and ATII. Data presented as mean ± SD.

Agonist (*n*)	Conc.	Δ Amplitude (mN)		*p*-value
		Control	+ fasudil	
Carbachol (8)	**1 µM**	**-4.40 ± 3.52**	**1.24 ± 1.55**	**0.006****
Histamine (8)	100 µM	1.39 ± 3.24	0.16 ± 1.64	0.46
5-HT (8)	**100 µM**	**-3.74 ± 2.11**	**-0.44 ± 3.78**	**0.03***
NKA (8)	300 nM	-0.86 ± 1.43	-0.67 ± 3.28	0.87
PGE2 (10)	10 µM	-0.05 ± 1.81	-0.13 ± 2.23	0.88
ATII (8)	100 nM	-0.94 ± 0.66	-0.49 ± 1.40	0.42

*$p < 0.05$, **$p < 0.01$ (paired Student's two-tailed *t*-test). Bold font designates significant difference.

4.4.3.2 Influence of Rho kinase inhibitor Y-27632 on spontaneous phasic contractions in U&LP

Baseline tension responses to agonists

To further explore the involvement of Rho kinase in mediating GPCR-induced contractions, the Rho kinase inhibitor Y-27632 was applied to the tissue prior to activation by any agonist. Y-27632 (1 μM) significantly inhibited the tonic contractions of the U&LP observed in response to tissue stimulation by all GPCR agonists. This inhibition to the baseline tension of contractions was around 50% (Figure 4-7).

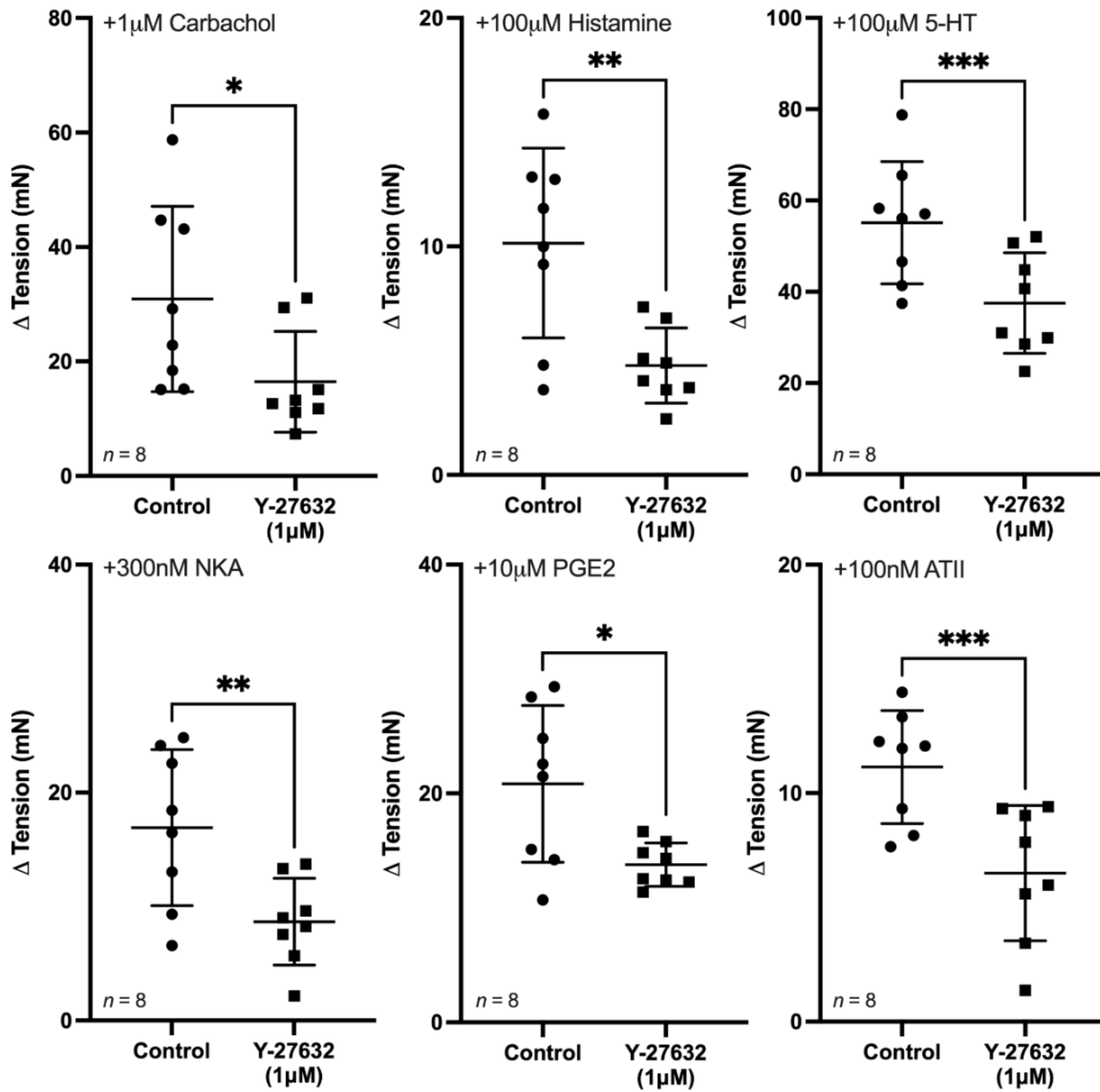

Figure 4-7: U&LP baseline tension responses (mean ± SD) to receptor agonists in the absence (control) and presence of the Rho kinase inhibitor Y-27632 (1 μM). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (paired Student's two-tailed *t*-test).

Frequency and amplitude of phasic contractions

The frequency of responses to the GPCR agonists (n = 8 for all) were not altered in the presence of Y-27632 (1 μM). In addition, there was no change in the amplitude of spontaneous phasic contractions with Rho kinase inhibition after the addition of 1 μM carbachol, 100 μM histamine, 100 μM 5-HT, 300nM NKA, and 100nM ATII (n = 8 for all). However, the amplitude of spontaneous phasic activity was significantly different after stimulation with PGE2 (control: Δ -3.47 ± 3.11 mN; with Y-27632: Δ -0.51 ± 1.69 mN; $p < 0.01$, n = 8).

4.4.3.3 Influence of GSK269962 on spontaneous phasic contractions in U&LP

The Rho kinase inhibitor GSK269962 (1 μM) inhibited baseline tension responses to all GPCR agonists except for 5-HT (Figure 4-8). The effect of GSK269962 on responses to carbachol, histamine, NKA, PGE2, and ATII appeared similar, inhibiting contractions by around 20-40%. GSK269962 (1 μM) had no effect on either the frequency or amplitude of spontaneous contractions in response to any of the agonists.

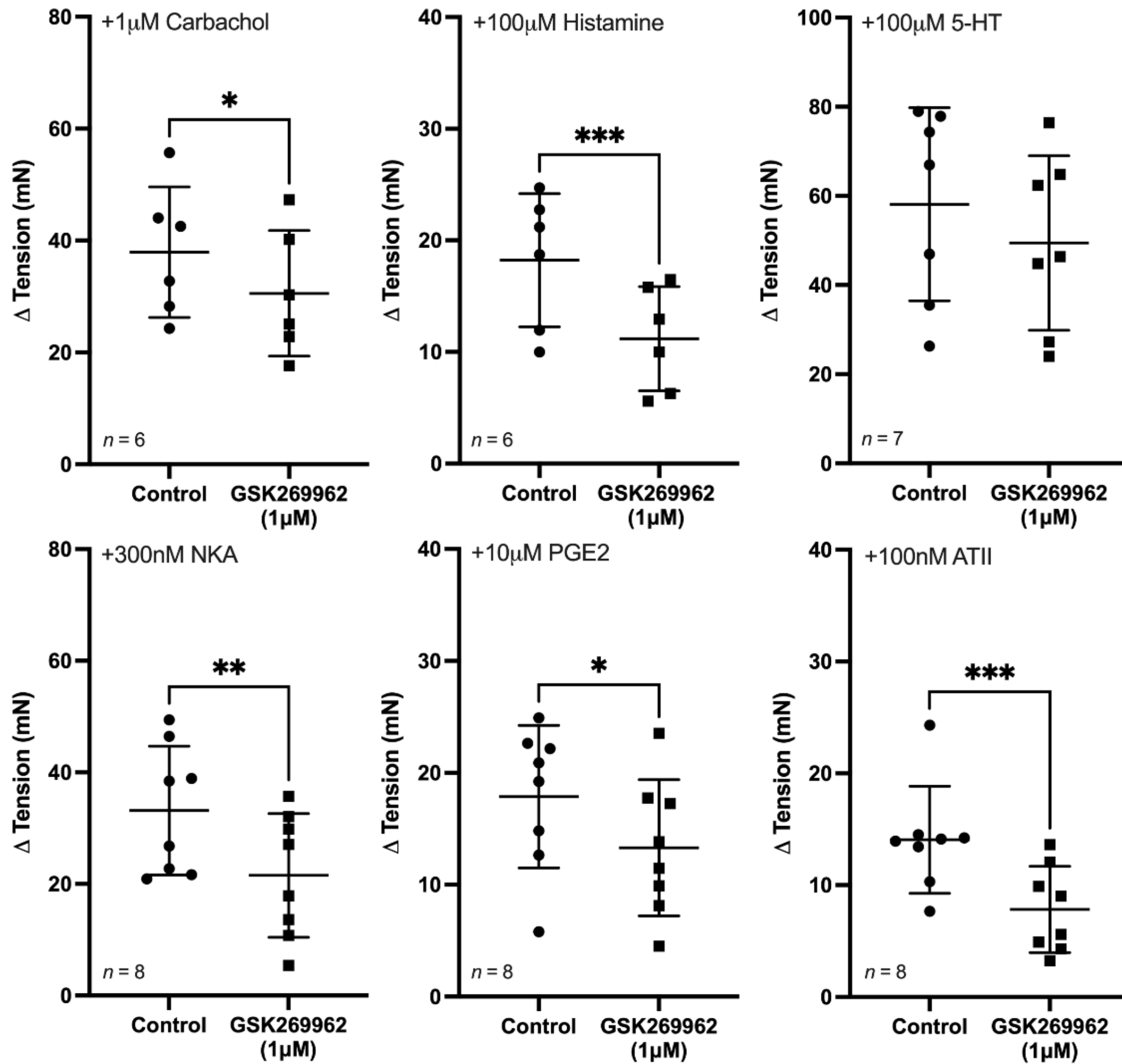

Figure 4-8: U&LP baseline tension responses (mean ± SD) to receptor agonists in the absence (control) and presence of GSK269962 (1 μM). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (paired Student's two-tailed *t*-test).

4.5 Discussion

In smooth muscle contraction, both extracellular Ca^{2+} influx across the plasma membrane and intracellular Ca^{2+} release from the sarcoplasmic reticulum contribute to the increase in cytosolic Ca^{2+} concentration (Anjum, 2018). In this current study, we support prior findings that the majority of contraction in the bladder U&LP is facilitated by the influx of extracellular Ca^{2+} (Phelps et al., 2022). However, we also identified the GPCR-mediated activation of the Rho kinase pathway to be a major contributor to U&LP contractility.

The $G_{q/11}$-coupled receptor subfamily has become a prominent area of research in urology. In particular, the urothelial and lamina propria M3 muscarinic (Moro et al., 2011), H1 histamine, 5-HT_{2A} (Moro et al., 2016), neurokinin-2 (Grundy et al., 2018), EP1 prostaglandin E2 (Stromberga et al., 2020c), and AT_1 angiotensin II (Lim et al., 2021) receptors. In each case, activation of these receptors stimulated U&LP tissue contractions. Although evidence exists that there is an additional coupling of some of these receptor systems to alternative pathways, such as G_i across a variety of species (Frazier et al., 2008), there remains the potential for a prominent contribution of $G_{q/11}$-coupled receptors in a range of bladder contractile diseases, such as idiopathic overactive bladder or underactive bladder.

The influence of Ca^{2+} and intracellular signalling pathways for receptor-mediated contractions has been widely reported in the detrusor smooth muscle of the urinary bladder. The release of Ca^{2+} from the SR is an important step in the activation of the detrusor muscle, as studies using blockers of SR function have demonstrated that nerve- (McCarthy et al., 2019), agonist- (Rivera & Brading, 2006), and stretch-induced contractions (Ji et al., 2002) of the urinary bladder are dependent on Ca^{2+} release from intracellular stores. Refilling of these intracellular

Ca^{2+} stores in the porcine lower urinary tract occurs predominantly via the influx of Ca^{2+} through L-type Ca^{2+} channels (Phelps et al., 2022; Rembetski et al., 2020). However, other pathways may be involved, such as where depletion of intracellular Ca^{2+} stores due to GPCR activation has been shown to activate the transient receptor potential canonical protein family (Sun et al., 2014). The highly innervated lamina propria, which forms the distinct connective tissue layer between the urothelium and detrusor smooth muscle, accommodates a number of cells that could contribute to contraction, including myofibroblasts (Sui et al., 2008), interstitial-like cells (Fry & McCloskey, 2019), pericytes (Hashitani et al., 2018; Lee et al., 2016), and muscularis mucosae (Heppner et al., 2011; Kushida & Fry, 2016). It is cells within this layer that are thought to be responsible for inducing the spontaneous phasic activity and generating spontaneous Ca^{2+} transients.

M3 muscarinic receptor stimulation of the urinary bladder detrusor muscle can increase the sensitivity to Ca^{2+}, leading to an influx of Ca^{2+} through L-type Ca^{2+} channels (Kishii et al., 1992). However, the contribution of extracellular Ca^{2+} influx and intracellular store release to muscarinic receptor stimulation often differs between studies (Batra et al., 1987; Rivera & Brading, 2006) and species (Wuest et al., 2007). Furthermore, other receptors systems, such as histamine (Rueda et al., 2002), neurokinin (Quinn et al., 2004), and purinergic receptors (Wu et al., 1999), have also been identified to depend on Ca^{2+} influences for urinary bladder contraction, however, there are inconsistencies in the reported contribution of extracellular and intracellular Ca^{2+} for these contractions. All previously reported literature has had a focus on the Ca^{2+} pathways controlling receptor-mediated contractions of the isolated detrusor smooth muscle layer or whole bladder preparations. Thus, this research is novel in reporting the influence of Ca^{2+} across a range of $G_{q/11}$-coupled receptors in the isolated porcine U&LP.

In a previous study (Phelps et al., 2022), we reported a strong dependence on extracellular Ca^{2+} for mediating muscarinic, histamine, 5-HT, neurokinin, prostaglandin, and angiotensin II contractions of the U&LP. This was shown to be consistent across two methods including inhibiting L-type Ca^{2+} channels with nifedipine as well as blocking Ca^{2+} entry into the U&LP with a nominally zero Ca^{2+} solution. The H1 histamine receptor, responsible for contraction in the porcine U&LP (Stromberga et al., 2019), may be of particular interest in future studies. Although most receptors identified in the current study had extracellular Ca^{2+} as the prominent source, histamine was unique, where baseline tension responses were inhibited by 60% from both 2-APB and ruthenium red. This suggests that the contraction to histamine receptor stimulation may be predominantly due to the influx of intracellular Ca^{2+}, potentially through IP3-induced Ca^{2+} release. This directly correlates with the finding in our past study that identified inhibition of 40-45% of the histamine response when extracellular sources of Ca^{2+} were removed.

CPA demonstrated minimal influence on the receptor-mediated activity of the U&LP. However, an interesting observation was the significant increase in baseline tension after the application of CPA in response to neurokinin-A receptor activation. A similar result was reported by Heppner et al. (2011), where SR store depletion by CPA increased baseline tension of the bladder U&LP in guinea pigs, reflecting an elevated intracellular Ca^{2+} level caused by blockade of the SR Ca^{2+}-ATPase pump. Although in our study we attempted to deplete intracellular Ca^{2+} levels with a wash-out method, neurokinin-induced contractile activity of the U&LP has demonstrated a strong dependence on both extracellular Ca^{2+}, and the Rho kinase pathway, for contraction.

There has been a longstanding interest in investigating the role of Rho kinase, a Ca^{2+}-independent mechanism, in receptor-mediated contractions (Peters et al., 2006; Tatsumiya et al., 2009), with this pathway hypothesized to present a potentially novel target in the future treatments of bladder contractile disorders (Joseph et al., 2022). In previous experiments, inhibition of extracellular Ca^{2+} influx reduced contractions, but not to a level where it was sufficient to entirely abolish the $G_{q/11}$-mediated U&LP responses. However, with our current study contributing to this data, finding that the release of Ca^{2+} from intracellular stores held a minimal influence on agonist responses, the role of a Ca^{2+}-independent pathway became of particular interest. It has been suggested that the U&LP may be involved in controlling carbachol-induced contractions of the bladder via the Rho kinase pathway, as fasudil demonstrates stronger inhibitory effects to contraction in urothelium-intact porcine tissue compared to denuded (Tatsumiya et al., 2009). In our study, we identified a prominent role for Rho kinase in mediating contractions in response to muscarinic, histamine, 5-HT, NKA, PGE2, and ATII receptor activation in the U&LP, which was demonstrated by inhibition of contractile responses in the presence of fasudil, Y-27632, and GSK. In particular, the influence of Rho kinase for receptor-mediated contractions was between 20-47% for carbachol, 39-67% for histamine, 17-32% for 5-HT, 35-49% for NKA, 25-34% for PGE2, and 42-47% for ATII. We suggest that this strong dependence on Rho kinase for $G_{q/11}$-coupled receptor contractions may indicate $G_{q/11}$ mediates RhoA activation in response to M3, H1, 5-HT_{2A}, NK2, EP1, and AT_1 stimulation in the U&LP. This is supported by the finding by Nakanishi et al. (2009) that the U&LP expresses higher levels of RhoA mRNA and RhoA enzyme than the detrusor in the urinary bladder. One hypothesis for this link may be the $G_{q/11}$-activated RhoGEF, p63RhoGEF, mediating RhoA activation (Momotani & Somlyo, 2012). The unique activity observed in the U&LP, compared to many other smooth muscles, may be due to differences in the mechanisms facilitating its contraction. However, it is uncertain whether the contractions are mediated by

myofibroblasts (Sui et al., 2008), interstitial-like cells (Fry & McCloskey, 2019), pericytes (Hashitani et al., 2018; Lee et al., 2016), muscularis mucosae (Heppner et al., 2011; Kushida & Fry, 2016), or a combination of these identified cells within the U&LP.

Across the three Rho kinase inhibitors, Y-27632 was the most effective and consistent inhibitor of the Ca^{2+} sensitization pathway, inhibiting responses to GPCR activation by 32%-53%. Fasudil also influenced all contractions (17% - 67%) except those to NKA or PGE2. This is consistent with past findings identifying Y-27632 as the inhibitor with the higher affinity to Rho kinase (Uehata et al., 1997) and selectivity across both ROCK-1 and ROCK-2 compared to fasudil. Alternatively, fasudil is less selective and comes with a risk of alternative actions on other kinases (Davies et al., 2000) as well as Ca^{2+} channels (Asano et al., 1987). More recently, a newer inhibitor has been developed with an even higher selectivity for ROCK, GSK269962 (Toyoda et al., 2017), and this was found to influence all contractions (20% - 44%) except those in response to 5-HT. Of particular interest to future studies may be the finding that fasudil, yet not Y-27632 or GSK269962, was effective at inhibiting increases to the frequency of spontaneous contractions in the presence of histamine, and altering the amplitude of spontaneous activity in response to carbachol and 5-HT. It is unclear which mechanisms may be involved in this, but one hypothesis may be a variation in the effects of phosphatase on myosin light chain phosphorylation. With detrusor overactivity due to abnormal spontaneous contractions of the whole urinary bladder during filling, it may be of interest to further identify the range of potential mechanisms underlying this response.

The role of Ca^{2+} and Rho kinase in the mediation of G protein-coupled receptor contractions presents the potential for new and novel targets to be investigated for the pharmacological management of bladder contractile dysfunctions, such as underactive bladder. In elderly people

with lower urinary tract symptoms, over 45% exhibit an underactive bladder, presenting this as an increasingly important and clinically relevant syndrome (Jeong et al., 2012). However, there are currently no outcome-validated effective therapeutics for the management, treatment, or prevention of underactive bladder (Moro et al., 2021b). In addition, underactive bladder can often coexist with overactive bladder, a disorder that has the most impact on patient quality of life among the lower urinary tract symptoms (Murukesu et al., 2019). However, until recently, there has been little research into the coexistent nature of overactive and underactive bladder (Mancini et al., 2020), and without an established diagnostic criterion, or well-understood urodynamic correlates for this syndrome, it presents a prominent issue for patients suffering from bladder contractile disorders.

4.5.1 *Limitations and future direction*

It should be noted that although three independent drugs were used to block the SR release of Ca^{2+} into the cytoplasm, there was a lack of inhibition on intracellular Ca^{2+} sources. The inhibitor 2-APB has been considered a reliable blocker of store-operated Ca^{2+} entry, however, may be an inconsistent inhibitor of IP3-induced Ca^{2+} release (Bootman et al., 2002). To accommodate this concern, instead of drawing conclusions solely from 2-APB, we also investigated the influence of CPA and ruthenium red. One additional treatment that could have also been considered is xestospongin C (Johnston et al., 2008), although due to a high procurement cost for the concentrations required, was not used in this study. It would also be of benefit to investigate the uniqueness of the U&LP responses compared to other smooth muscles, as well as identify which mechanisms might be responsible for the different influence of fasudil compared to Y-27632 or GSK269962 to histamine, carbachol, and 5-HT. There was some observed influence on contractile velocity in the study, for example, with 5-HT appearing

to peak slower than other agonists, which would also be of interest for future studies to investigate.

4.6 Conclusions

Ca^{2+}-mediated contraction of the U&LP is typically stimulated by both extracellular Ca^{2+} influx and release from intracellular stores. However, in the urinary bladder U&LP, responses to G protein-coupled receptor stimulation are more sensitive to extracellular Ca^{2+} for the mediation of contractions, supported by the lack of any discernible impact on receptor agonist-induced contractions of the U&LP to 2-APB, CPA, and ruthenium red. An alternative mechanism of action for GPCR-mediated contraction for the muscarinic, histamine, 5-HT, neurokinin, prostaglandin, and angiotensin receptors was identified to be the activation of Rho kinase. The unique activity of the receptors investigated in this study presents novel directions for future research into the mechanisms underlying bladder contraction, and how these systems may be altered in disease states. The results suggest that Ca^{2+} regulation is important for activating the contractile machinery of the urinary bladder and highlight an equally important and complementary role for Rho kinase pathways. This presents potential novel targets for the pharmaceutical management of bladder disorders, in particular, overactive and underactive bladder.

Chapter 5

The urothelium and lamina propria in ageing: A scoping review of animal model studies

PUBLISHED CHAPTER

Phelps, C., & Moro, C. (2023). Ageing and the bladder mucosa: A scoping review of animal model studies from the last five years. *The Australian and New Zealand Continence Journal, 28*(4): 77-83. https://doi.org/10.33235/anzcj.28.4.77-83

Preface

The previous thesis chapters solely focused on juvenile porcine samples, however, there is a suggestion that ageing influences the work of G protein-coupled receptor systems in the urinary bladder urothelium and lamina propria. To assess the literature in this field, the following chapter outlines a scoping review.

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Formatting and terminology changes have been made to maintain consistency throughout this thesis. In particular, note the title of this chapter has been changed from the original publication for consistency (i.e., changing mucosa to urothelium and lamina propria as required by the journal reviewers). The final version of this manuscript can be accessed at: https://journals.cambridgemedia.com.au/anzcj/volume-28-number-3-1/ageing-and-bladder-mucosa-scoping-review-recent-animal-model-studies.

All data presented in this manuscript are the sole work of Charlotte Phelps.

5.1 Abstract

The incidence of bladder dysfunction increases with age. People with bladder disorders who are older than 60 years are more likely to comply and persist with treatment regimens, such as antimuscarinic medications for overactive bladder. Suggestions for these differences include lifestyle factors, greater compliance with medication prescription, or pharmacological and physiological changes to the receptors within the bladder tissue itself. This scoping review focused on the latter and sought to identify if there is recent evidence to support a prominent role for the internal lining of the urinary bladder, the bladder urothelium (mucosa), in age-related alterations. Although there continues to be substantial urological research in humans, animal models remain highly important for the assessment of physiological and pharmacological changes in the bladder. Recent work in this field brings insights into the overall understanding of bladder function and dysfunction. The PUBMED database was searched for studies published between 2018 and 2022. From the 25 articles identified, 10 were eligible for inclusion in the evaluation, and a risk of bias assessment performed on these studies. Studies reported a variety of age-related alterations in the bladder U&LP. The most pronounced changes appear to be an inhibition of the U&LP barrier function and signalling pathways with ageing. Ageing also appeared to inhibit receptor systems associated with contraction inhibition, potentially leading to enhanced contractions. The overall evidence suggests that the increased prevalence of bladder contractile disorders seen in ageing may be due, in some part, to physiological alterations occurring within the bladder tissue itself.

5.2 Introduction

During ageing, the bladder undergoes various anatomical and physiological changes that may alter its ability to maintain normal function (Ellsworth et al., 2013). Marked changes in the urinary bladder include impaired contractility, sensitivity, and storage (Suskind, 2017; Wehrberger et al., 2012). As a result, there is an increase in the prevalence of lower urinary tract symptoms (LUTS), including urinary incontinence, overactive bladder, nocturia, urgency, and underactive bladder (Suskind, 2017; Wehrberger et al., 2012). The risk of bladder dysfunction tends to increase linearly with age, and becomes particularly problematic in frail elderly patients (Richters et al., 2020), with an estimated 70% of the elderly population presenting with at least one LUTS (Przydacz et al., 2020). The pathophysiology of age-related LUTS includes comorbid medical illnesses (Coyne et al., 2013), lifestyle factors (Smith et al., 2014), and medicine use (Hashimoto et al., 2015). With the ageing population in Australia, the prevalence of lower urinary tract dysfunction as it affects the elderly is expected to place a significant burden on health services and the economy (Powell et al., 2018). Interestingly, 48% of Australians consider incontinence to be an inevitable consequence of ageing (Continence Foundation of Australia, 2020).

Current front-line pharmaceutical treatment options for bladder contractile disorders target the muscarinic receptors in the urinary bladder, for example, antimuscarinics such as fesoterodine, oxybutynin, propiverine, solifenacin, tolterodine, and trospium for the treatment of overactive bladder (OAB) (Robinson & Cardozo, 2019). Despite this, a lack of persistence with antimuscarinics has been identified as a major problem when treating OAB, with between 65% and 86% of patients discontinuing therapy within one year, depending on the antimuscarinic prescribed (Wagg et al., 2012). This has been attributed to the occurrence of adverse side effects

and lower-than-expected treatment outcomes (Yeowell et al., 2018), which are also found with the use of parasympathomimetics (muscarinic agonists) in the treatment of underactive bladder (Moro et al., 2021b). A determinant in persistence and adherence to antimuscarinics is age, where older people are more likely to persist with antimuscarinic treatments for longer (Carlson et al., 2021; Yeowell et al., 2018). This may be due to pharmacological changes to the receptors or systems within the bladder tissue that respond to agonists and antagonists, or even due to alterations of cells within the tissue itself, however, this remains largely unclear.

The lack of compliance with treatment regimens due to the low effectiveness of current pharmaceutical options and the occurrence of adverse side effects highlights that a better understanding of the mechanisms underlying bladder contractions is warranted. Furthermore, the demonstrated successes for combination therapies, such as the use of an antimuscarinic with an α-adrenoceptor antagonist (Yamanishi et al., 2004), or an antimuscarinic with a β3-adrenoceptor agonist (Lightner et al., 2019) highlights the importance of continuing research in this field.

Animal models, in particular from pig and rodent tissues, have offered insights into potential future medications, the receptors to target, and other directions for clinical application. The research question underpinning this study was: is there a clinical application from the knowledge of overactive and underactive bladder gained from animal model studies published within the last five years? The objective of this review was to investigate the impacts of ageing on the physiology of the urinary bladder U&LP, using information obtained from animal investigations to draw conclusions.

5.3 Methods

The Preferred Reporting Items for Systematic Reviews and Meta-Analyses extension for Scoping Reviews (PRISMA-ScR) was followed for the review's outline (Tricco et al., 2018). Prior to commencement, an *a priori* protocol was prepared but not published. A comprehensive review of peer-reviewed English-language full-text articles published in the last five years (January 2018 to September 2022) was searched. Studies were identified through the available literature via PubMed (MEDLINE database) to identify original research papers on age-related physiological changes to the urinary bladder in animal models. All studies assessing any measurement of U&LP function with ageing were included. Both in vitro and in vivo studies were included, and all studies on bladder U&LP were included, with no requirement to have a formal control animal for assessments.

5.3.1 *Search strategy*

The following search strategy (formatted for the PUBMED database) was employed: (ageing[tiab] OR aging[tiab] OR "aging"[mesh] OR "age-related"[tiab]) AND (bladder[tiab] OR "urinary bladder"[tiab] OR "urinary bladder"[mesh]) AND (urothelium[tiab] OR mucosa[tiab] OR "lamina propria"[tiab]) AND (physiolog*[tiab] OR receptor*[tiab] OR function*[tiab] OR "muscle contraction"[tiab] OR "muscle contraction"[mesh] OR "overactive bladder"[tiab] OR "underactive bladder"[tiab]). A forward-backwards search of reference lists of retrieved articles was also undertaken (C.P.) to identify additional studies not captured from the database search.

5.3.2 *Study selection*

Screening of the literature was conducted by two authors independently (C.P. and C.M.), first by title/abstract, and subsequently in full text. Discrepancies were to be referred to an additional academic if required, although this was not necessary for this study.

5.3.3 *Risk of bias*

SYRCLE's risk of bias tool for animal studies (Hooijmans et al., 2014), an adapted version of the Cochrane Risk of Bias tool, was used for the critical appraisal of the sources of evidence. Each study was assessed to each criterion in the risk of bias tool by the first author (C.P.) with reference to the second author (C.M.) to collaborate on grading any ambiguities. No contact was required to any included publication authors to obtain more data or information regarding the presented results. Each potential source of bias from the 10-item SYRCLE assessment was graded as low risk, high risk, or unclear risk.

5.4 Results

5.4.1 *Search results*

The electronic search identified 22 articles, and the forward and backwards citation searches identified an additional three articles. After a title and abstract search, the full texts for 13 articles were obtained and analysed. From this collection, two were excluded due to wrong animal species (both on humans), and one was excluded due to wrong tissue (intact with detrusor rather than isolated mucosa). The results from the remaining 10 articles informed the recommendations in this review (Cheng et al., 2018; de Oliveira et al., 2019; de Rijk et al.,

2022; Erzar et al., 2020; Kim et al., 2021; Nishikawa et al., 2020; Roberts et al., 2020; Stromberga et al., 2020a; Truschel et al., 2018; Wen et al., 2018) (Figure 5-1, Table 5-1).

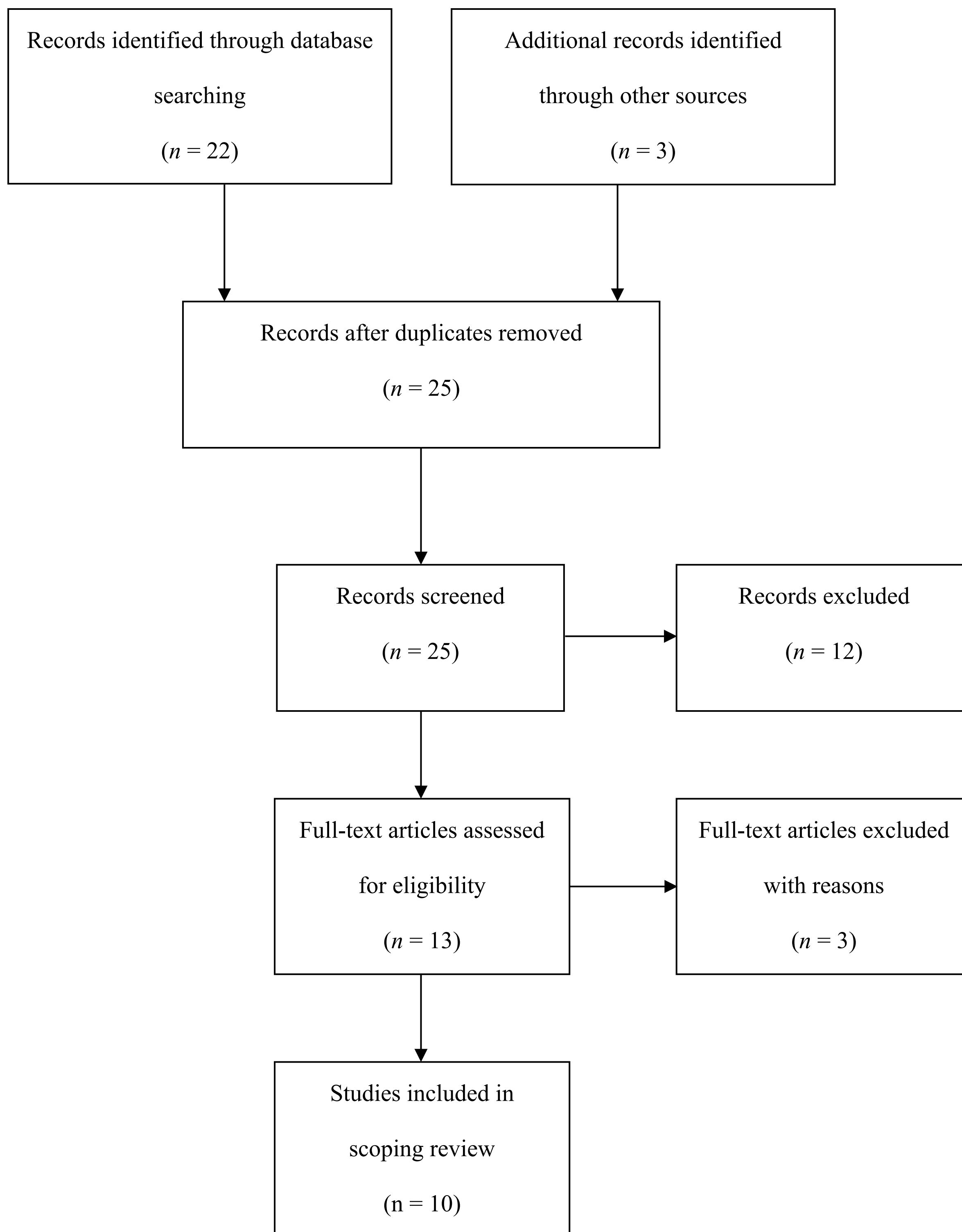

Figure 5-1: PRISMA Flow Diagram for the identification of studies via databases and registers.

Table 5-1: Characteristics of included studies.

Reference	Animal model	Strain	Sex	Body weight	Age	*n*	Methods
Cheng et al. (2018)	Rat	F344	Male	-	Adult (12 months); Aged (21-24 months)	4 adult; 5 aged	Mechanical testing; bladder compliance and capacity; loading curve; multiphoton imaging; immunohistochemistry.
de Oliveira et al. (2019)	Mouse	C57/BL6	Female	Young (23.2 ± 1.5 g); Old (30.9 ± 3.2 g)	Young (3 months); Aged (18 months)	30 young; 30 aged	Urodynamic assessment; functional assays; electrical field stimulation; real-time polymerase chain reaction; cyclic adenosine monophosphate determination.
de Rijk et al. (2022)	Rat	F344	Female	-	Young (3–4 months); Mature (25–30 months)	4 young; 4 aged	Western immunoblotting; cell culture; cellular respiration; mitochondrial bioenergetics; ATP release.
Erzar et al. (2020)	Mouse	C57/BL6-JOLaHsd	Female	-	Young (8-16 weeks); Aged (24 months)	Ex vivo (5 young; 5 aged); In vivo (35 young; 20 aged)	Transepithelial electrical resistance; immunohistochemistry; quantitative RNA analysis.
Kim et al. (2021)	Rat	Sprague-Dawley	Female	-	Young (12 weeks); Aged (80 weeks)	15 young; 15 aged	Cystometry; immunohistochemistry; Western blotting.

Nishikawa et al. (2020)	Mouse	C57/BL6	Male	-	Young (9-12 weeks); Aged (24 months)	18 young; 21 old	Contraction studies; electrical field stimulation; histology.
Roberts et al. (2020)	Guinea pig	Dunkin-Hartley	Male	-	Young (2-5 months); Aged (24-36 months)	8 young; 14 aged	Contraction studies; ATP measurement; epifluorescence microscopy; immunohistochemistry; Western blot; chemiluminescence.
Stromberga et al. (2020a)	Pig	Large-White-Landrace-Duroc	-	Young (80kg); Aged (120kg)	Young (6 months); Aged (2-3 years)	159 young; 59 aged	Functional organ bath contraction studies.
Truschel et al. (2018)	Rat	F344	-	-	Young (3 months); Mature (12 months); Aged (26 months)	3 young; 3 mature; 3 aged	Transmission electron microscopy; stereological analysis.
Wen et al. (2018)	Rat	Sprague-Dawley	Female	Young (225 g); Old (316 g)	Young (2-3 months); Aged (12-15 months)	20 young; 18 aged	Metabolic cage study; cystometry; contraction studies; electrical field stimulation; cell culture; Ca^{2+} imaging; immunohistochemistry.

5.4.2 *Risk of Bias*

Eight of the 10 studies clearly reported that the baseline characteristics of animals were balanced, including animal model and gender. All studies had a formal control group, where 'young' was compared with 'old'. Due to the nature of the studies, and limited information provided, only one study (Stromberga et al., 2020a) reported random housing of animals during the experiment, one study (Stromberga et al., 2020a) reported random selection of animals for outcome assessment, and one study (Wen et al., 2018) reported blinding of investigators. All studies were free of other problems that could result in high risk of bias (Figure 5-2).

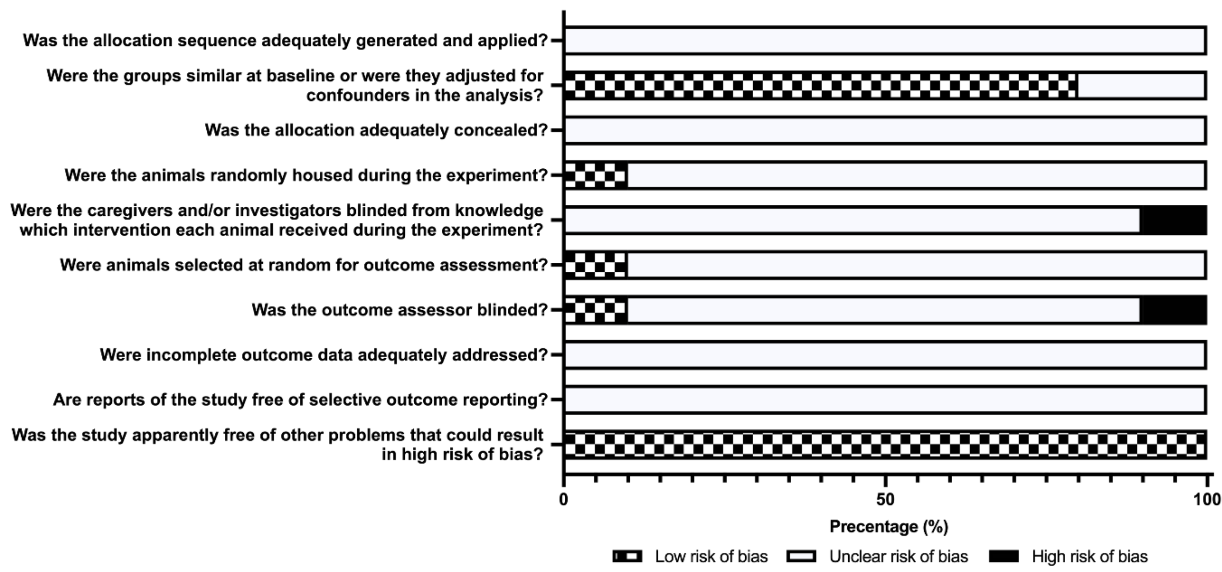

Figure 5-2: Risk of bias assessment graph: authors' judgments about each risk of bias item presented as percentages across all included studies.

5.5 Evaluation of the literature

The developing body of research into the role of the urothelium with lamina propria in overall bladder contractile activity highlights the potential role of this layer as a target for the development of novel therapeutics. Previously characterised for its protective functions, the bladder U&LP is now understood to play a key role in regulating, mediating, and modulating overall bladder function through its signalling properties. Isolated U&LP tissue is capable of developing spontaneous phasic activity (Moro & Phelps, 2022b), suggesting mechanisms where it might underlie bladder contractile disorders. Although past research on the U&LP has focussed on traditional mediators of contraction, such as acetylcholine (Moro et al., 2011), noradrenaline (Moro & Chess-Williams, 2012), and nitric oxide (Moro et al., 2012), recent research has considered histamine (Stromberga et al., 2019), prostaglandins (Stromberga et al., 2020b; Stromberga et al., 2020c; Stromberga & Moro, 2020), neurokinin-A (Grundy et al., 2018), and angiotensin II (Lim et al., 2021) as key mediators of contraction in the U&LP. However, the impact of ageing on these receptor systems is not often included in research outcomes.

Insights from animal studies have identified that the activity of contractile mediators of the urinary bladder may be altered throughout the lifespan. Stromberga and colleagues (2020a) investigated the effects of age on the function of the histaminergic receptor system in porcine U&LP. The findings identified the histamine receptor system as having the potential to induce diseased states of the bladder due to its alterations by ageing. In particular, although the H1 receptor was responsible for contraction (Stromberga et al., 2019), results suggest that the H2 receptor may have an inhibitory role. However, this was only in juvenile bladders, and aged bladders did not exhibit any response from H2 receptor activation. This could mean that the

H2 receptor subtype plays an active part in maintaining bladder contractile stability in the young, and reduced activity with ageing could stimulate enhanced contractions, which would be particularly noticeable during the filling phase (Stromberga et al., 2019). This potentially age-dependent alteration to the histamine receptor system highlights the potential for further research into this pathway as a possible future therapeutic target.

Other studies show the release of stretch-induced and stimulation-induced adenosine triphosphate (ATP) in the intact U&LP decreases with ageing, which could be a contributing factor to symptoms of urgency and overactive bladder in the elderly (Nishikawa et al., 2020). The ageing process can deteriorate the barrier and signalling properties of the bladder U&LP, as shown by significant increases in cellular senescence and oxidate stress (de Rijk et al., 2022). Age-related bladder dysfunction could also be caused by a decrease in the cell signalling processes caveolin 1 and 2, which was found by an immunohistochemical study in the urothelium of aged rat bladders (Kim et al., 2021). The study also reported cystometric results, showing that aged bladders exhibited a decreased voiding interval and pressure (Kim et al., 2021), symptoms commensurate with overactive bladder, which is highly prevalent in the ageing population. In addition, Truschel and colleagues (Truschel et al., 2018) found an age-related decrease in lysosomal function in the urothelium of rats, which may have significant effects on bladder function. The authors reported this could alter the homeostatic chemical balance of the urothelium and cellular communication with underlying layers, manifesting various clinical conditions observed in the elderly (Truschel et al., 2018). The regenerative capability of the urothelium in aged mice bladders is slightly delayed compared to younger models, but there is no difference in the restoration of urothelial function (Erzar et al., 2020). However, some regenerative functions appear to deplete during ageing. For example, reduced recruitment of collagen fibres across the lamina propria occurs with age, impacting the overall

elasticity of the bladder wall (Cheng et al., 2018). This could also have a role in diminishing sensitivity in the U&LP and its ability to release mediators when required.

Underactive bladder is an increasingly prevalent problem in the elderly population. Age-related alterations to bladder function leading to the development of an underactive bladder include sensory impairment, decreased voiding efficiency, and impaired bladder contractility (Santos-Pereira & Charrua, 2020). The developing interest in the sensory role of the U&LP has led to the recognition of sensory molecules in these tissue layers. One is the transient receptor potential vanilloid-4 (TRPV4) channel, a key sensory protein in the bladder urothelium involved in stretch-induced ATP release from the urothelium, which modulates afferent nerve activity in response to bladder filling (Wu et al., 2021). A recent study found that TRPV4 expression was reduced in the bladder urothelium of older female mice compared to young mice, which may play a role in the pathophysiology of underactive bladder (de Oliveira et al., 2019). Similarly, Wen and colleagues (2018) reported a down-regulation of urothelial TRPV4 expression in older female rats accompanied by diminished sensory functioning of the bladder. However, in aged male guinea-pig bladders, the expression of TRPV4 was shown to be upregulated in the U&LP, with an increase in the quantity of urothelial ATP release, which could potentially become a contributing factor in overactive bladder (Roberts et al., 2020). These insights highlight that TRPV4 signalling in the bladder U&LP may be altered with age; however, there may be gender and species differences.

5.5.1 *Limitations of the review*

Overall, the findings of the 10 studies reviewed related to alterations in the bladder U&LP with age highlight this as an area of developing interest. Although limited by the short five-year

timeframe, and inclusion of only English language articles, this scoping review has identified a variety of age-related alterations in the U&LP, which could contribute to bladder dysfunction.

5.6 Conclusion

Understanding the physiological mechanisms that are impaired in the urinary bladder U&LP with age can provide insights into novel therapeutic targets to manage bladder contractile disorders. Marked changes in receptor systems, barrier properties, and signalling pathways are shown in animal studies to play a significant role in age-related bladder dysfunction. These findings are of importance as there is an increasing interest in finding potential targets to use for combination therapies in the treatment of contractile disorders. Further research into using animal models to investigate additional impacts of ageing on bladder dysfunction is needed as we continue to build an understanding of the variations across bladder mechanisms which may present insights into age-specific treatment options for older people presenting with lower urinary tract dysfunctions.

Chapter 6

Ageing influences detrusor contractions to prostaglandin, angiotensin, histamine and 5-HT (serotonin), independent to the Rho kinase and extracellular calcium pathways

PUBLISHED CHAPTER

Phelps, C., Chess-Williams, R., & Moro, C. (2023). Ageing influences detrusor contractions to prostaglandin, angiotensin, histamine and 5-HT (serotonin), independent to the Rho kinase and extracellular calcium pathways. *Scientific Reports*, *13*. https://doi.org/10.1038/s41598-023-44916-8

Additional published abstracts and conference presentations arising from this chapter:

Phelps, C., Chess-Williams, R., & Moro, C. (2023, November). Smooth muscle contractions in the urinary bladder: Alterations between juvenile and adult detrusor and the influences of G protein-coupled receptor stimulation. *Australian Physiological Society (AuPS) 2023 Meeting.* Melbourne, Victoria.

Preface

The outcome of the scoping review in the previous chapter identified various changes in the urinary bladder urothelium and lamina propria with age. The scoping review presented in Chapter 5 guided this final thesis chapter for the following reasons: 1) age-related alterations to urinary bladder function is a variable that should be investigated; 2) most of the observed clinical benefits and research undertaken are on the detrusor smooth muscle. As such, this final chapter identified ageing as an important concept of the scoping review and focused on the detrusor smooth muscle as an important mediator of urinary bladder contraction. While the previous original results chapters are focussed on the urothelium and lamina propria, the main target in the pharmaceutical management of bladder disorders is the detrusor smooth muscle, so this chapter aims to identify whether similar effects obtained in the first two chapters also occurred in the detrusor. In addition, an aged model was used in this study to see if there are any differences between a juvenile (pre-pubescent, 6-month-old) and developed (post-pubescent, 2+ years old) sample.

This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International License (CC BY 4.0 DEED): https://creativecommons.org/licenses/by/4.0/. Formatting and terminology changes have been made to maintain consistency throughout this thesis. The final version of this manuscript can be accessed at: https://www.nature.com/articles/s41598-023-44916-8. All data presented in this manuscript are the sole work of Charlotte Phelps.

6.1 Abstract

Ageing is associated with deteriorating urinary bladder function and an increasing prevalence of disorders such as underactive bladder. There are suggestions that G protein-coupled receptor (GPCR) second messenger pathways are altered during ageing, rather than the receptor proteins themselves. The aim of this study was to identify age-related variations in GPCR activation systems in urinary bladder smooth muscle (detrusor). Isolated porcine detrusor strips were mounted in organ baths and contractile responses induced by receptor agonists were assessed and compared between juvenile (6 months) and adult (2 years) animals. The effects of drugs disrupting intracellular calcium signalling were also studied. Adult tissue was far more sensitive to stimulation by 5-hydroxytryptamine (42% greater increase than juvenile), prostaglandin-E2 (26% greater increase), and angiotensin-II (39% greater increase), however less sensitive to histamine. Although nifedipine and Y-27632 impacted the contraction to all agonists, there were no significant differences between juvenile and adult detrusor. Impairment of IP3-mediated calcium release by 2-aminoethyl diphenylborinate had no effect on any contractile activity, except for neurokinin-A which inhibited both juvenile and adult detrusor, and prostaglandin-E2 which inhibited juvenile. Carbachol, histamine, 5-hydroxytryptamine, and angiotensin-II were not affected by the application of 2-aminoethyl diphenylborinate. In conclusion, the contractile responses to all the GPCR agonists involved extracellular calcium influx and calcium sensitisation, but for prostaglandin-E2 the dependence on calcium from intracellular sources was greater in the younger animals.

6.2 Introduction

Underactive bladder (UAB) is a prevalent disorder affecting males and females of all ages. Despite significant impacts on quality of life, UAB is frequently misdiagnosed due to the lack of a detailed definition and diagnostic criteria in clinical practice, with the pathophysiology remaining poorly understood (Yu & Jeong, 2017). As a result, there are currently no outcome-validated effective therapeutics for the management or prevention of UAB (Moro et al., 2021b). The front-line pharmaceutical treatment for UAB is parasympathomimetics (muscarinic agonists), which act to directly stimulate muscarinic receptors or inhibit anticholinesterase to increase cholinergic neurotransmission (Moro et al., 2021b). However, there is a paucity of quality evidence suggesting long-term benefits for this class of medicine (Chancellor et al., 2020; Osman et al., 2018), and there are a range of adverse effects associated with parasympathomimetic use, such as nausea, vomiting, diarrhea, sweating, and salivation, which lead to low adherence rates (Osman & Chapple, 2018).

Diminished urinary bladder function and control is a frequent occurrence among elderly populations (Suskind, 2017). Ageing is associated with various alterations in urinary bladder structure and function, resulting in changes to bladder storage, contraction, emptying, and sensitivity (Jiang & Kuo, 2017; Kiba et al., 2022). These age-induced alterations contribute to the increasing prevalence of lower urinary tract symptoms (LUTS) with progressing age (Suskind, 2017). In elderly men and women with LUTS, over 45% exhibit an underactive bladder, presenting this as an increasingly prevalent problem that commonly results in urinary retention, incomplete bladder emptying, and other bothersome urinary symptoms, such as nocturia, urgency, hesitancy, and incontinence (Jeong et al., 2012; Taylor & Kuchel, 2006). UAB is often a result of detrusor underactivity, which is a urodynamic diagnosis characterised

by a reduced contraction strength or length of prolonged voiding, resulting in incomplete bladder emptying (Osman et al., 2018). However, the direct mechanisms underlying UAB are complex to diagnose and manage, and as ageing is a predictor of developing UAB, this highlights the need to further enhance understanding of age-related changes in bladder contraction. Furthermore, persistence and adherence to some pharmaceutical therapies for bladder contractile disorders increase with age, suggesting variations in bladder responses to receptor agonists (Carlson et al., 2021; Yeowell et al., 2018).

The detrusor smooth muscle remains the primary target in the pharmaceutical therapy of bladder contractile disorders (Joseph et al., 2022). For UAB patients, the primary treatment goal is to enhance the contractile activity of the urinary bladder to increase the strength and duration of contractions to complete bladder emptying (Bayrak & Dmochowski, 2019). Smooth muscle contraction is stimulated by an increase in intracellular calcium (Ca^{2+}) via an influx of extracellular Ca^{2+} through voltage-gated Ca^{2+} channels or triggering its release from intracellular sarcoplasmic reticulum stores. The influx of intracellular Ca^{2+} activates myosin light chain kinase, which phosphorylates myosin light chain and promotes the interaction of myosin with actin and subsequent contraction. G protein-coupled receptor (GPCR) activation is a primary stimulus for increases in intracellular Ca^{2+} concentration (Phelps et al., 2022). Agonists binding to GPCRs activate voltage-dependent Ca^{2+} channels to increase intracellular Ca^{2+}, which increases the strength and force of contraction with the subsequent influx of extracellular Ca^{2+} (Kishii et al., 1992). Further, $G_{q/11}$-coupled receptors activate phospholipase C with the subsequent formation of inositol trisphosphate (IP3) and diacylglycerol to release Ca^{2+} from intracellular stores (Phelps et al., 2023b). Smooth muscle contraction is also initiated by a Ca^{2+}-independent pathway via activation of Rho kinase, achieved by inhibiting the

counteracting enzyme myosin light chain phosphatase leading to sustained contraction (Anjum, 2018).

Pharmaceutical treatments for UAB target the $G_{q/11}$-coupled M3 muscarinic receptors within the urinary bladder wall (Moro et al., 2021b). However, due to low adherence rates from lower-than-expected outcomes and side effects associated with parasympathomimetic use, there has been increasing interest in novel targets in the urinary bladder for future treatment development (Moro et al., 2021b). Of particular interest are the $G_{q/11}$-coupled receptors, which mediate contractions in the bladder. Recent research has highlighted the role of histamine, 5-hydroxytryptamine (5-HT, serotonin), neurokinin-A (NKA), prostaglandin E2 (PGE2), and angiotensin-II (ATII) receptors within the various layers of the bladder wall contributing to contraction (Phelps et al., 2022).

A range of $G_{q/11}$ receptors have been observed to mediate contraction in healthy urinary bladders, with responses influenced by Ca^{2+} influx from extracellular sources (Phelps et al., 2022), as well as Rho kinase Ca^{2+}-sensitisation pathways (Phelps et al., 2023b). Studies have also shown age-related alterations in GPCR-mediated contractile responses in the urinary bladder, for example, muscarinic (Yu et al., 1997), histamine (Stromberga et al., 2020a), 5-HT (Saito et al., 1993a), and prostaglandin (Morita et al., 1987). Inhibited GPCR-induced contractions may be related to decreased receptor density or down-regulation by ageing (Mansfield et al., 2005), or may be caused by alterations downstream of smooth muscle receptors such as depressed intracellular pathways involved in contraction (Durlu-Kandilci et al., 2015). It has been suggested that it is not the contractile or cytoskeleton proteins that are affected by ageing (Sjuve et al., 1997), but it is more likely due to alterations in the intracellular mechanisms involving secondary messengers that may affect the response of the bladder to

agonists (Durlu-Kandilci et al., 2015). Ageing has been shown to increase the dependence of muscarinic receptor-mediated detrusor smooth muscle contractions on extracellular Ca^{2+} influx (Gomez-Pinilla et al., 2011), and alter Ca^{2+}-dependent and -independent contraction pathways (Gomez-Pinilla et al., 2008). There are also suggestions that Rho kinase activity may be altered with ageing, affecting carbachol-induced contractions of the detrusor smooth muscle (Kirschstein et al., 2014). However, age-related alterations to these pathways and their influence on smooth muscle contraction have not been well-defined across the various other receptor systems.

6.3 Aims

Porcine tissue has been increasingly used as a research model for healthy human tissue. Although there is currently no validated model of underactive bladder that replicates human presentations, assessing the influence of development between juvenile and adult porcine bladders is likely to provide insights into how age can influence receptor activation and contractility in the urinary bladder. This study aimed to assess the influence of prominent $G_{q/11}$ receptor signalling pathways, chosen for their ability to induce strong contractions of detrusor smooth muscle, between juvenile and adult urinary bladders.

6.4 Methods

6.4.1 *Tissue collection*

Porcine urinary bladders from Large White-Landrace-Duroc cross-bred pigs (*Suf scrofa domestica*) were obtained from the local abattoir after slaughter for the routine commercial provision of food. Juvenile samples were taken from prepubescent pigs aged 6 months old at

80kg live weight, and adult samples from sow pigs aged 2-3 years old at 200kg live weight. After collection from the abattoir, tissues were transported in a portable cooler in cold Krebs-Henseleit bicarbonate solution (Krebs, composition in mM: NaCl 118.4; $NaHCO_3$ 24.9; D-glucose 11.7; KCl 4.6; $MgSO_4$ 2.41; $CaCl_2$ 1.9; and KH_2PO_4 1.18) maintained at 4°C to the University research facilities and used within three hours of the animal's slaughter.

6.4.2 *Tissue preparation*

Strips measuring 2.0cm x 0.5cm were dissected longitudinally from the urinary bladder dome. The detrusor smooth muscle was carefully isolated from the urothelium, lamina propria, and serosal tissue layers using fine scissors (Moro & Phelps, 2022b). Adjacent paired tissue strips were mounted and suspended in 10mL organ baths (Labglass, Brisbane, Australia) containing Krebs bicarbonate solution at a constant temperature of 37°C and gassed with carbogen (95% oxygen and 5% carbon dioxide). After mounting, tissues were equilibrated for 15 minutes, and each bath was washed through with warmed Krebs a total of three times prior to the addition of any pharmaceuticals. The tension placed on the tissues was manually adjusted to 20mN using a moveable isometric force transducer (MCT050/D, ADInstruments, Castle Hill, Australia) with a fine adjustment level. At the conclusion of each experiment, tissue strips were dried and weighed, and the mean net weight of porcine detrusor tissue for juvenile samples was 0.39 ± 0.01 g (n = 288) and for adult samples was 0.52 ± 0.01 g (n = 288), with significant differences between specimens ($p < 0.01$, unpaired Student's two-tailed t-test). As such, data has been presented as millinewton force per gram tissue weight (mN/g) to accommodate for the different weights. The number of tissues (n) is quoted from paired tissue strips, therefore the number of animals (N) used can be calculated by using $n \div 2$.

6.4.3 *Measurements and data collection*

After equilibration, selective inhibitors of extracellular Ca^{2+} influx, intracellular Ca^{2+} release, or Rho kinase were added separately to tissues for 30 minutes and contractile responses were assessed by performing single-dose agonist studies. After equilibration, a single dose of a selective GPCR agonist was added to both the control and experimental tissues after equilibration. Baseline tension was measured with an isometric force transducer and recorded on a Powerlab system using Labchart v7 software (ADInstruments). Changes in baseline tension were expressed as millinewton force per gram tissue weight (mN/g).

6.4.4 *Pharmaceutical agents*

Carbamylcholine chloride (carbachol), histamine dihydrochloride (histamine), and 2-aminoethyl diphenylborinate (2-APB) were obtained from Sigma-Aldrich (Missouri, US). Neurokinin-A (NKA) and nifedipine were from Tocris Bioscience (Bristol, UK). Serotonin hydrochloride (5-HT) was from Toronto Research Centre (Toronto, CA). Angiotensin II (ATII) and prostaglandin E2 (PGE2) were obtained from Cayman Chemicals (Michigan, US), and Y-27632 hydrochloride (Y-27632) from AdooQ BioScience (Irvine, CA). PGE2 and nifedipine were dissolved in 100% ethanol, 2-APB was dissolved in dimethyl sulfate, and all other pharmaceutical agents were soluble in distilled water. Nifedipine was stored in the dark until the final application in the organ bath to ensure no adverse light impacts and experiments were concluded within a 30-minute period. Concentrations chosen for the agonists were selected based on their selectivity at each receptor to produce a submaximal contraction (~80%) and consistent with concentrations used in previous studies using porcine tissue.

6.4.5 *Statistical analysis*

Statistical analysis of data was undertaken using GraphPad Prism version 10 (San Diego, CA). Results were presented as mean ± standard error of the mean (SEM). A paired Student's two-tailed *t*-test was used to compare tissue responses to their direct controls, and an unpaired Student's two-tailed *t*-test was applied to make comparisons between juvenile and adult groups. In all cases, $p < 0.05$ was considered statistically significant.

6.4.6 *Ethics*

As no animals were bred, harmed, culled, interfered, or interacted with as part of this research project, animal ethics approval was not required. Experimental protocols remained in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (Queensland Government, 2015).

6.5 Results

6.5.1 *GPCR agonist stimulation of detrusor contractile activity*

The mean ± SEM baseline tension in the absence of any stimulation for juvenile detrusor tissues was 44.57 ± 0.92 mN/g ($n = 144$) and for adult tissues was 38.25 ± 0.93 mN/g ($n = 144$). Table 1 presents the change in baseline tension for juvenile and adult detrusor smooth muscle in response to activation of GPCRs with the agonists carbachol (1µM), histamine (100µM), 5-HT (100µM), NKA (300nM), PGE2 (10µM), and ATII (100nM). Juvenile tissues showed a significantly greater tension response to histamine (100µM) compared to adult tissues ($n = 22$, $p = 0.04$). In detrusor preparations from adult pigs, 5-HT (100µM, Figure 6-1), PGE2 (10µM), and ATII (100nM) elicited significantly greater tension responses when compared to tissues

from juvenile animals (Table 6-1). There was no significant difference between juvenile and adult responses to muscarinic and NKA receptor activation.

Table 6-1: Comparison of responses to GPCR activation between juvenile and adult detrusor tissues. Data reported as mean ± SEM.

	Δ Tension (mN)			
Agonist (conc.)	Juvenile	Adult	*n*	*p*-value
Carbachol (1μM)	112.95 ± 12.75	128.18 ± 17.39	24	0.48
Histamine (100μM)	**15.35 ± 2.94**	**8.63 ± 1.27**	**22**	**0.04**
5-HT (100μM)	**26.09 ± 4.53**	**44.82 ± 7.54**	**22**	**0.04**
NKA (300nM)	35.99 ± 6.65	52.32 ± 8.50	24	0.14
PGE2 (10μM)	**22.93 ± 1.81**	**31.15 ± 3.21**	**24**	**0.03**
ATII (100nM)	**6.40 ± 1.25**	**10.52 ± 1.50**	**24**	**0.04**

Bold font indicates statistical significance ($p < 0.05$, unpaired Student's two-tailed t-test).

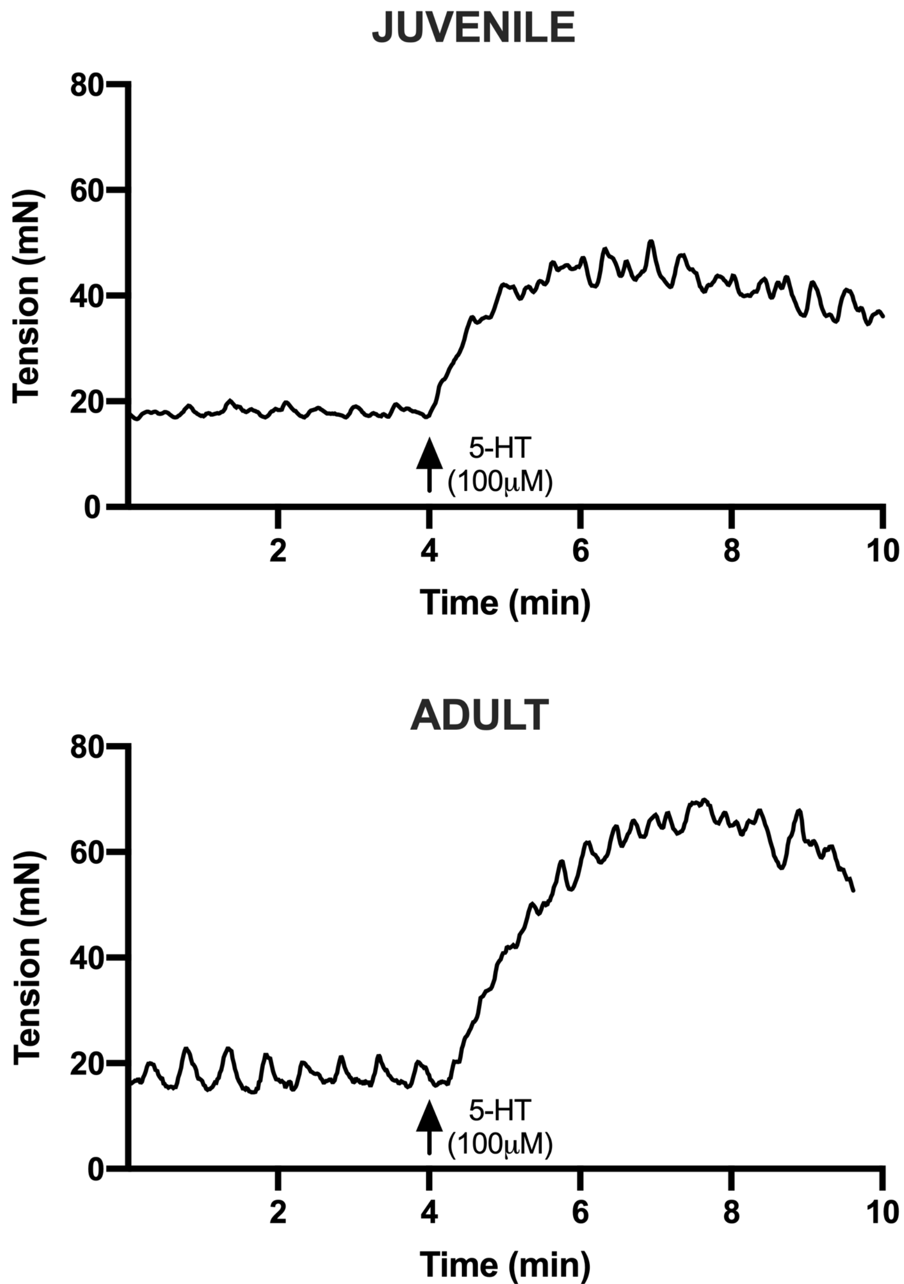

Figure 6-1: Representative experimental trace comparing juvenile (*upper trace*) and adult (*lower trace*) detrusor tension response to 5-HT (100 µM). Note the significantly greater response in the adult detrusor compared to the juvenile detrusor.

6.5.2 *Extracellular Ca^{2+} for GPCR activation of juvenile and adult tissues*

In both juvenile and adult detrusor smooth muscle, blocking extracellular Ca^{2+} influx with nifedipine (1μM) reduced all agonist responses. In juvenile detrusor, all responses were significantly inhibited by nifedipine ($p < 0.05$ for all, paired Student's two-tailed *t*-test, Figure 6-2), for carbachol (Δ 238.59 ± 60.73 mN/g, 1μM, $n = 8$), histamine (Δ 38.82 ± 10.66 mN/g, 100μM, $n = 8$), 5-HT (Δ 75.56 ± 22.83 mN/g. 100μM, $n = 8$), NKA (Δ 22.54 ± 5.91 mN/g, 300nM, $n = 8$), PGE2 (Δ 29.30 ± 4.95 mN/g, 10μM, $n = 8$), and ATII (Δ 9.56 ± 3.73 mN/g, 100nM, $n = 8$). The depressions were also consistent with the adult detrusor, where the application of nifedipine (1μM, $p < 0.05$ for all, Figure 6-3) impacted responses to carbachol (Δ 122.80 ± 39.70 mN/g, 1μM, $n = 8$), histamine (Δ 10.49 ± 1.48 mN/g, 100μM, $n = 8$), 5-HT (Δ 26.01 ± 9.65 mN/g, 100μM, $n = 8$), NKA (Δ 28.49 ± 3.57 mN/g, 300nM, $n = 8$), PGE2 (Δ 17.87 ± 5.92 mN/g, 10μM, $n = 8$), and ATII (Δ 15.70 ± 6.27 mN/g, 100nM, $n = 8$).

Comparison between juvenile and adult

Responses to each agonist for both age groups were significantly depressed after the application of nifedipine. When comparing the magnitude of depression between juvenile and adult tissues, the impact of nifedipine was similar between age groups, with no significant differences for any of the agonists (p = NSD for all, unpaired Student's two-tailed *t*-test).

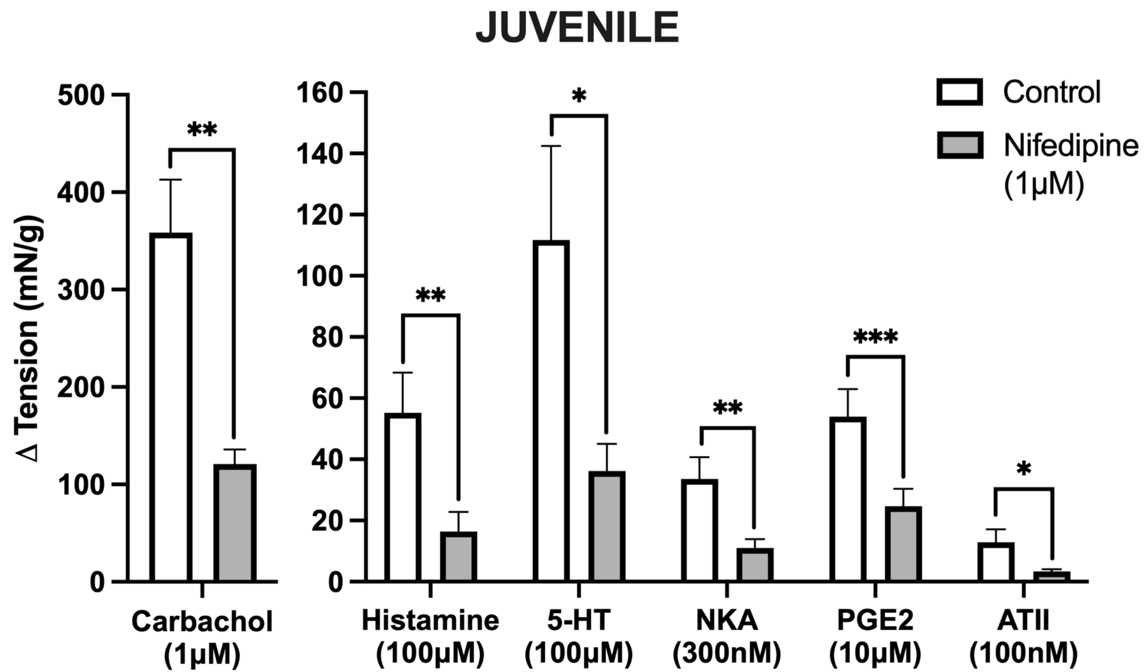

Figure 6-2: The influence of nifedipine (1 μM) on GPCR-mediated contractile responses in juvenile detrusor smooth muscle (n = 8 for all). *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ (paired Student's two-tailed t-test). Data reported as mean ± SEM. As carbachol induced a stronger contraction than the other receptors, the y-axis for this data set was expanded.

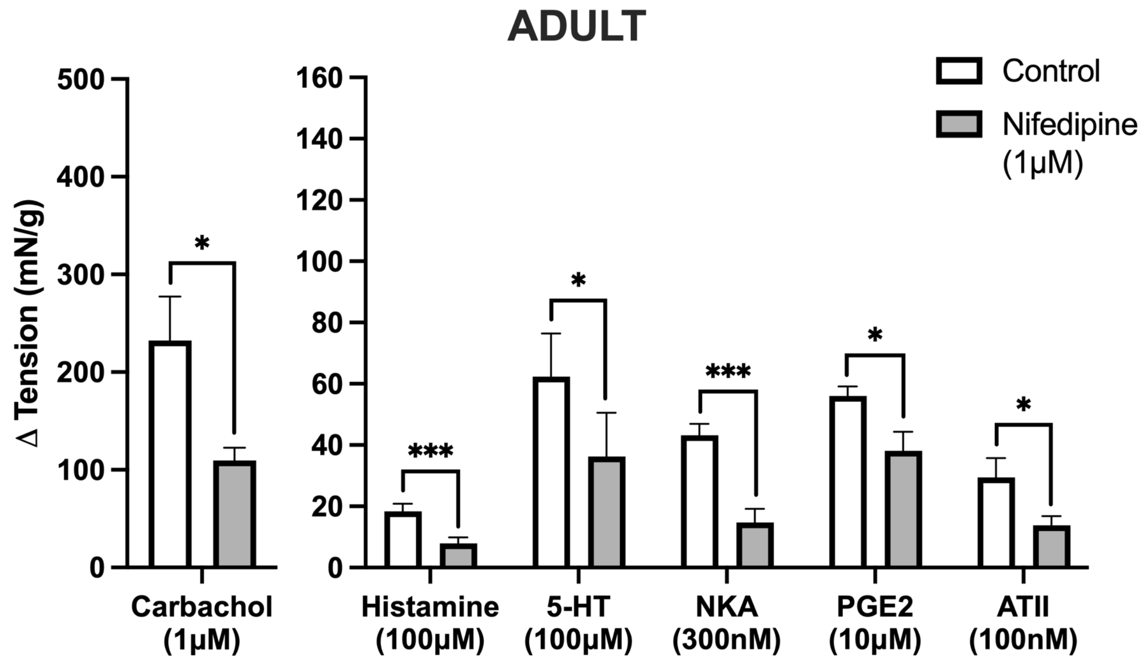

Figure 6-3: The influence of nifedipine (1 μM) on GPCR-mediated contractile responses in adult detrusor smooth muscle (n = 8 for all). *$p < 0.05$, ***$p < 0.001$ (paired Student's two-tailed t-test). Data reported as mean ± SEM. As carbachol induced a stronger contraction than the other receptors, the y-axis for this data set was expanded.

6.5.3 *Intracellular Ca^{2+} for GPCR activation of juvenile and adult tissues*

After application of an inhibitor of IP3-induced Ca^{2+} release, 2-APB (300μM), there was no significant difference in the change in baseline tension for responses to carbachol (1μM, n = 8), histamine (100μM, n = 8), 5-HT (100μM, n = 8), and ATII (100nM, n = 8) for both juvenile (Figure 6-4) and adult (Figure 6-5) detrusor smooth muscle. GPCR-mediated contraction in the presence of 2-APB compared to controls was reduced in both juvenile and adult detrusor tissues in response to NKA (300nM) by 38% (n = 8, p = 0.04) and 31% (n = 8, p = 0.004) respectively, with no significant difference between the two groups. However, after the application of 2-APB, a difference *was* recorded between juvenile and adult responses to PGE2 (10μM, p = 0.03, unpaired Student's two-tailed t-test). While the response to PGE2 activation was inhibited in juvenile samples by 35% (n = 8, p = 0.02), there was no inhibition in aged samples (Figure 6-4).

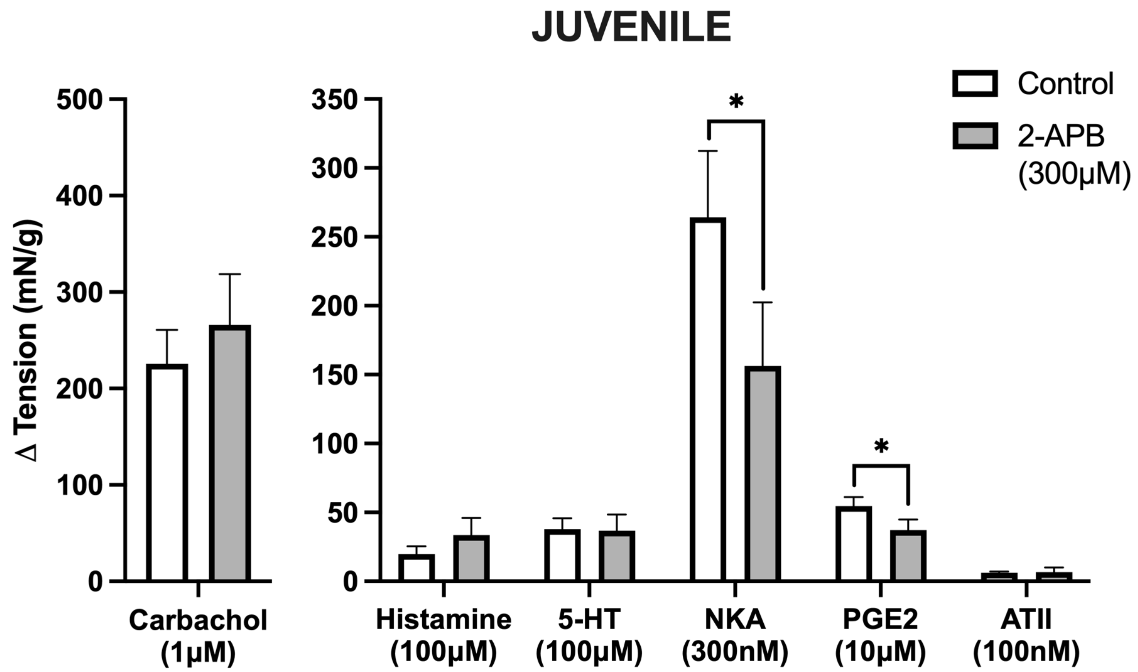

Figure 6-4: The influence of 2-APB (300 µM) on GPCR-mediated contractile responses in juvenile detrusor smooth muscle (n = 8 for all). *$p > 0.05$ (paired Student's two-tailed t-test). Data reported as mean ± SEM. As carbachol induced a stronger contraction than the other receptors, the y-axis for this data set was expanded.

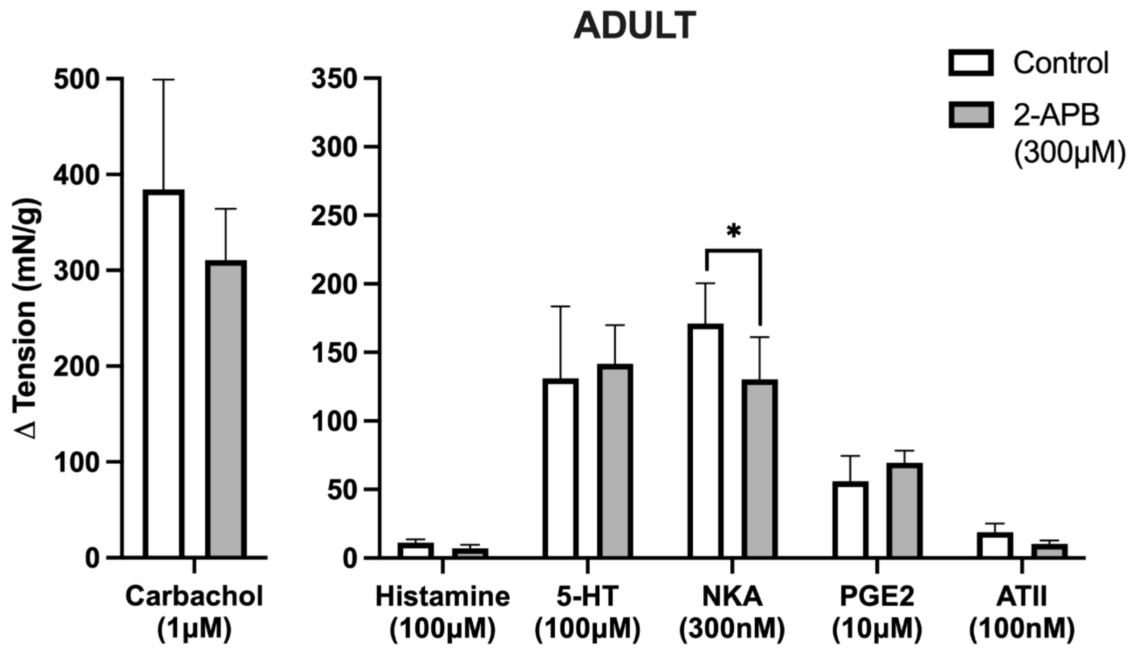

Figure 6-5: The influence of 2-APB (300 μM) on GPCR-mediated contractile responses in adult detrusor smooth muscle (n = 8 for all). *$p > 0.05$ (paired Student's two-tailed t-test). Data reported as mean ± SEM. As carbachol induced a stronger contraction than the other receptors, the y-axis for this data set was expanded.

6.5.4 *Rho kinase pathway for GPCR activation of juvenile and adult tissues*

After activation of the six GPCRs with agonists, in the presence of the Rho kinase inhibitor, Y-27632 (1μM), increases in the baseline tension of detrusor smooth muscle tissues for both juvenile (Figure 6-6) and adult (Figure 6-7) samples was significantly reduced compared to controls ($p < 0.05$ for all, paired Student's two-tailed *t*-test). Inhibition of GPCR-mediated contractions by Y-27632 for juvenile and adult detrusor samples, respectively, was: 1μM carbachol by 42% and 34% ($n = 8$); 100μM histamine by 66% and 61% (n = 6); 100μM 5-HT by 60% and 52% ($n = 6$); 300nM NKA by 51% and 52% ($n = 8$); 10μM PGE2 by 47% and 43% ($n = 8$); and 100nM ATII by 78% and 54% ($n = 8$). When comparing the difference between juvenile and adult responses to the inhibition of Rho kinase, Y-27632 was equally as effective at inhibiting contractile activity between the two age groups, with no significant differences (unpaired Student's two-tailed *t*-test) between the magnitude of responses to any of the agonists.

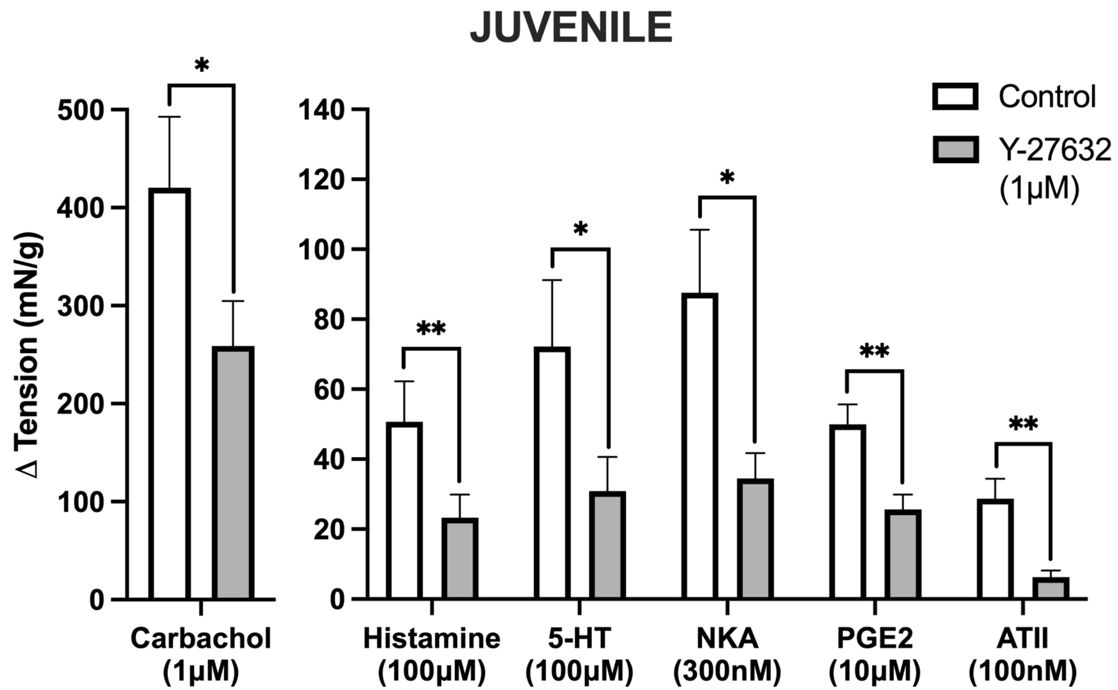

Figure 6-6: The influence of Y-27632 (1 µM) on GPCR-mediated contractile responses in juvenile detrusor smooth muscle (n = 8 for all). *$p > 0.05$, ** $p > 0.01$ (paired Student's two-tailed t-test). Data reported as mean ± SEM. As carbachol induced a stronger contraction than the other receptors, the y-axis for this data set was expanded.

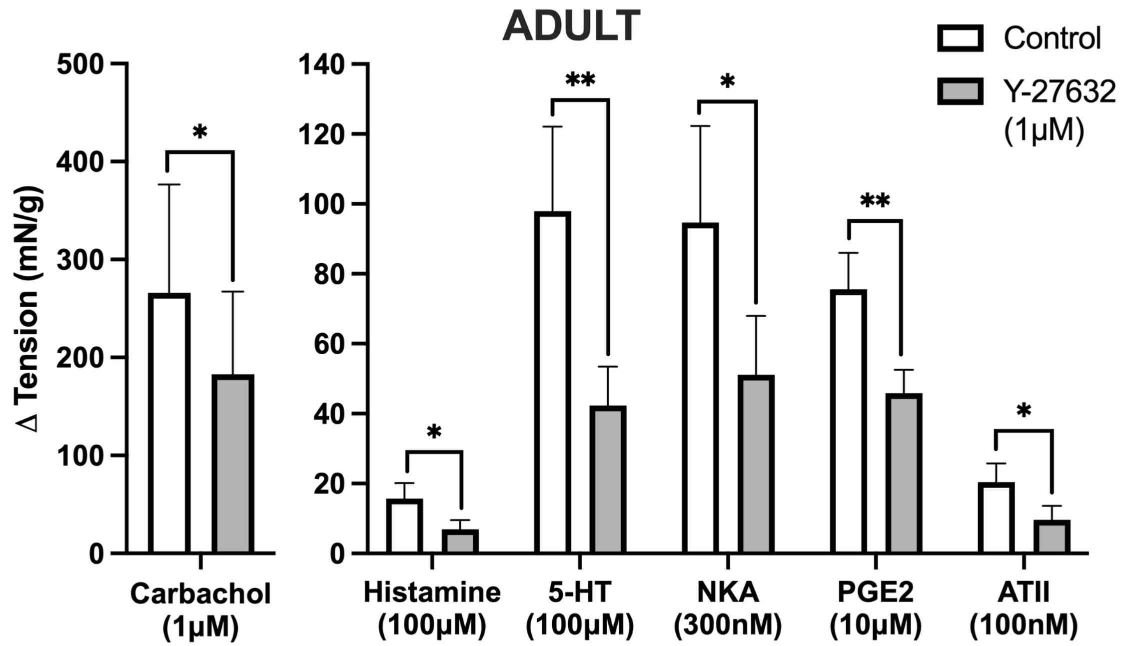

Figure 6-7: The influence of Y-27632 (1 μM) on GPCR-mediated contractile responses in adult detrusor smooth muscle (n = 8 for all). *$p > 0.05$, ** $p > 0.01$ (paired Student's two-tailed t-test). Data reported as mean ± SEM. As carbachol induced a stronger contraction than the other receptors, the y-axis for this data set was expanded.

6.6 Discussion

As the prevalence of underactive bladder increases with age, this study sought to investigate different time points to see how receptor-mediated contractile activity of the urinary bladder differs. Using porcine detrusor smooth muscle, various G protein-coupled receptor systems and their downstream signalling pathways that contribute to detrusor smooth muscle contractions were explored. The findings from this research highlight the varying responses to key mediators of contraction between juvenile and adult urinary bladder smooth muscle. Whilst the alterations do not appear to be related to $G_{q/11}$-coupled second messenger pathways, particularly extracellular Ca^{2+} or Rho kinase, there were differences in the sensitivity to intracellular Ca^{2+}.

The activation of the muscarinic, histamine, 5-HT, NKA, PGE2, or ATII receptors increased the force of contraction in both juvenile and adult urinary bladder detrusor smooth muscle. Muscarinic receptors, in particular the M3 muscarinic receptor remain the most widely studied receptor in the detrusor (Chess-Williams, 2002) and urothelium/lamina propria. (Moro et al., 2011), as they are primarily responsible for mediating contractions through cholinergic activation, and therefore remain the main target for treating bladder contractile disorders (Veer et al., 2023). However, due to the side effects and lower-than-expected treatment outcomes associated with muscarinic receptor therapeutics (Moro et al., 2021b), there is a growing interest in additional receptor systems in the urinary bladder that may present novel targets for future pharmaceutical development. Of note, the H1 histamine (Stromberga et al., 2019), 5-HT_{2A} (Moro et al., 2016), neurokinin-2 (Grundy et al., 2018), EP1 prostaglandin E2 (Stromberga et al., 2020c), and AT_1 angiotensin II (Phelps et al., 2022) receptors are known to cause contractions in the urinary bladder, and therapeutics that target these $G_{q/11}$-coupled

receptors could be effective in the treatment of bladder contractile disorders, such as underactive bladder.

Across the GPCRs explored, developmental differences were identified in detrusor contractile activity in response to some of the agonists. Contractile responses to histamine in the adult detrusor were significantly smaller when compared to contractions observed in juveniles, which is consistent with findings by Stromberga et al. (2020a). This could be explained by reduced compliance in the adult detrusor as a result of a greater deposition of collagen in the aged bladder (Lepor et al., 1992; Nordling, 2002). In contrast, the urinary bladder of adult detrusor responded to a variety of agonists, including 5-HT, PGE2, and ATII, to a higher degree than the juvenile detrusor. It is known that these agonists are involved in bladder contraction, and the finding that the detrusor contractile response is more sensitive with age may indicate involvement in bladder dysfunction.

Beyond receptor activation, intracellular signalling mechanisms contributing to contraction could also be accessible targets for pharmaceutical development (Christ & Andersson, 2007), and these may also be altered with age. Extracellular Ca^{2+}, intracellular Ca^{2+}, and Rho kinase are important components of the $G_{q/11}$-coupled contraction pathway (Mizuno & Itoh, 2009), and alterations to the availability of these molecules for contraction may contribute to the development of bladder contraction disorders.

The contribution of extracellular Ca^{2+} and Rho kinase for contractions was consistent across both juvenile and adult detrusor, indicating that extracellular Ca^{2+} entry into the tissue via voltage-gated Ca^{2+} channels and Ca^{2+} sensitisation via the Rho kinase pathway is unlikely to be impaired with age for various $G_{q/11}$ receptors known to cause contraction. The influence of

Ca^{2+} from intracellular sources for agonist-induced contractions of tissues was minimal, and this was similar for the two age groups. This highlights that there is a stronger dependence on extracellular Ca^{2+} and Rho kinase for mediating muscarinic, histamine, 5-HT, and ATII detrusor contractions. Impairment of IP3-mediated Ca^{2+} release with 2-APB inhibited contractions induced by NKA for both juvenile and adult detrusor samples and juvenile samples inhibited in response to PGE2. These findings are unique to these receptors in the detrusor, as the influence of intracellular Ca^{2+} release was found to be minimal for neurokinin and prostaglandin receptors in the urothelium and lamina propria (Phelps et al., 2023b). The finding that there was decreasing sensitivity to intracellular Ca^{2+} in response to PGE2-mediated contractions during ageing indicates an age-related difference in the $G_{q/11}$ second messenger pathway. The EP1 prostaglandin E2 receptor may be of particular interest in future studies, as the magnitude of inhibition was significantly greater in juvenile detrusor than adult detrusor. This may be supported by findings that urinary bladder PGE2 levels are negatively correlated with age (Aoki et al., 2009; Kuhlik et al., 1995), and as such, further investigations focussing on the receptor expressions could assist. One hypothesis for this age-related difference is that the juvenile detrusor may be more dependent on IP3-mediated Ca^{2+} release for PGE2-induced contractions, whereas activation of the prostaglandin receptors in the adult detrusor relies on an increase in extracellular Ca^{2+}, which may activate ryanodine receptors to induce intracellular Ca^{2+} release (Shibuya et al., 1999).

6.6.1 *Limitations and future directions*

Although a viable and well-validated model, porcine tissue may not directly correlate to human function, and experiments that replicate this study on human tissue would be of interest. It is also unclear which age would be equivalent to a two-year-old sow. These animals are past their reproductive prime, and often referred to as the older pigs. However, it would likely not be

correct to classify these animals as “elderly”. As such, the terms juvenile and adult were used throughout, and future studies could investigate different age groups. Further, finding the molecular mechanisms involved in the differences observed, and any morphological changes, such as receptor density, tissue morphology, and collagen deposition, would present an interesting direction for future investigations. Lastly, future studies could explore age-related alterations in the signalling pathways associated with GPCR-mediated contractions across the various tissue layers in the urinary bladder, such as the urothelium and lamina propria.

6.7 Conclusions

The increased contraction to 5-HT, PGE2, and ATII, and decreased sensitivity to histamine in older tissue, may provide insights into potential systems that could contribute to dysfunction in the contractility of the urinary bladder. In most cases, these observed alterations do not appear to be related to $G_{q/11}$-coupled second messenger pathways that involve extracellular Ca^{2+} or Rho kinase, highlighting a potentially important age-related alteration. Alternatively, the decreased sensitivity to intracellular Ca^{2+} in response to PGE2-mediated contractions in aged tissues, compared to juvenile samples may also warrant further investigation. Overall, juvenile and adult bladders demonstrate clear differences in their ability to contract in response to agonist stimulation, suggesting additional mechanisms that may contribute to bladder contractile dysfunction.

Chapter 7

General discussion

This thesis investigated the G protein-coupled receptors mediating prominent contractions within the urinary bladder urothelium, lamina propria, and detrusor isolated tissues. The results identified the receptor signalling pathways mediating responses to muscarinic, histamine, 5-hydroxytryptamine, neurokinin, prostaglandin, and angiotensin II receptor activation. An additional outcome of this study was the investigation of age-related alterations to the signalling pathways underlying GPCR-mediated contractions within the urinary bladder. Uncovering insights into the mechanisms modulating these receptor systems presents novel therapeutic targets for future pharmacological therapies that could be used in the management of bladder dysfunction and provides further understanding of the pathophysiology underlying some common bladder presentations.

Recent therapeutics for bladder dysfunction have a particular focus on the GPCRs lining the surface of urinary bladder cells. While the main mediator of contraction appears to be the M3 muscarinic receptors (Chess-Williams, 2002), when prescribed antimuscarinic therapies, up to 90% of people cease their treatment regimens due to lower-than-expected treatment benefits or adverse side effects (Nazir et al., 2018; Yeowell et al., 2018). This has stimulated an interest towards investigating alternative receptors that could contribute to contractile dysfunctions such as underactive and overactive bladder. This consideration was the focus of this thesis, using the porcine model to provide insights into which of the GPCRs and associated second messenger systems might be integral and potential treatments in the future management of bladder dysfunction.

7.1 G protein-coupled receptors in the urinary bladder

GPCRs are the largest and most versatile receptor family, comprising over 800 members that contribute to a range of important physiological and pathophysiological processes (Robert et al., 2003; Yang et al., 2021). This family of receptors are some of the most successful targets for investigating and developing pharmaceutical treatments (Addis et al., 2023), and is the target for 34% of approved drugs used today (Hauser et al., 2017).

Prominent GPCRs that could mediate urinary bladder function identified in previous literature were the M3 muscarinic (Moro et al., 2011), H1 histamine (Stromberga et al., 2019), 5-HT_{2A} (Moro et al., 2016), neurokinin-2 (Grundy et al., 2018), prostaglandin E2 (Stromberga et al., 2020c), and angiotensin II type 1 (Lim et al., 2021) receptors. As such, these became the focus of this thesis. Activation of the urinary bladder with each of these receptor agonists induced strong tonic contractions in the U&LP and detrusor smooth muscle. The frequency of spontaneous contractions in the U&LP also significantly increased in response to these receptor agonists. As such, this thesis has identified a potential for these novel mediators of contraction to be involved in bladder contractions. While muscarinic stimulation remains the front-line target for pharmaceutical treatments, identifying commensurate strong instant contractions to histamine, 5-HT, NKA, PGE2, and ATII presents alternative systems that could be considered in future treatments. In humans, while parasympathomimetics are unsuccessful in enhancing contraction in the urinary bladder (Moro et al., 2021b), this finding certainly provides future directions for novel therapies and treatments for underactive bladder (Chapters 3 and 4).

Aside from muscarinic antagonists, the search for alternate targets that can be used in pharmaceutical treatments has been ongoing. In recent years, success has been found with the introduction of combination therapies for the management of overactive bladder. The most common involves the use of both an antimuscarinic in conjunction with a beta-3 adrenergic agonist (Babin et al., 2023). Some success has also been observed from the use of alpha-1 adrenergic antagonists (Kim et al., 2019). This highlights a willingness for both clinicians and patients to trial alternate pharmaceutical therapies, and the successes have paved the way for potential gains from investigating other receptor systems. The prior findings that the activation of histamine (Stromberga et al., 2019), 5-HT (Moro et al., 2016), neurokinin (Grundy et al., 2018), prostaglandin (Stromberga et al., 2020c), and angiotensin (Lim et al., 2021) receptors causes relatively strong contractions in bladder tissue is of particular interest. Dysfunction of these receptor systems, or abnormal secretion of their agonists and ligands, might be responsible for some presentations of overactive bladder. Upon activation, these receptors not only induce tonic contractions, but also increase the frequency of spontaneous phasic activity, suggesting a further link to disorders such as urinary urgency, where spontaneous contractions, or heightened contractile frequency, can be involved. Furthermore, if they do play a role in the maintenance of normal bladder function, it is also likely that dysfunction, or inhibition of these receptor systems could result in an inhibited bladder contractile capacity, such as in the case with underactive bladder (Aizawa & Igawa, 2017).

7.1.1 *Ca^{2+} involvement in GPCR-mediated contractions*

All assessed GPCRs in this thesis were highly sensitive to extracellular Ca^{2+}, although this varied between receptors. Inhibiting extracellular Ca^{2+} influx through various methods (Chapter 3) inhibited baseline tension responses to agonists carbachol by 39-54%, histamine by 45%, 5-HT by 28%, NKA by 22-49%, PGE2 by 29-32%, and ATII by 43-47%. Removing extracellular Ca^{2+} mechanisms was far more impactful than any impact of intracellular Ca^{2+} (Chapter 4), with minimal impact on the receptors. Of interest, was the sensitivity of all assessed receptors to Rho kinase (Chapter 4), which has been identified as a clear Ca^{2+}-independent second messenger pathway mediating bladder contraction.

Along with presenting a range of GPCR systems within the layers of the urinary bladder wall that could present as future therapeutic targets for underactive bladder, extracellular Ca^{2+} directly influences the contractile activity of the U&LP and detrusor to various agonists, highlighting a target for UAB treatments to enhance this system. Ca^{2+} channel blockers have frequently been suggested in the literature as a potential target for overactive bladder treatments (Joseph et al., 2022), and more specifically, there are recent suggestions for targeting the L-type voltage-gated Ca^{2+} channels as a promising novel therapy for bladder dysfunction (Yu, 2022). This would inhibit the entry of Ca^{2+} ions into the cell through voltage-gated Ca^{2+} channels from the exterior of the cell and lead to a fall in intracellular Ca^{2+} levels and muscle relaxation.

This finding is supported by previous studies on the detrusor smooth muscle of guinea pigs, where spontaneous action potentials and their corresponding Ca^{2+} transients were blocked by nifedipine, but not by intracellular Ca^{2+} store inhibitors (Hashitani & Brading, 2003b; Hashitani et al., 2001). This highlights an important role of Ca^{2+} entry from the extracellular

fluid through L-type Ca^{2+} channels to induce spontaneous activity in the urinary bladder, which may be similar in both the urothelium and lamina propria and detrusor tissue layers.

7.1.2 *Rho kinase involvement in GPCR-mediated contractions*

The well-established dependence on Ca^{2+} for smooth muscle contractions has been the focus of many of the findings reported in the literature (Masters et al., 1999). However, agonist-induced activation of GPCRs can also lead to smooth muscle contraction without changes to intracellular Ca^{2+} concentrations via the Rho kinase pathway (Somlyo & Somlyo, 2003). This Ca^{2+} sensitisation pathway was investigated in U&LP contractions in Chapter 4 of this thesis, as well as in the detrusor smooth muscle in Chapter 6. A strong dependence on Rho kinase for contractions was shown by inhibition of agonist-induced GPCR contractions in the presence of the Rho kinase inhibitor Y-27632, fasudil, and GSK629962. This may present Rho kinase as an attractive target for treatment development as it is involved in the contraction elicited by a wide variety of agonists at muscarinic histamine, 5-HT, neurokinin, prostaglandin, and ATII receptors.

Preclinical studies have investigated the contribution of Rho kinase in the contraction of the detrusor smooth muscle, highlighting this as a potential therapeutic target (Joseph et al., 2022). The finding in this thesis that Rho kinase contributes to receptor-mediated contractions in both the detrusor and U&LP to a similar degree highlights the further potential for this pathway to be targeted in the treatment of bladder contractile dysfunction. This supports previous suggestions where Ca^{2+} and Rho kinase have both been postulated as future targets in the treatment of bladder dysfunction (Joseph et al., 2022), but the potential for side effects such as those associated with muscarinic receptor targets remains to be clarified.

7.2 Age-related alterations to GPCRs in the bladder

The influence of ageing on bladder dysfunction is of increasing interest as the world's population ages (Batmani et al., 2021). While clinicians certainly take the patients' age and capabilities into account (Wagg, 2011), age is not always a considered factor in issues such as the recommended dosage of antimuscarinics (Geoffrion, 2012). This is likely due to a paucity of understanding of how age impacts the urinary bladder itself (Siroky, 2004). There is the potential for the concentration or efficacy of receptors to deplete, for the tissue itself to become less (or more) contractile, or for fibrosis and hardening to set in, impacting contraction. This means that an aged bladder may act quite differently from that of a younger person (Stromberga et al., 2020a). Having an enhanced knowledge of how ageing impacts the tissue itself can provide insights into how certain medications or treatment options may be more targeted, effective, and helpful to the individual patient.

With the identification of key GPCRs mediating contraction in the porcine urinary bladder, it was important to consider if these would alter or change during development. In humans, ageing is associated with an increased prevalence of bladder dysfunction. Overactive bladder affects around 16% of the adult population (Reynolds et al., 2016) and underactive bladder prevalence is 9-28% (Osman et al., 2018). These prevalence rates of bladder contractile disorders increase up to about 50% in elderly men and women (Przydacz et al., 2023; Suskind, 2017). In Chapter, 5, upon scoping the literature to identify any insights from animal models, it was apparent that clear physiological alterations were occurring within the bladder tissue itself, potentially meaning the treatments could be considered on an age and developmental basis.

There is some evidence that current therapeutics for underactive bladder are effective, however, side effects are still common for patients. Age-related symptoms are particularly apparent for the elderly. For example, older patients may be more sensitive to the cholinergic effects of antimuscarinics and are often prescribed multiple agents with anticholinergic effects, which may have significant cognitive effects in the long-term (Dantas et al., 2022; Staskin, 2005). As bladder instability is a frequent complaint among the elderly population, insights into the age-related alterations to receptor systems in the urinary bladder may help us better understand the mechanisms underlying contractile disorders. Chapter 6 of this thesis investigated different time points to see how receptor-mediated contractile activity of the bladder differs.

Previous research has reported various responses to agonist-induced contractions in the bladder that differ with age. Saito et al. (1993a) showed that the contractile response to 5-HT, ATP, and noradrenaline are increased in ageing, but no age-related change was observed to the contractile response upon activation of the muscarinic, prostaglandin F2 alpha, and ATII receptors in female mice. In response to agonists 5-HT, PGE2 and ATII, adult tissue was more sensitive to stimulation, resulting in greater increased baseline tension responses of 42%, 26%, and 39%, respectively, when compared to juvenile tissue. The finding that ageing presents heightened sensitivities to agonists for these GPCRs may present receptors of interest for age-specific treatment development. Alternatively, perhaps the increased sensitivity to neurotransmitters, inflammatory mediators, and peptide hormones could present contributing factors to the development of overactive bladder in the elderly. Another observation was the response to histamine, which was observed to have a greater increase (44%) in contraction when stimulated in the juvenile tissue compared to adult tissue.

The unique age-related alterations to different receptor systems in the bladder also have further implications for future pharmaceutical treatment development for dysfunctions underlying bladder contractions. Intracellular contraction mechanisms could be accessible targets for pharmacological manipulation (Kirschstein et al., 2015), specifically targeting extracellular Ca^{2+} channels and Rho kinase, which plays a key role in the influence of GPCR-mediated contraction of the various tissue layers in the bladder. There are suggestions that ageing can lead to various alterations in the urinary bladder, downstream of the receptors themselves (Durlu-Kandilci et al., 2015). Therefore, investigating the signalling systems underlying receptor responses was of interest. Previous studies highlight age-related alterations on the dependence of Ca^{2+} for detrusor contractions, including increased dependence on extracellular Ca^{2+} influx for contractions (Yu et al., 1996), reduction to Ca^{2+} extrusion activity (Gomez-Pinilla et al., 2007), alterations to Ca^{2+}-independent pathways (Gomez-Pinilla et al., 2008), and impairment of detrusor contractions in response to cholinergic stimulation (Gomez-Pinilla et al., 2011). Chapter 6 of this thesis fills the identified gap of knowledge in the literature where the dependence of extracellular Ca^{2+} on receptor-mediated contractions was identified and evaluated, and this thesis found no change to the impact of these Ca^{2+} disturbances in the contraction of adult detrusor smooth muscle compared to juvenile in porcine urinary bladder.

7.3 Future direction

This thesis has identified a range of G protein-coupled receptors that contribute to the contractile activity of the porcine urinary bladder urothelium, lamina propria, and detrusor smooth muscle layers. It was observed that both extracellular Ca^{2+} and Rho kinase are involved in the signalling pathways of muscarinic, histamine, 5-HT, NKA, PGE2, and ATII receptors, and stand as key mediators of contraction. Intracellular Ca^{2+} was also involved for some

receptors, however to a lesser extent. This presents a range of potential avenues for future research into these receptor systems. Although assessed in pigs, this study presents further insights into the role of these second messengers and their potential use for future treatment. Recommendations for future research in this area are to replicate these studies on human urinary bladder tissue and consider any applications in the clinical setting.

It was also observed that ageing impacts multiple receptor systems in the detrusor smooth muscle, which could be a potential cause of underlying dysfunction of the urinary bladder contractile activity. Intracellular Ca^{2+} was found to have the most significant age-related difference between the receptor systems investigated. The influence of extracellular Ca^{2+} and Rho kinase in these age-related differences was minimal, highlighting that age may not affect the underlying signalling properties of these receptors. This also presents an area for future research to identify the density and expression of these receptors across various age groups.

7.4 Concluding remarks

The identification of alternative receptors contributing to overall urinary bladder contractions provides exciting opportunities for non-muscarinic treatments for bladder contractile disorders. It is apparent that histamine, 5-hydroxytryptamine (serotonin), neurokinin, prostaglandin, and angiotensin II receptors are important mediators of contraction in both the urothelium, lamina propria, and detrusor smooth muscle. Avenues for future research should investigate the potential for a contribution of these receptors to human urinary bladder function and investigate the clinical application for these novel systems.

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