DOCTORAL THESIS

The role of the inflammatory mediators in modulating bladder contractile activity.

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Award date:
2021

BOND UNIVERSITY

FACULTY OF HEALTH SCIENCES & MEDICINE

The role of the inflammatory mediators in modulating bladder contractile activity

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Submitted in total fulfilment of the requirements of the degree of Doctor of Philosophy

July 2020

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This research was supported by an Australian Government Research Training Program Scholarship

Abstract

Urinary bladder inflammation has been observed in various lower urinary tract disorders, including overactive bladder (OAB) and interstitial cystitis/bladder pain syndrome (IC/BPS). However, the mechanisms underlying these conditions are not fully understood. It is apparent that acetylcholine release is involved, yet other mediators and regulator chemicals may also influence bladder function and sensation. There is a particular interest in identifying which receptors other than muscarinic are functional within the bladder wall and capable of mediating overall contractility. Furthermore, there are reports of an increased presence of inflammatory mediators within the bladder and urine of patients suffering from OAB and IC/BPS. Therefore, the involvement of immune cells and the various inflammatory mediators released at sites of inflammation is an important avenue to explore. Understanding the actions of these mediators and the associated receptor systems may reveal future therapeutic targets for lower urinary tract dysfunction. This thesis aimed to determine the effect of histamine and the five primary prostaglandins on tonic contractions and phasic activity of the urinary bladder. This led to an additional aim to investigate the age-associated contractile responses observed to histamine and prostaglandin E_2. Isolated tissue baths containing adjacent strips of urothelium with lamina propria or detrusor were used to examine the key receptor systems involved in the contractions observed in response to stimulation with histamine or prostaglandin.

In response to histamine, tonic contractions and spontaneous phasic activity were significantly enhanced. The receptor subtype involved in mediating this response was determined to be the H1 receptor with no involvement of the H3 and H4 receptors. The treatment with the H2 receptor antagonist enhanced urothelium with lamina propria (U&LP) tonic contractions to histamine, whereas stimulation with the receptor agonist-induced relaxation. In detrusor, the H1 receptor was also involved in mediating the responses to histamine. However, no involvement of the H2, H3 or H4 receptors was determined. Further studies assessing an older animal model revealed that ageing impacts the responses to histamine in detrusor, although it does not influence U&LP tissue. Additional differences between the two age groups included the involvement of the H2 receptor in U&LP responses. In juvenile animals, the H2 receptor stimulation relaxed the U&LP preparations, whereas this receptor had no influence on

contraction in adult animals. The inhibition observed from H1 receptor antagonists as consistent in reducing tonic contractions and phasic activity in both juvenile and adult animal models.

All five prostaglandins stimulated contractions in both U&LP and detrusor with an identified potency of $PGE_2 > PGF_{2\alpha} > TXA_2 > PGD_2 > PGI_2$. Only 34% of isolated detrusor preparations developed an initial phasic activity in the absence of any stimulation. However, the application of all prostaglandin agonists induced this phasic activity in the majority of the remaining preparations. Further investigation of the PGE_2 receptor subtypes revealed that increases in contractions to PGE_2 were not mediated by any of the EP receptor subtypes. Other receptor systems, such as the purinergic and cholinergic, were also shown to be not involved in this response. The presence of an FP receptor antagonist significantly inhibited increases to tonic contractions in U&LP and detrusor in response to both PGE_2 and $PGF_{2\alpha}$. Therefore, the contractile response to PGE_2 appears to be mediated, at least partially, via the FP receptor in both U&LP and detrusor, suggesting some conversion of PGE_2 to $PGF_{2\alpha}$ upon contact with tissue.

The findings in this thesis demonstrate that both histamine and prostaglandin receptor systems are capable of modulating tonic and phasic contractions of detrusor smooth muscle, and urothelium with lamina propria. Across all mediators considered, histamine, prostaglandin E_2 and $F_{2\alpha}$ exhibited the most pronounced effects on tonic contractions and phasic activities in both layers of the urinary bladder. Therefore, it is feasible that these receptor systems within the bladder wall could act as novel future therapeutic targets in the treatment of lower urinary tract disorders.

Keywords: urinary bladder, urothelium, lamina propria, detrusor, contractility, spontaneous contractions, ageing, inflammatory mediators, histamine, prostaglandins

Declaration by author

This thesis is submitted to Bond University in fulfilment of the requirements of the degree of Doctor of Philosophy (PhD).

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.

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Zane Štromberga

Research impact

Peer-reviewed publications arising from the data presented within this thesis

1. Stromberga, Z., Chess-Williams, R. & Moro, C. (2020). Alterations in histamine responses between juvenile and adult urinary bladder urothelium, lamina propria and detrusor tissues. *Scientific Reports*, *10*(1), 4116. doi:10.1038/s41598-020-60967-7

2. Stromberga, Z., Chess-Williams, R., & Moro, C. (2020). Prostaglandin E2 and F2alpha modulate urinary bladder urothelium, lamina propria and detrusor contractility via the FP receptor. *Frontiers in Physiology*, 11, 705. doi:10.3389/fphys.2020.00705

3. Stromberga, Z., Chess-Williams, R., & Moro, C. (2020). The five primary prostaglandins stimulate contractions and phasic activity of the urinary bladder urothelium, lamina propria and detrusor. *BMC Urology, 20*(1). doi: 10.1186/s12894-020-00619-0

4. Stromberga, Z., & Moro, C. (2020). Which of the primary prostaglandin receptors might play a role in lower urinary tract dysfunction? A narrative review. *Australian and New Zealand Continence Journal*, 26(*3*), 58-60.

5. Stromberga, Z., Chess-Williams, R., & Moro, C. (2019). Histamine modulation of urinary bladder urothelium, lamina propria and detrusor contractile activity via H1 and H2 receptors. *Scientific reports*, *9*(1), 3899. doi:10.1038/s41598-019-40384-1

Published conference abstracts

1. Stromberga, Z., Smith, J., Tan, J., & Moro, C. (2020, July). Influences of the mast cell degranulates histamine and prostaglandins on urinary bladder contractile activity. *Future Physiology 2020, virtual conference.* Can be accessed on https://static.physoc.org/app/uploads/2020/01/25092350/Future-Physiology-2020-programme-and-abstracts.pdf

2. Stromberga, Z., Chess-Williams, R., & Moro, C. (2020, July). Inflammatory mediators as contributors to age-related urinary bladder dysfunction. *Future Physiology 2020, virtual conference.* Can be accessed on https://static.physoc.org/app/uploads/2020/01/25092350/Future-Physiology-2020-programme-and-abstracts.pdf

3. Stromberga, Z., Chess-Williams, R., & Moro, C. (2019, November). Age-related changes in histamine receptor mediated contractions of the urinary bladder. *Proceedings of the ASCEPT-PAGANZ joint scientific meeting, Queenstown, New Zealand.* Can be accessed on https://www.asceptasm.com/wp-content/uploads/2019/11/ASCEPT-PAGANZ-2019-oral-abstracts-updated.pdf

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5. Moro, C., West, E., Stromberga, Z., & Chess-Williams, R. (2019, November). The impact of ageing on urinary bladder muscarinic receptor activity. *Proceedings of the ASCEPT-PAGANZ joint scientific meeting, Queenstown, New Zealand.* Can be accessed on https://www.asceptasm.com/wp-content/uploads/2019/11/Ascept-Paganz-2019-poster-abstracts.pdf

6. Stromberga, Z., Chess-Williams, R., & Moro, C. (2018, November). The effects of age on contractile activity of the bladder urothelium and detrusor. *Proceedings of the 27th National Conference on Incontinence, Hobart, Australia.*

7. Stromberga, Z., Chess-Williams, R., & Moro, C. (2018, November). The involvement of histamine receptors in modulating bladder spontaneous contractile activity. *Proceedings of the 27th National Conference on Incontinence, Hobart, Australia.*

8. Stromberga, Z., Chess-Williams, R., & Moro, C. (2018, July). Histamine H1 and H2 receptors as regulators of urinary bladder urothelium/lamina propria and detrusor contractile activity. *Proceedings of the 18th World Congress of Basic and Clinical Pharmacology, Kyoto, Japan.* Can be accessed on https://www.micenavi.jp/wcp2018/search/detail_program/id:503

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11. Stromberga, Z., Chess-Williams, R., & Moro, C. (2017, December). Histamine receptors as regulators of urothelial and detrusor contractile activity. *Proceedings of the APSA-ASCEPT joint scientific meeting, Brisbane, Australia.* Can be accessed on https://www.asceptasm.com/wp-content/uploads/2017/12/APSA-ASCEPT-Poster-51217.pdf

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13. Stromberga, Z., Chess-Williams, R., & Moro, C. (2017, November). Histamine as a potential mediator in bladder contractile disorders. *Proceedings of the Gold Coast Health Research Week Conference 2017, Gold Coast, Australia.*

14. Stromberga, Z., Chess-Williams, R., & Moro, C. (2017, November). The role of inflammatory mediators in modulating urothelial contractile activity. *Proceedings of the 26th National Conference on Incontinence, Sydney, Australia.*

15. Stromberga, Z., Chess-Williams, R., & Moro, C. (2017, November). The role of histamine in modulating urothelial contractile activity. *Proceedings of the 26th National Conference on Incontinence, Sydney, Australia.*

Travel grants

1. Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists (ASCEPT) 2019, 25 – 29 November, Queenstown, New Zealand – student travel grant.

Contributions to other peer-reviewed publications during candidature

1. Moro, C., Phelps, C., Redmond, C., & Stromberga, Z. (2020). HoloLens and mobile augmented reality in medical and health science education: A randomised controlled trial. British Journal of Educational Technology. doi:10.1111/bjet.13049

2. Moro, C., Phelps, C., & Stromberga, Z. (2020). Utilizing serious games for physiology and anatomy learning and revision. *Advances in Physiology Education,* 44(*3*), 505-507. doi:10.1152/advan.00074.2020

3. Moro, C. & Stromberga, Z. (2020). Enhancing variety through gamified, interactive learning experiences. *Medical Education*, 1-2. doi:10.1111/medu.14251

4. Moro, C., Phelps, C., Jones, D., & Stromberga, Z. (2020). Using Holograms to Enhance Learning in Health Sciences and Medicine. Medical Science Educator, 30, 1351-1352. doi:10.1007/s40670-020-01051-7

5. Stromberga, Z., Phelps, C., Smith, J., & Moro, C. (2020). Teaching with disruptive technology: The use of augmented, virtual, and mixed reality (HoloLens) for disease education. In *Biomedical Visualisation* (in press). Springer, Cham.

6. Moro, C., Stromberga, Z., & Moreland, A. (2020). Enhancing teaching in biomedical, health and exercise science with real-time physiological visualisations. In Rea P.M. (ed.) *Biomedical Visualisation*. Springer, Cham. doi:10.1007/978-3-030-47483-6_1

7. Moro C., Stromberga Z., & Birt J. (2020). Technology Considerations in Health Professions and Clinical Education. In Nestel D., Reedy G., McKenna L., Gough S. (eds) *Clinical Education for the Health Professions*. Springer, Singapore

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1. Moro, C., Phelps, C., Stromberga, Z., & Finch, E. (2020, November). Can Augmented Reality be utilised for disease education in health sciences and medicine? *ASCALITE 2020 Virtual Conference.*

2. Phelps, C., Stromberga, Z., & Moro, C. (2020, November). Delivering health sciences and medicine online: Does live quizzing translate well to digital teaching? *ASCALITE 2020 Virtual Conference.*

3. Phelps, C., Stromberga, Z., & Moro, C. (2020, November). Adjusting curricula for the modern age: Can face-to-face live polling be transferred effectively to an online learning environment in physiology education? *The Australian Physiological Society (AuPS) 2020 Virtual Education Forum.*

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5. Moro, C., & Stromberga, Z. (2020, July). Online engagement with interactive quizzes. *Virtual ASCEPT Education Forum.*

6. Moro, C., Phelps, C., & Stromberga, Z. (2020). Enhancing physiology teaching with holograms in health, biomedical sciences and medicine. *Future Physiology 2020, virtual conference.*

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8. Stromberga, Z. (2019, November). Using Kahoot to engage students in tutorials. *Proceedings of the ASCEPT-PAGANZ joint scientific meeting, Queenstown, New Zealand.*

9. Moro, C., Stromberga, Z., Raikos, A., & Stirling, A. (2016, December). Combining Virtual (Oculus Rift & Gear VR) and Augmented Reality with interactive applications to enhance tertiary medical and biomedical curricula. *Siggraph Asia: 16th Symposium on Education, Macau.*

10. Stromberga, Z., Raikos, A., Stirling, A., & Moro, C. (2016, November). Utilising Virtual and Augmented Reality to enhance medical and healthcare education. *Proceedings of the 55th Australian Society for Medical Research Annual Scientific Meeting, Gold Coast.*

Acknowledgements

I would like to express my utmost gratitude to Bond University for supporting my international journey and the continuous encouragement received from the wonderful academics and professional staff. My experience here has been nothing short of amazing. I am grateful for receiving the Australian Government Research Training Program Scholarship, as well as university's support in attending many fantastic conferences across both Australia and international locations.

To Dr Christian Moro – thank you for taking a chance on me while I was still an undergraduate student and guiding me throughout my career. Your mentorship has helped tremendously and will continuously help shape my professional life. My fondest memories of PhD are our 'Ultimate Plan' days and all the amazing research projects you have given me the chance to be involved in. To Professor Russ Chess-Williams, I am incredibly grateful for your enthusiasm, encouragement and helping me become a better researcher. I am so lucky to have gone through this journey with your support.

My research would have been impossible without the aid and support of the wonderful laboratory staff – Marion, Jan and Katie. You were always so patient with me and offered a helping hand whenever I needed it. I would also like to thank the amazing research development manager, Tanya Forbes. You have never missed any of my talks, and I have been so lucky to be supported by you in my research journey.

To the anonymous peer-reviewers that supported me through many submissions and re-submissions – thank you! While your feedback was extensive at times, it helped me create the best version of my results chapters, and for that, I am very endlessly thankful.

To all my students, teaching you and sharing your university journey was the greatest privilege. This position made me realise how much I enjoy sharing science with those around me.

My fellow HDR students and Urology group colleagues, I would like to thank you for listening, always encouraging, and landing a helping hand whenever I needed it. Especially Jamie-Lee – we have shared this academic path from the first day of our undergraduate degree. Doing puzzles and playing board games was always the greatest distraction. I would also like to acknowledge and thank Jess Smith and Charlotte Phelps for making this journey such a wonderful experience.

To Macarena – thank you for your endless positivity and support. Some of the best work I have done for this thesis was done in your living room in one of our 45-minute productivity sessions.

I want to express a special thank you to Dayne, who has been there for every challenge and success. You encouraged me when things got tough, made sure I was always fed and displayed amazing patience. I could not have achieved this without your support.

I would like to finish with those in Latvia, which I will always hold in my heart. I have an amazing family that supported me unconditionally throughout these years. My endless gratitude to my parents, who supported me even though it meant having their only daughter on the other side of the world who kept saying that she will stay there for "just another degree".

Table of contents

List of tables

Note: All tables and their contents are original work produced by the author, Zane Stromberga.

List of figures

Note: All figures and their contents are original work produced by the author (Zane Stromberga) unless otherwise stated in the figure description.

List of abbreviations

ATP: adenosine 5’-triphosphate

cAMP: 3’-5’-cyclic adenosine monophosphate

COX: cyclooxygenase

DMSO: dimethyl sulphoxide

GAG: glycosaminoglycans

IC/BPS: interstitial cystitis/bladder pain syndrome

IP3: inositol triphosphate

NA: noradrenaline

NO: nitric oxide

OAB: overactive bladder

PG: prostaglandin

SEM: standard error of the mean

U&LP: urothelium with lamina propria

LUT: lower urinary tract

Chapter 1

General introduction

1.1. Anatomy of the lower urinary tract

The lower urinary tract (LUT) consists of the urinary bladder, urethra and prostate in males. The urinary bladder is a hollow organ divided into two main areas: the bladder body and the bladder base. In females, the urinary bladder is positioned anterior to the vagina (Figure 1-1), whereas in males it is situated superior to the prostate gland and directly anterior to the rectum (Figure 1-2). As it originates from the urogenital sinus, it remains loosely associated with the anterior abdominal wall via uracal and median umbilical ligaments (Ramakrishnan and Eswara, 2020).

The ureters convey urine from the renal pelvis using peristaltic contractions (Brenner, 2019) and enter the bladder via their corresponding ureteric orifices, forming the two corners of the triangle known as the bladder trigone. This structure is responsible for guiding urine down the bladder neck into the proximal urethra. The ureteric orifices possess a one-way mucosal flap that prevents the reflux of urine back into the ureters. The base of the bladder has a thick musculature and therefore is less distensible during the filling of urine; however, the dome is relatively thin and more capable of being distended (Keane and O'Sullivan, 2000).

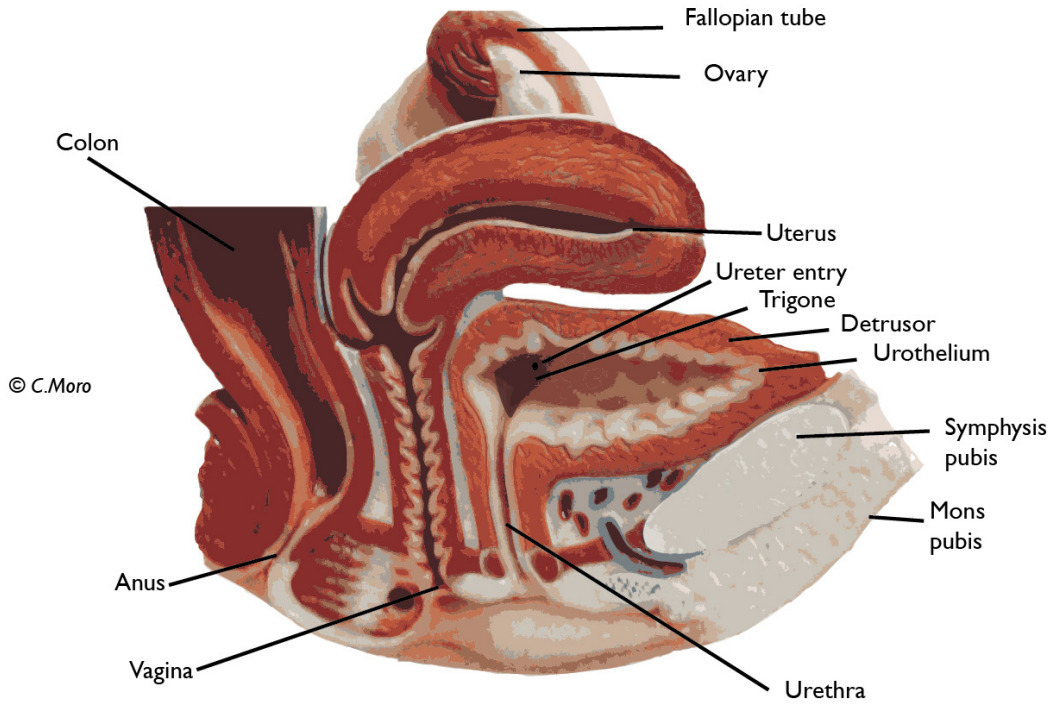

Figure 1-1: Illustration of the female urinary bladder and associated anatomical structures. This figure was produced by Christian Moro.

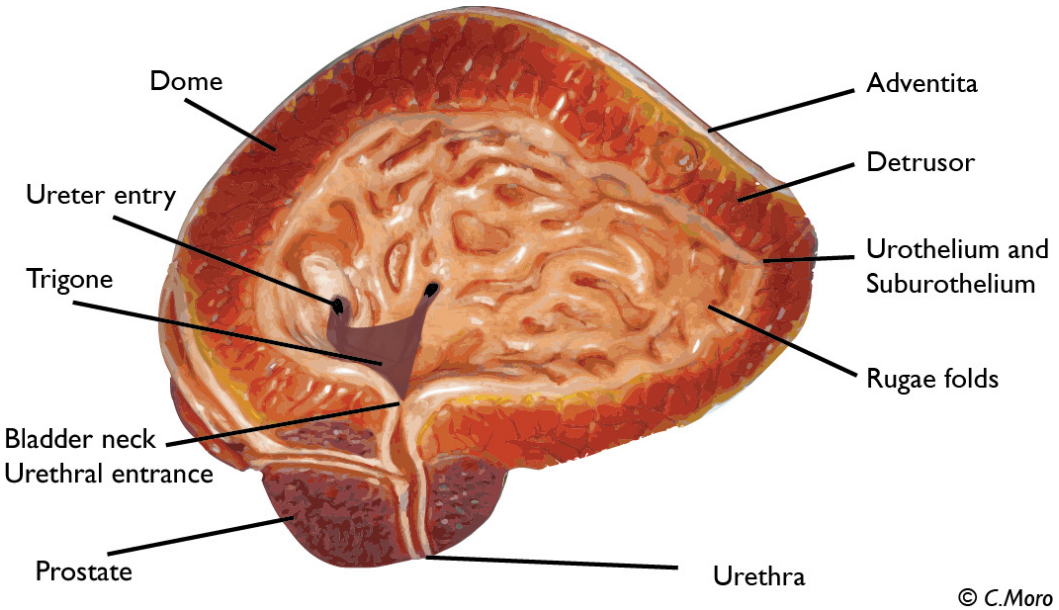

Figure 1-2: Illustration of the male urinary bladder and associated anatomical structures. This figure was produced by Christian Moro.

The urethra is a small tube lined with mucous membrane that connects the urinary bladder to the external genitalia for the removal of urine (Mistry et al., 2020). In males, the urethra passes through the centre of the prostate gland, carrying both semen and urine. The mean urethral length of healthy adult males is 22.3 cm, but it can vary between 15 cm and 29 cm (Kohler et al., 2008). In females, the urethra is between 1.9 and 4.5 cm long and lies behind the symphysis pubis, where it passes from the bladder to the external urinary meatus (Pomian et al., 2018). A group of striated muscles surrounding the urethra at the base of the bladder forms the urethral sphincter.

The urethral sphincter plays an essential role in maintaining urinary continence and preventing urine leakage (Keller et al., 2018). There are two urethral sphincter muscles present: the internal urethral sphincter, which is made of smooth muscle and under involuntary control, and the external urethral sphincter, which is made of striated muscle and under voluntary control (Stoker et al., 2002). Malfunction of the urethral sphincter can cause debilitating disorders of the lower urinary tract, such as urinary incontinence which is characterised by an involuntary loss of urine (Heesakkers and Gerretsen, 2004). The internal urethral sphincter is located at the junction of the urethra and urinary bladder and is a continuation of the detrusor smooth muscle. It functions to control the flow of urine by contracting around the internal urethral orifice. As the internal urethral sphincter is comprised of smooth muscle cells, it cannot be activated voluntarily and thereby is under the control of the autonomic nervous system (Jung et al., 2012). The sympathetic nervous system maintains tonic contractions of the internal urethral sphincter muscle, whereas parasympathetic nervous system relaxes the muscle during micturition (Griffiths, 2015). The secondary sphincter involved in the control of urine flow through the urethra is the external urethral sphincter. It is located in the inferior distal end of the urinary bladder in females

(Hudson et al., 2002) and at the membranous or intermediate part of the urethra in males (Karam et al., 2005). As the external urethral sphincter is composed of skeletal muscle, it is under somatic control and can be activated voluntarily (Shefchyk, 2001).

In males, the prostate gland forms a part of the lower urinary tract. It is situated anterior to the rectum and inferior to the urinary bladder, surrounding the urethra. The human prostate is a composite organ, composed of three glandular zones (transition, central and peripheral) and a fourth non-glandular region called anterior fibromuscular stroma (Fine and Reuter, 2012). The transition zone surrounds the urethra proximal to the ejaculatory ducts, central zone projects under the bladder base and surrounds the ejaculatory ducts and the peripheral zone makes up most of the apical, posterior and lateral aspects of the prostate (Verze et al., 2016). Benign prostatic hyperplasia occurs when stromal and epithelial cells of the transition zone surrounding the urethra proliferate (Roehrborn, 2008). This constriction of the urethra causes resistance to the urine flow, also known as bladder outlet obstruction, which can lead to changes in the bladder function, such as detrusor muscle overactivity or underactivity.

1.2. Urinary bladder wall

The urinary bladder wall consists of four distinct layers (inside to outside): urothelium, lamina propria (LP), detrusor (smooth muscle) and the adventitia (Figure 1-3). The urothelium is a specialised transitional epithelium lining the inside of the bladder and is composed of three morphologically distinct cell layers (Jackson et al., 2020). The glycosaminoglycan (GAG) layer lines the luminal surface of the urothelium, and the basal membrane separates urothelium from the underlying connective tissue layer. It is one of the

most effective permeability barriers to protect the body from the toxic substances of the urine, with a high transepithelial electrical resistance and ability to accommodate significant changes in the surface area during bladder filling (Wu et al., 2009). Studies have shown that both afferent and efferent nerves are localised close to the urothelium (Dixon and Gosling, 1983, Jen et al., 1995, Kunze et al., 2006). Furthermore, urothelium is involved in the afferent signalling through which information is conveyed to the central nervous system (Kanai and Andersson, 2010).

The lamina propria lies between the basement membrane of the urothelium and the detrusor muscle. It is mainly composed of connective tissue, but also contains myofibroblasts, muscularis mucosae, lymphatic vessels, blood vessels and nerve fibres (Aitken and Bagli, 2009). Smooth muscle cells forming the muscularis mucosae are structurally different from those found in the detrusor, consisting of relatively small, irregularly arranged cells containing non-specific cholinesterase and glycogen (Dixon and Gosling, 1983).

In most species, the mucosal layer can be easily separated from the underlying detrusor by blunt dissection. *In vitro*, isolated mucosa strips are capable of generating spontaneous phasic contractions in the absence of any stimulation and increases in baseline tension in response to electrical field stimulation (Moro et al., 2012) and muscarinic receptor agonists (Moro et al., 2011). There is increasing evidence that this spontaneous activity developed in the mucosa is propagated by the muscularis mucosae cells (Fry and Vahabi, 2016, Drake et al., 2018, Mitsui et al., 2019) which is thought to occur as a means to prevent the stretching of the microvasculature upon bladder distension (Lee et al., 2016). Therefore, chemical mediators capable of modulating the spontaneous activity that occurs within the mucosa are

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

The most substantial part of the urinary bladder is formed by the detrusor layer, which is made up of smooth muscle bundles separated by connective tissue and interstitial cells (Fry and Vahabi, 2016). These muscle bundles can combine in both circular and longitudinal directions. However, they are not as evident as those found in the gastrointestinal tract or in the urethra where two separate layers can be observed. Finally, a serosa covers the detrusor and protects underlying tissues in the superior and upper lateral parts of the bladder wall, whereas the remainder of the bladder is protected by adventitia merged with other organs of the pelvic floor (Fry and McCloskey, 2019).

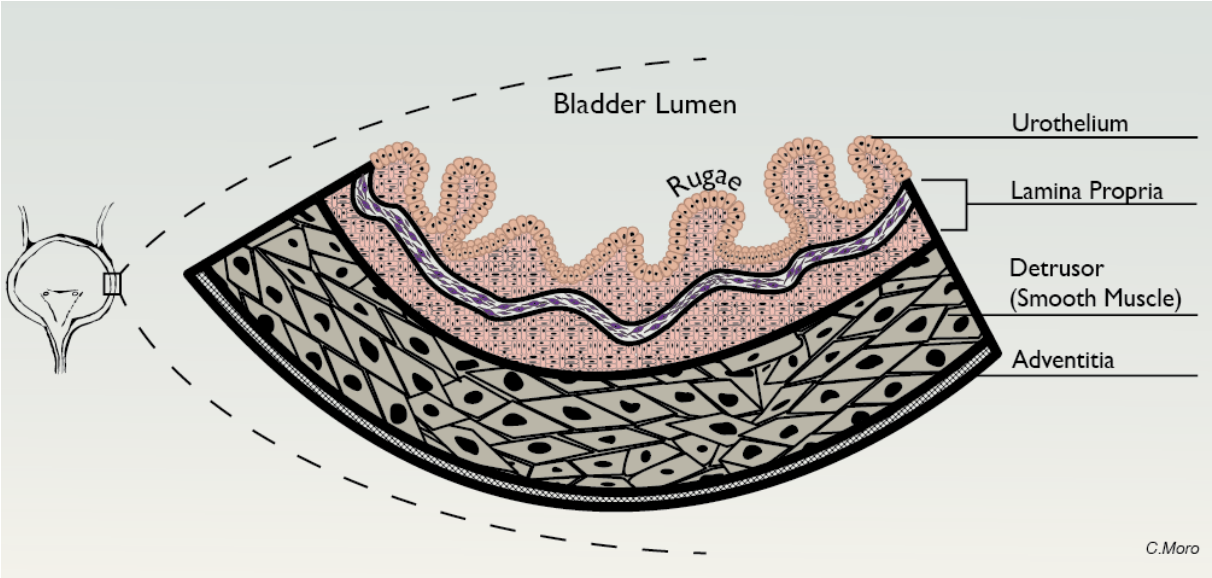

Figure 1-3: Illustration of the urinary bladder wall. It consists of four distinct layers: urothelium, lamina propria, detrusor and adventitia. This Figure was produced by Christian Moro.

1.2.1. Urothelium

The urothelium is a transitional epithelium with a morphology that is considered to be between pseudostratified and stratified epithelia (Martin, 1972) depending upon the volume of urine present in the bladder. In an empty bladder, the urothelial cells appear to be roughly cuboidal, whereas, in a filled bladder, they become stretched and resemble squamous cells (Truschel et al., 2002). During bladder filling, the urothelium appears thinned and retains a three-layer appearance with an increased surface area. When the bladder is empty, the mucosa layer becomes highly folded, and urothelium appears to have 6 – 7 cell layers. In men, the urothelium lines the renal pelvis, ureters, bladder, upper urethra, and the glandular ducts of the prostate. It forms the boundary between the urine and the underlying connective, muscular and nervous tissue (Khandelwal et al., 2009).

The urothelium is composed of three cell layers: the umbrella, intermediate and basal cell layers. The basal cells (5 – 10 μm in cell diameter) form a single layer that is in direct contact with the underlying connective tissue and capillary bed, and functions as a precursor for other cell layers (Apodaca, 2004). These cells exhibit properties similar to stem cells, such as having a slow turnover rate (Martin, 1972). The cells of the intermediate layer (20 μm in cell diameter) are pear-shaped and of different size and height. The cells in this layer are highly proliferative, therefore providing a rapid response to urothelial injury or infection (Osborn and Kurzrock, 2015). The superficial layer of urothelium is composed of umbrella cells, which are large (50 – 150 μm in cell diameter) polyhedral cells that extend over several smaller cells of the underlying layers. They are bound to each other by tight junctions composed of multiple proteins, such as claudins, which prevent urine from seeping into the deeper layers (Koss and Hoda, 2012). Both autonomic efferent and sensory afferent nerves

are located in the suburothelial plexus in close proximity to urothelium and in between urothelial cells (Jen et al., 1995, Birder et al., 2002, Kunze et al., 2006).

Approximately 90% of the apical surface of the umbrella cells is covered by proteins called uroplakins, which are assembled into rigid-looking hexagonal plaques visible only by electron microscopy (Liang et al., 2001, Wu et al., 2009). These proteins function to maintain the integrity and strength of the urothelium, prevent ruptures during the filling phase, protect against toxic substances and contribute to the low permeability of water and solutes (Negrete et al., 1996, Hu et al., 2002).

The GAG layer covers the luminal surface of the urothelium and contributes to the maintenance of the barrier from harmful substances found in urine (Hurst et al., 1987, Parsons et al., 1991, Birder and Andersson, 2013). Damage to the GAG layer can lead to exposure of urine to the underlying epithelial cells (Hurst et al., 1987) and is thought to be involved in the development of bladder disorders such as interstitial cystitis (Morales et al., 1996) and common urinary tract infections (Constantinides et al., 2004).

The urothelium has one of the slowest cycling epithelia in the body, with a turnover rate of approximately 200 days (Hicks, 1975, Wu et al., 2009). This slow turnover rate is essential to fulfil its protective function, as the urothelium needs to continually act as a barrier against toxic urinary substances. However, in response to urothelial injury, these cells can regenerate rapidly in a matter of days (Koss and Lavin, 1970, Romih et al., 2001, Veranic et al., 2009). Cell division occurs in any of the three cell layers, and it is thought that the umbrella cells are formed as a result of intermediate cell fusion (Hicks, 1975).

The urothelium functions to accommodate changes in the urine volume and facilitate substance exchange between urine and the blood supply (Apodaca, 2004). Due to its high electrical resistance, the urothelium is classified as a "tight" epithelium, allowing only active ion transport between urine and blood (Lewis and Diamond, 1975). The urothelium's barrier function is maintained through a layer of umbrella cells lining the lumen of the bladder, with high-resistance tight junctions between the epithelial cells (Acharya et al., 2004). The GAG layer protects the bladder from many potentially harmful substances in the urine (Hurst et al., 1987).

Urothelial cells express a variety of receptors and channels capable of responding to physical and chemical stimuli. They can also secrete a variety of chemicals capable of modulating, activating or inhibiting other cells present within the bladder wall. Channels expressed in urothelial cells include sodium channels (Smith et al., 1998) and various transient receptor potential (TRP) channels (Yu et al., 2011). TRP channels are non-specific cation channels that are generally permeable to calcium (Moran et al., 2011) and have been suggested to be involved as sensors of stretch or chemical irritation in the lower urinary tract (Araki, 2011). Many types of TRP channel have been suggested to be involved in lower urinary tract disorders, such as overactive bladder and interstitial cystitis (Nilius et al., 2007). Du et al. (2008) suggested that TRPA1 receptors are involved in the bladder sensory transduction and may contribute to overactive bladder caused by outlet obstruction.

Some of the receptors expressed in urothelial cells include nicotinic (Beckel et al., 2006), purinergic (Moro and Chess-Williams, 2012, Chess-Williams et al., 2019), adrenergic (Yanase et al., 2008, Moro et al., 2013), cholinergic (Chess-Williams, 2002), bradykinin (Chopra et al., 2005) and protease-activated (Ossovskaya and Bunnett, 2004) receptors.

Chemical mediators that are released from the urothelium in response to chemical or mechanical stress include ATP (Ferguson et al., 1997, Wang et al., 2005), nitric oxide (Birder et al., 1998, Moro et al., 2012), acetylcholine (Moro et al., 2011, McLatchie et al., 2014), adenosine (Yu et al., 2006) and prostaglandins (Jeremy et al., 1987) which can interact with a wide range of receptors localised throughout the bladder wall. The release of these mediators can alter the excitability of the afferent nerves and affect the contractility of the detrusor smooth muscle (Maggi et al., 1987, Birder and de Groat, 2007). Mechanical stress occurs as a result of changes in the bladder pressure, lateral tension, movement of visceral organs and urine composition or tonicity. In contrast, chemical stress that impacts the function of urothelium can be induced by changes in the level of trophic factors or steroid hormones (Birder et al., 2012).

1.2.2. Lamina propria

The lamina propria is a connective tissue layer that lies between the urothelium and detrusor smooth muscle. Urothelium together with the lamina propria forms the mucosa layer of the bladder (Birder and Andersson, 2013) which in this thesis is referred to as the urothelium with lamina propria (U&LP). It is made up of loose fibroelastic connective tissue and contains different cell types, including interstitial cells, myofibroblasts and adipocytes (Wiseman et al., 2003). It also has a rich blood supply, nerve axons with varicosities, lymphatic vessels and smooth muscle fibres termed muscularis mucosae (Dixon and Gosling, 1983, Aitken and Bagli, 2009). The cells forming the muscularis mucosae are morphologically different from those found in the underlying detrusor muscle and is noted to be discontinuous and less defined than those found in the gastrointestinal tract (Dixon and Gosling, 1983). The muscle fibres within the muscularis mucosae are generally thin and

wispy, arranged parallel to the urothelium and are accompanied by a row of large blood vessels (Ro et al., 1987). The lamina propria layer contains both afferent and efferent nerves. The specific distribution of these nerves was described by Gabella and Davis (1998) using whole-mount preparation of rat bladders using immunohistochemistry for calcitonin gene-related peptide. The authors found that afferent nerve axons were distributed in four main regions: at the base and inside the urothelium, on blood vessels (arteries and veins) and along the muscularis mucosae muscle bundles.

Myofibroblasts play an essential role in integrating signals and responses in the bladder wall (Sui et al., 2002), and are important in bladder function and dysfunction. In the literature, myofibroblasts have also been referred to as urinary tract Interstitial cells of Cajal (ICCs) (McCloskey, 2011), ICC-like cells (ICC-LCs) (Hashitani and Lang, 2010), and c-kit+ interstitial cells (Koh et al., 2017). They are widely dispersed within the gastrointestinal tract and stomach where they serve as pacemakers that depolarise smooth muscle cells around them to initiate contractions (Sanders, 1996). They are also known to be involved in the transmission of signals from the nervous system to the smooth muscle cells (Albertí et al., 2007). As the name suggests, myofibroblasts have characteristics of both the smooth muscle cells and fibroblasts. These cells express several membrane receptors, including M3 muscarinic receptors that are involved in smooth muscle contraction (Matsui et al., 2002, Grol et al., 2009) and prostaglandin EP1 and EP2 receptors (Rahnama'i et al., 2010). It has been proposed that myofibroblasts are involved in relaying sensory information to regions of the bladder that are poorly innervated (Nile and Gillespie, 2012). They also play an essential role in the inflammatory response, as they can secrete pro-inflammatory mediators such as cytokines, chemokines and growth factors (Powell et al., 1999). Therefore, these

cells may be involved in bladder disorders that are associated with inflammation, such as interstitial cystitis.

Additionally, these cells contain smooth muscle myosin isoforms and alpha-smooth muscle actin that are required for a contraction to occur (Powell et al., 1999). In patients with overactive bladder and detrusor overactivity, the number of myofibroblasts is enhanced and the cells are more excitable, which results in an overall enhancement of bladder excitability (Juszczak et al., 2013). However, there is an ongoing discussion about the presence of true myofibroblasts in the urinary bladder. Recent studies involving 3D-electron microscopy have suggested that there are two types of interstitial cells within the lamina propria: fibroblast-type IC and myoid-type IC (Neuhaus et al., 2018). The myoid ICs are thought to resemble myofibroblasts as both of these cell types contain fibronexus, whereas fibroblast type ICs have more pronounced cell bodies and cellular protrusions. Fibronexus is a cell-to-matrix junction, which consists of a myofilament bundle and fibronectin fibril. It functions to connect cell to matrix through a point found on the cell surface (Eyden, 2008) and is also a characteristic marker of myofibroblasts (Eyden, 2001). The presence of contractile units within these cells make them a potential target in the mediation of bladder contractility and a potential pharmaceutical target for future therapies.

The presence of interstitial cells in the lamina propria region is often established through the use of *c-kit* markers, which detect the expression of tyrosine kinase receptors on the surface of myofibroblasts in the urinary bladder (McCloskey and Gurney, 2002, Davidson and McCloskey, 2005) or through immunohistochemical staining (Sadananda et al., 2008). Although *c-kit* has been used to identify ICC-like cells, it must be noted that the mast cells found in the lamina propria and detrusor layers (Christmas and Rode, 1991) also express *c-*

kit (Koh et al., 2017). As such, Gevaert et al. (2017) suggested that many prior studies had incorrectly classified myofibroblasts cells in this region when the cells in question were, in fact, mast cells that were identified through additional staining with mast cell tryptase. These findings further depict that mast cells are of particular interest in bladder physiology and pathophysiology due to their widespread localisation throughout the bladder layers. There is also uncertainty whether the urinary bladder actually contains *c-kit* positive ICC-like cells. While their presence in the lamina propria has been documented by Gevaert et al. (2017) further research by the same group did not subsequently find these cells (Neuhaus et al., 2018). Instead, the authors found a unique type of cell termed branched IC, which shares some ultrastructural features with ICCs. Other identification methods for myofibroblasts include antibodies to vimentin and more recently antibodies against platelet-derived growth factor receptor-α together with confocal microscopy (Koh et al., 2012, Monaghan et al., 2012).

1.2.3. Detrusor smooth muscle

The detrusor layer is made of smooth muscle fibres that are organised in three distinct layers: two outer layers in which the cells are oriented longitudinally and one layer in the middle, where cells are orientated circularly (Andersson and Arner, 2004, Mangera et al., 2013). Human detrusor cells are arranged in muscle bundles which are large and composed of several smaller sub-bundles (Brading, 1987) that run in all directions. The cells present in the detrusor are typical smooth muscle cells that are long and spindle-shaped with a central nucleus (DeLancey et al., 2002). Detrusor smooth muscle cells express stretch-activated, non-selective cation channels that are activated when they elongate as the bladder fills up and may constitute a pacemaking mechanism (Wellner and Isenberg, 1993). The activation

of these channels initiates inward depolarisation of smooth muscle cells and enhances the opening of voltage-dependant calcium channels that results in generation of action potentials and subsequent contractions.

Detrusor cells have intricate mechanisms of relaxation and contraction in order to fulfil the function of storing urine for extended periods and rapid expulsion during micturition. During the filling phase, the detrusor cells relax and elongate in order to accommodate a large volume of urine by becoming flattened. During the micturition phase, enough force has to be generated in order to synchronously contract the muscle cells of the whole bladder (Andersson and Arner, 2004). Contractions observed in porcine detrusor smooth muscle strips have shown to initially consist of phasic contractions, which are then followed by much smaller tonic contractions (Uchida et al., 1994) thought to be in place to ensure bladder emptying (DeLancey et al., 2002). Detrusor cells are also capable of developing spontaneous phasic contractions in the absence of stimulation, as shown in strips of human, porcine and rabbit detrusor (Sibley, 1984, Moro et al., 2011). These contractions have variable amplitudes and do not represent the maximal force they can generate.

The presence of interstitial cells has also been confirmed in the detrusor layer using ultrastructural studies (McCloskey and Gurney, 2002) and through immunohistochemical labelling (Davidson and McCloskey, 2005). This is achieved by using antibodies to the kit receptor encoded by the proto-oncogene *c-kit*, which is extensively used for identifying ICC cells in the gastrointestinal tract. Cells with myofibroblast characteristics were found along the edges and within the muscle bundles located in the detrusor layer (Rasmussen et al., 2009). They are thought to contribute to the stabilisation of detrusor membrane potential (Koh et al., 2017). Upregulation of these cells has been suggested to be involved in several

bladder conditions, including overactive bladder (McCloskey, 2010). An *in silico* model of detrusor smooth muscles cells and myofibroblasts coupled through gap junctions was created in order to test this theory (Rosenberg et al., 2016). This study found that the myofibroblasts of the bladder significantly contributed to the contractility of the detrusor muscle. Therefore, when researching bladder contractile disorders, one should consider not only the involvement of smooth muscle cells but also the role of myofibroblasts in the development of bladder dysfunction.

1.2.4. Extracellular matrix

1.2.4.1. Collagen fibres

The bladder extracellular matrix (ECM) plays an essential function in the bladder, as it not only provides structural support but also generates cell signalling responses and cytoskeletal tension. The ECM continuously undergoes remodelling to which cells attach and respond, leading to cell adhesion, production of matrix components, growth, migration or differentiation (Aitken and Bagli, 2009). Collagen I and III form the bulk of matrix proteins found in the bladder (Macarak and Howard, 1997). In healthy bladders, the composition of collagen is approximately 25% type III collagen and 75% type I collagen. These proteins are mainly localised in the lamina propria and in the endomysium that surrounds bladder smooth muscle cells, providing structure, tensile strength and compliance (Ewalt et al., 1992).

The exact arrangement of collagen fibres was described in the study by Murakumo et al. (1995), where nine human urinary bladders were examined using scanning electron microscopy with chemical digestion methods. According to the arrangement of collagen, the

mucosal layer is divided into three portions: superficial, middle and deep. The superficial layer consists of a dense collagen layer where individual collagen fibres are not organised in bundles, but instead run in various directions. The middle layer is much thicker and composed of flat, tape-like bundles of parallel collagen fibres that are loosely interwoven with each other in two-dimensional directions. The deepest portion is found just beneath the muscularis mucosae, consisting of a loose network of collagen bundles that appear to be twisted in strands. In the detrusor layer, the smooth muscle fascicles are covered with collagen sheets, with each muscle cell surrounded by a thin sheath of collagen fibres. Finally, the serosal layer that covers the outside of the bladder consists of wavy collagen bundles in a sheet interwoven by clusters of adipose cells.

Deposition and arrangement of these fibres are of particular importance, as it is believed that volume accommodation as the bladder fills up with urine is achieved through changes in the arrangement of collagen type III fibres (Chang et al., 1998). Furthermore, correct deposition of collagen type I fibres is essential in bladder function, as a study involving women with stress incontinence determined that these patients had a significant reduction in collagen type I fibres (Liapis et al., 2000).

1.2.4.2. Elastic fibres

Elastin fibres are sparser when compared to collagen fibres. Nevertheless, they can be found in all layers of the bladder. These fibres are long-lasting and only have a 1% turnover rate per year. Their primary function is to allow the bladder to recoil to its original shape after micturition, and deficiencies in the production of elastic fibres can affect the overall bladder function (Aitken and Bagli, 2009). The exact arrangement of elastic fibres was determined

by Murakumo et al. (1995) using a scanning electron microscopy approach. They were mostly found to form a loose network on the surface of the detrusor smooth muscle fascicles and in the border between smooth muscle and the serosal layer. Elastic fibres appeared to be sparse in the mucosal layer, mainly present around large blood vessels and muscularis mucosae forming loose networks of interwoven strands. Further immunohistochemical analysis using anti-elastin staining revealed that elastin in the mucosal layer appears to be arranged parallel to the urothelial lining (Rosenbloom et al., 1995).

1.2.5 Vasculature

The arterial supply of the urinary bladder was first described by Braithwaite (1952) and Shehata (1976) based on a careful dissection of bladders obtained from 35 males with injected blood vessels. The main arterial blood supply to the detrusor comes from the internal iliac artery on each side of the bladder. The blood enters the detrusor through between one and four superior vesical arteries, a single inferior vesical artery and a vesiculo-deferential artery. The base of the bladder and the urethra receives separate blood supply from branches of the inferior vesical arteries. Furthermore, the bladder is also variably supplied by small branches of other arteries arising from the internal iliac arteries. The microanatomy of blood vessels was studied in male and female bladders obtained from post-mortem examinations that were deemed to be disease-free (Sarma, 1981). The author used radiographs of thin slices of the bladder wall after injecting the vessels with radiopaque contrast and described three plexuses of blood vessels that are interconnected across the bladder wall: an intramural, and extramural and a suburothelial plexus. The winding arrangement of the blood vessels prevents them from being stretched longitudinally during bladder filling, thereby maintaining their diameter (Sarma, 1981). Bladder blood flow differs based on the

micturition cycle; during the filling phase, bladder perfusion can increase, whereas as it continually fills with urine, compression of the blood vessels within the bladder wall can occur. Overall, the vascular architecture of the bladder can be effectively adapted based on the changes associated with the phasic contractions and changes in the inner and outer surface area (Miodoński and Litwin, 1999). As the barrier function of the urothelium and the contractile function of detrusor smooth muscle are primarily dependent on the adequate blood supply of oxygen and nutrients, blood vessels in the bladder wall must be highly adaptive to the spatial changes resulting from the micturition cycle without impacting the blood flow during filling (Andersson et al., 2017).

The arrangement of the suburothelial blood capillary network has been studied using scanning electron microscopy in the rat (Inoue and Gabella, 1991) and human (Congiu et al., 2004) bladders. In rat bladders, the capillaries were located within the grooves of the basal surface of the urothelium and surrounded by urothelial cells, whereas in human bladders a well-developed capillary plexus was present just below the basal lamina. Using an antibody against von Willebrand factor, Brading et al. (1999) selectively stained endothelial cells of pig and human blood vessels in order to study the microanatomy. The authors noted that the density of blood vessels was higher in the lamina propria than in the detrusor. Furthermore, both arteries and veins were found in the connective tissue between muscle bundles and capillaries between the smooth muscle fascicles. This distribution of blood vessels was also found in other studies involving human (Miodoński and Litwin, 1999) and rabbit bladders (Hossler and Monson, 1995).

Bladder blood flow is influenced by several factors, namely muscle contractions, compression of urine content and stretch during distension. Venules, which are located in

the lamina propria, exhibit spontaneous phasic contractions that may be involved in maintaining venular drainage and blood flow to the surrounding cells (Hashitani et al., 2011). Further calcium imaging and electron microscopy studies revealed that the venular wall of the mouse bladder consists of a pericyte network thought to be contractile and capable of regulating capillary blood flow (Hashitani et al., 2012).

1.3. Innervation of the lower urinary tract

The voluntary control of the lower urinary tract involves interactions between the central nervous system (supraspinal and spinal) and the peripheral nervous system (autonomic and somatic) divisions. Experimental studies involving electrical stimulation (Holstege et al., 1986, Mallory et al., 1991) and brain lesioning techniques (Barrington, 1925) in cats has revealed that the pontine micturition centre (also known as Barrington's nucleus) is the primary region involved in normal micturition reflexes, exerting its function on the activity of detrusor smooth muscle and urethral sphincter muscles. The pontine micturition centre receives input from several centres of the brain, including the basal ganglia, periaqueductal gray, thalamus and hypothalamus (Fowler et al., 2008).

The lower urinary tract is innervated by three nerves arising from the spinal cord, involving the autonomic nervous system (mediated by sympathetic and parasympathetic nerves) and the somatic nervous system (mediated by pudendal nerves). Parasympathetic nerves originate in S2-S4 sacral region of the spinal cord and function to contract bladder smooth muscle and relax the urethra. Sympathetic nerves originate in the T11-L2 segment of the spinal cord and act to relax the bladder smooth muscle and contract the bladder base and

urethra. There is also an input from the pudendal nerves, which work to contract the external urethral sphincter at the base of the urinary bladder.

1.3.1. Efferent innervation

The urinary bladder detrusor smooth muscle is primarily innervated by parasympathetic nerves, whereas sympathetic nerves innervate the bladder neck, urethra and the internal sphincter surrounding the urethra. The skeletal muscle forming the external urethral sphincter receives an input from somatic nerves.

1.3.1.1. Parasympathetic nerves

The parasympathetic nerve pathway provides the excitatory innervation of the bladder detrusor smooth muscle (Yoshimura and de Groat, 1997). Parasympathetic nerves not only stimulate bladder smooth muscle but also exert their function on urethral smooth muscle, causing relaxation via the release of nitric oxide (Bennett et al., 1995). Preganglionic parasympathetic axons originate at the sacral region of the spinal cord (S2-S4) and synapse in the pelvic ganglia as well as in the small ganglia of the bladder wall, resulting in the release of acetylcholine. At the ganglion, acetylcholine exerts its function on nicotinic acetylcholine receptors located on the post-ganglionic neurons. After the activation of these neurons, the signal travels a short distance down post-ganglionic axons, terminating in the detrusor layer and releasing acetylcholine that binds to muscarinic acetylcholine receptors on detrusor cells. The stimulation of these receptors results in the contraction of the bladder (Yoshimura and de Groat, 1997), with the M3 subtype being the primary receptor involved in the stimulation of detrusor contractions (Chapple et al., 2002). In addition to acetylcholine,

post-ganglionic nerves are capable of releasing non-adrenergic, non-cholinergic neurotransmitters (Moro et al., 2012).

The release of ATP as a neurotransmitter responsible for non-adrenergic, non-cholinergic neurotransmission was first established in 1972 by Burnstock (1972) and termed purinergic signalling. Purinergic stimulation of the detrusor is considered to be a minor contributor to the normal function of the bladder (Yoshimura and de Groat, 1997). The presence of purinergic receptor P2X has been demonstrated in both diseased and healthy human bladders using the real-time quantitative reverse transcription-polymerase chain reaction method, which detects and analyses RNA (O'Reilly et al., 2001). Seven P2X genes in the total RNA were determined, with the P2X1 subtype being the most predominant purinoreceptor present in the human bladder. Furthermore, the authors found that the density of P2X1 receptors was significantly greater in diseased bladders when compared to controls, indicating upregulation of purinergic receptors during pathological conditions.

In addition to neuronal ATP release, results from both animal studies involving pigs (Cheng et al., 2011), rabbits (Ferguson et al., 1997), rats (Munoz et al., 2010) and human studies (Kumar et al., 2004) show that ATP can also be released from the bladder urothelium in response to stretch. In addition to ATP released from urothelial cells, Cheng et al. (2011) suggested that myofibroblasts present in the lamina propria may also be capable of releasing ATP in response to stretch.

1.3.1.2. Sympathetic nerves

Sympathetic postganglionic nerves (hypogastric nerve) release noradrenaline, which exerts its function on β-adrenergic receptors responsible for detrusor smooth muscle relaxation and on α-adrenergic receptors in the urethra and the bladder neck to cause relaxation (Moro et al., 2013). Preganglionic sympathetic neurons originate in the intermediolateral column of the thoracolumbar cord segment (T10 – L2) (Yoshimura and de Groat, 1997). The majority of the pre-ganglionic fibres synapse in the mesenteric ganglia where acetylcholine is released to act on the nicotinic receptors located on the post-ganglionic neurons.. From there, post-ganglionic neurons travel via the hypogastric nerve to release noradrenaline (NA) at the terminals found in the urethra, bladder neck and bladder body. Specifically, NA stimulates the contractions of urethra and bladder neck via α1-adrenoceptors and relaxation of detrusor via β_2-adrenoceptors and β_3-adrenoceptors.

1.3.1.3. Somatic nerves

Somatic nerves provide excitatory innervation to skeletal muscle in the external urethral sphincter and the pelvic floor musculature. These somatic neurons that originate in the Onuf's nucleus of the anterior horn of the S2 to S4 spinal cord release acetylcholine that acts on nicotinic receptors to induce muscle contraction (Yoshimura and de Groat, 1997) and maintain the closure of the external urethral sphincter.

1.3.2. Afferent innervation

The pelvic, hypogastric and pudendal nerves carry sensory information from the urinary bladder back to the spinal cord via afferent fibres (Yoshimura and de Groat, 1997) which

gives rise to local sensations. These nerves provide sensations about bladder fullness to the spinal cord, which subsequently activates areas in the brain responsible for micturition (Kanai and Andersson, 2010). In human bladders, the cell bodies of pelvic and pudendal nerves are located in the S2-S4 dorsal root ganglion, whereas the cell bodies of the hypogastric nerve are located at the T11-L2 levels (Kanai and Andersson, 2010).

There are two different types of afferent nerve fibres in the urinary tract: small myelinated Aδ fibres and unmyelinated C fibres (de Groat et al., 2011). Aδ fibres are mainly located in the detrusor smooth muscle layer, where they respond to stretching of detrusor as it fills with urine, conveying the sense of fullness via mechano-transduction to the central nervous system (Kanai and Andersson, 2010). Unmyelinated C fibres are found in the detrusor layer close to urothelium and lamina propria, as well as directly adjacent to urothelial cells. These fibres are minimally active, with a conduction velocity of less than 2.5 m/s (Vera and Nadelhaft, 1990). However, they can become activated by harmful chemicals, mechanical stimuli and inflammation of the urinary bladder (Habler et al., 1990).

The distribution of afferent axons in the bladder has been identified through immunohistochemistry for calcitonin-gene-related-peptide in frozen sections and preparations of mucosa and detrusor layers in rats (Gabella and Davis, 1998). Afferent axons are distributed at the base of and inside the urothelium, on arteries and veins, and along the smooth muscle bundles. In the urothelium, afferent nerves are either inside the layer or in the subepithelial plexus, close to the basal surface of the urothelium. Nerve fibres in the detrusor layer run parallel to the muscles, lamina propria and urothelium. The pelvic nerve conveys information to the central nervous system about the bladder volume during the storage phase and the amplitude of bladder contractions during urination. Thereby, these

sensory nerves are involved in initiating the micturition reflex as well as reinforcing bladder contractions in order to empty the bladder. Animal studies have demonstrated the presence of bladder mechanoreceptors that respond to changes in bladder pressure and volume (Habler et al., 1990). However, it is unclear which mechanisms underly the mechanosensory activation of pelvic and hypogastric nerves. There are two potential mechanosensory transduction pathways involved in activating bladder afferent nerves in response to filling. The first is the direct mechanism, which relies on mechanically-gated ion channels expressed on afferent nerve terminals. Possible receptor candidates include ENaC/ASIC/degenerin Na^+ channels and transient receptor potential (TRP) cation channels. The second proposed mechanism involves indirect mechanosensory transduction that relies on the interaction of chemical mediators (e.g. ATP) released by non-neuronal cells (e.g. cells of urothelium and detrusor smooth muscle) (Sun et al., 2010).

1.4. Urinary bladder function

The bladder has two primary functions: storage and the periodic elimination of urine. In order to fulfil these functions there is a complex involvement of the nervous system, smooth muscle fibres, skeletal muscle fibres, the urethra and urethral sphincter. These structures are regulated by three peripheral nerves: sacral parasympathetic (pelvic nerves); thoracolumbar sympathetic (hypogastric nerves) and somatic nerves (pudendal nerves) (de Groat, 1986). The storage reflexes are activated during the filling phase of the bladder and are organised mainly in the spinal cord, whereas micturition or bladder voiding reflex mechanisms are organised in the brain (Fowler et al., 2008). Stimulation from parasympathetic nerves contract the bladder and relax the urethra, sympathetic nerves relax the bladder and contract

the urethra and somatic nerves contract the external urethral sphincter. A good understanding of the mechanisms involved in the storage and micturition mechanisms is necessary to target the underlying cause of bladder dysfunction, particularly those related to contractile disorders.

1.4.1. Storage

The primary function of the urinary bladder is to store urine for short periods of time while maintaining the composition of urine that is similar to that delivered by the kidneys (Lewis, 2000). During the storage phase, the outlet (bladder neck and urethra) is closed and the detrusor smooth muscle is relaxed, allowing intravesical pressure to remain low to accommodate increasing bladder volumes via sympathetic nerve stimulation (Coolsaet, 1985, Fowler, 2006). Until the volume of urine reaches a critical threshold for voiding, the bladder has a low and relatively constant level of internal pressure (Yoshimura and de Groat, 1997). The average bladder capacity in healthy adults is between 300 and 600 mL (Fitzgerald et al., 2002, Latini et al., 2004, Pauwels et al., 2004). The release of noradrenaline from hypogastric sympathetic nerves inhibit contractile responses via α- and β-adrenergic receptors (Åmark et al., 1986). The activation of β-receptors in the detrusor smooth muscle relaxes the bladder, whereas activation of α-receptors on urethra leads to contraction of the smooth muscle, thereby preventing the leakage of urine. The gradual filling of urine and increase in intravesical pressure activates afferent sensory nerves that convey the sense of fullness to the central nervous system (de Groat and Yoshimura, 2009). Abnormal signal transduction has been suggested as a contributor to bladder dysfunction (Araki et al., 2008). However, the exact mechanisms involved are still not fully understood.

1.4.2. Micturition

Bladder voiding, or micturition, involves contraction of the bladder smooth muscle and relaxation of the urethra and urethral sphincters, which function to prevent urine leakage during the filling phase (de Groat and Yoshimura, 2001). In infants, micturition is initiated when the bladder volume reaches a critical condition. In adults, the individual has sensory awareness of a full bladder, and a guarding reflex that prevents involuntary urination is in place until voluntary elimination is possible (Chancellor and Yoshimura, 2004).

The mechanisms involved in micturition are driven by the spinal autonomic nervous system, which involves parasympathetic activation followed by the subsequent contraction of the detrusor smooth muscle and inhibition of sympathetic action on the internal smooth muscle sphincter (de Groat and Yoshimura, 2001). Molecular studies have demonstrated the presence of all five muscarinic receptor subtypes in the bladder areas that are involved in micturition, wherein M2 and M3 receptors are the most predominant (Sigala et al., 2002). It is generally accepted that M3 receptors are responsible for the contraction of the detrusor smooth muscle (Chapple, 2000, Chess-Williams, 2002, Fetscher et al., 2002). All muscarinic receptor subtypes couple to G proteins, but their mechanisms of signal transduction differ. M1, M3 and M5 receptors couple to $G_{q/11,}$ causing a contraction through phosphoinositol hydrolysis, which leads to intracellular calcium mobilisation that is used to drive the contraction. M2 and M4 receptors couple to $G_{i/o}$, leading to inhibition of adenylyl cyclase, which then lowers cAMP levels thereby inhibiting β-adrenergic receptor-mediated bladder relaxation (Hegde et al., 1997). In addition to cholinergic activation, purinergic signalling contributes to the contractile response in the bladder through the binding of ATP to the P2X receptor in the detrusor smooth muscle (Burnstock, 1972). These two neurotransmitters act

together to initiate and maintain contraction. ATP induces a fast contraction and is thought to be responsible for the initiation of micturition (Chancellor et al., 1992).

In contrast, acetylcholine stimulates receptors to induce a slower, more prolonged contraction to maintain the voiding of the bladder and urine flow (Theobald, 1995). Release of nitric oxide from parasympathetic nerves leads to relaxation of the urethral smooth muscle (Andersson and Arner, 2004) that prevents involuntary leaking of urine during the storage phase. Other factors such as the stretching of the muscle cells during filling can activate the micturition reflex. The stretching of the cells activates non-selective cation channels which initiates inward current, depolarising the smooth muscle cells and opening voltage-dependent calcium channels (Wellner and Isenberg, 1993). The opening of these channels trigger calcium influx from the outside of the cell, leading to the release of calcium from intracellular stores which causes a contraction of the smooth muscle cells. The mechanisms that are involved in the premature contraction of the smooth muscle cells are not fully understood and require further investigation.

1.5. Effects of ageing on urinary bladder function

1.5.1. Structural changes

An anatomical study of aged human bladders from subjects with detrusor overactivity has revealed an atypical pattern that was characterized by widened spaces between cells, reduction in intermediate cell junctions and increases in protrusion junctions (Elbadawi et al., 1993). Additionally, there have been numerous studies that evaluated age-related

changes to bladder function using animal models. In the rat, increases in the urine output and frequency of micturition were observed with the advancing of age (Chun et al., 1988, Chun et al., 1989). The authors also found that intravesical pressure during micturition was higher in older animals. However, these increases in intravesical pressure were not observed in a later study by Hotta et al. (1995), demonstrating some uncertainties in this area.

Histological analysis of the human bladder lamina propria performed by Levy and Wight (1990) revealed several ways in which normal bladder histology changes as an individual ages. Firstly, collagen fibres that typically form intricate networks throughout the lamina propria separate into individual fibres that no longer form fascicles as ageing progresses. Secondly, smooth muscle cells of the muscularis mucosae layer are dispersed throughout the whole lamina propria layer during adolescence, yet are rarely seen after the age of sixty (Levy and Wight, 1990). Age-associated structural changes have also been reported in a mice model (Schueth et al., 2016). In the mucosal layer, detachment, degeneration and loss of cytoplasm of urothelial cells were observed, suggesting loss of urothelial barrier function and an increased permeability to harmful matter. In the detrusor layer, the authors noted intermingling of connective and muscle tissue, whereas in younger mice models these layers were separated (Schueth et al., 2016). Other studies have also established a strong association between ageing and a relative increase in detrusor fibrosis (Lepor et al., 1992, Al-Motabagani, 2005), deposition and crosslinking of collagen with elastin (Hald and Horn, 1998) and the presence of an unusual spontaneous bladder activity during filling (Lluel et al., 2000).

1.5.2. Changes in neurotransmission

Age-related changes in the bladder structure, voiding patterns and neurotransmitter release have been studied to some extent, but they often provide conflicting evidence. Specifically, contractile responses to endogenous chemicals, noradrenaline, ATP and 5-HT increase with age (Saito et al., 1993). Relaxation responses to a beta-adrenoceptor agonist, isoproterenol, were significantly inhibited in comparison to younger age groups, although the authors found no significant age-related differences in the contractile responses to acetylcholine, prostaglandin $F_{2\alpha}$, angiotensin II, vasoactive intestinal polypeptide or potassium chloride. Additional functional studies involving human tissue also revealed that there is a non-neuronal release of acetylcholine from both the human U&LP and detrusor, which increases during ageing (Yoshida et al., 2004) and is capable of impacting the overall contractility of the bladder.

Furthermore, in a mouse model, Daly et al. (2014) found that ageing was associated with increases in voiding frequency, the release of ATP, frequency of spontaneous detrusor contractions, contractile responses of detrusor to muscarinic and purinergic agonists and afferent nerve activity, while urothelial acetylcholine release was reduced. Based on these contractile changes in response to different endogenous chemicals and pharmacological agents, it is likely that other receptor systems that are capable of inducing contractile changes in the bladder may also be affected by age and, therefore, an important avenue to explore further.

1.6. Urinary bladder dysfunction

1.6.1. Overactive bladder

Overactive bladder (OAB) is a lower urinary tract disorder associated with a set of chronic symptoms that significantly impacts the lives of those affected. It is highly prevalent in both men and women of all ages (Wein and Rovner, 1999), with a marked increase after 40 years of age (Stewart et al., 2003). Various epidemiological studies have estimated that between 12% and 17% of people worldwide suffer from this disorder (Nitti, 2002, Stewart et al., 2003, Irwin et al., 2006, Lawrence et al., 2008). There is an overlap between the definitions of overactive bladder and detrusor overactivity in the literature. Detrusor overactivity is defined as a urodynamic observation that is characterised by involuntary contractions of the detrusor smooth muscle during the filling phase of the bladder that can be spontaneous or provoked (Abrams, 2003). OAB is considered to be a symptom complex defined as urinary urgency with or without urge incontinence, which is often accompanied by frequency and nocturia in the absence of any infection or other pathological condition (Abrams et al., 2002). The principal symptom associated with this bladder dysfunction is urinary urgency, which is defined as a sudden and irresistible desire to void that is difficult to defer. Detrusor overactivity is generally regarded within the underlying pathophysiology of OAB rather than a disorder in itself (Al-Ghazo et al., 2011).

OAB significantly impacts the quality of life of those affected, as it has a direct physical and psychological effect (Basra and Kelleher, 2007) and carries a significant socioeconomic burden (Sacco et al., 2010). This condition interferes with many daily activities and social

functioning. People with OAB are less likely to attend social events, engage in physical activities due to the fear of having an accident (Sexton et al., 2011), which has direct implications on their mental health as it can make them feel isolated, lonely and depressed. Additionally, it influences their job performance due to a large number of bathroom trips and frequent hospital visits (Willis-Gray et al., 2016). However, a large proportion of the sufferers have not been formally diagnosed with OAB or have been underdiagnosed. As such, these individuals currently do not receive any treatment to alleviate the symptoms associated with OAB.

Despite numerous studies that have tried to unravel the mechanisms involved in the pathogenesis of OAB, the cause and subsequent development of this disorder is poorly understood. Some of the proposed mechanisms involved in this disorder include increased afferent signal activity, decreased capacity of the central nervous system to process afferent signals and increased sensitivity of contraction-mediating transmitters in the bladder (Andersson, 2004). Furthermore, the pathology of OAB is thought to involve myogenic, neurogenic and urotheliogenic factors. The myogenic hypothesis (Brading, 1997) suggests that partial denervation of the detrusor tissue causes an alteration of smooth muscle activity, leading to involuntary contraction and an increase in the intravesical pressure observed in OAB. Additionally, the partial denervation can also cause enhanced sensitivity to neurotransmitters, which subsequently increases the response to stimulation (Sibley, 1997). The neurogenic hypothesis suggests that changes to the central nervous system causes imbalance which leads to increased bladder excitation, reduced inhibition and increased afferent output (Foon and Drake, 2010). Therefore, damage to the central inhibitory pathway or sensitisation of the afferent terminals in the bladder wall can unveil the voiding reflexes that trigger detrusor overactivity (de Groat, 1997).

1.6.2. Interstitial cystitis/Bladder Pain Syndrome

Interstitial cystitis/bladder pain syndrome (IC/BPS) is a syndrome characterised by urinary bladder pain and irritative symptoms that persist for more than six months (Grover et al., 2011). This syndrome has also previously been referred to as interstitial cystitis/painful bladder syndrome and painful bladder syndrome/interstitial cystitis. To avoid confusion, the European Society for the study of Interstitial Cystitis proposed a new nomenclature and classification system (van de Merwe et al., 2008). The authors agreed to name the disease bladder pain syndrome and diagnose it based on chronic pelvic pain, pressure or discomfort perceived to be related to the urinary bladder and accompanied by at least one other urinary symptom (e.g. persistent urge to void or frequency). The prevalence of this syndrome is relatively low (Curhan et al., 1999). However, the true prevalence might be underestimated as a result of varying criteria used when establishing a diagnosis.

The most typical symptom of this syndrome is pelvic pain that occurs when a small amount of urine is present within the bladder (Erickson and Davies, 1998), which implies changes in the afferent nervous system and processing of bladder sensations (Ottem and Teichman, 2005). Indeed, there is a significantly enhanced number of nerve fibres observed in the lamina propria and detrusor muscle layers in patients with IC than in healthy subjects (Christmas et al., 1990). The pain associated with IC/BPS can be relieved by emptying of the bladder; however, the symptoms return as the bladder starts filling up with urine. On average, healthy individuals empty their bladders 6.5 times a day, whereas patients with IC average 16.5 times (Parsons and Koprowski, 1991) and even up to 40 times in 24 hours (Erickson and Davies, 1998). Furthermore, the consistent urge to void is always present and remains even after emptying of the bladder. In healthy individuals, the mean bladder capacity

is estimated to be 586 mL, with the first filling sensation occurring at 163 mL and first voiding desire at a mean volume of 315 mL (Pauwels et al., 2004). In patients with IC, the mean bladder capacity is estimated at 265 mL and the first voiding desire at only 74 mL (Steinkohl and Leach, 1989).

IC/BPS patients can be categorised into two general subtypes based upon cystoscopic findings: IC/BPS with and without Hunner's lesions (Peters et al., 2011). Recently, it has been proposed that IC/BPS with Hunner's lesions is a clinically distinct disease entity with different aetiology (Akiyama and Hanno, 2019, Whitmore et al., 2019, Akiyama et al., 2020). Hunner's lesions occur in the mucosa and are accompanied by abnormal capillary structures. Patients with these lesions tend to be older (Braunstein et al., 2008), have lower bladder capacity and increased urinary frequency (Koziol et al., 1996). IC/BPS patients without Hunner's lesions frequently exhibit non-bladder symptoms, including other common systemic pain problems, psychosocial problems and emotional dysregulation (Warren et al., 2009). Furthermore, fewer histological changes are observed in these patients (Logadottir et al., 2014, Maeda et al., 2015). These findings suggest that IC/BPS with Hunner's lesions is a true chronic inflammatory disorder of the urinary bladder, whereas IC/BPS without Hunner's lesions is a non-inflammatory disorder with no apparent cause (Akiyama et al., 2020).

The aetiology of IC/BPS remains unknown. Several pathophysiological mechanisms underlying this syndrome have been suggested: urothelial dysfunction (Parsons et al., 1991), mast cell activation, neurogenic inflammation, autoimmunity and occult infection (Chancellor and Yoshimura, 2004). However, none of these aetiologies has been proven. Urothelial impairment is considered to be the primary cause of IC/BPS (Parsons et al., 1991).

The impairment of the barrier function can lead to the migration of urinary solutes, particularly potassium, which can depolarise nerves and muscles, thereby causing tissue injury (Teichman and Moldwin, 2007, Parsons, 2011). In patients with IC/BPS with Hunner's lesions, the urothelium is almost completely denuded at lesion sites (Akiyama et al., 2019). Mast cell infiltration in IC/BPS has been widely documented in the literature (Larsen et al., 1982, Kastrup et al., 1983, Aldenborg et al., 1986, Feltis et al., 1987, Lynes et al., 1987, Johansson and Fall, 1990, Theoharides et al., 1990, Christmas and Rode, 1991, Theoharides et al., 1995, Peeker et al., 2000, Yamada et al., 2000, Logadottir et al., 2014, Malik et al., 2018). However, more recent research (Akiyama et al., 2018) provides conflicting evidence and suggests that mast cell infiltration is not a histological feature of IC/BPS. Nonetheless, the presence of inflammation in bladders of IC/BPS patients and its association with various immune cells of the body still highlights the importance of determining the effects of pro-inflammatory mediators on urinary bladder contractility.

It has also been proposed that IC/BPS could be an allergic or autoimmune disorder due to the high prevalence of these comorbidities (Peeker et al., 2003). A case report of a 28-year-old female reported improvement in urinary symptoms by treating the patient with a combination of specific immunotherapy (SIT) and anti-immunoglobulin E antibody (anti-IgE) therapy (Lee et al., 2006). Anti-IgE therapy eliminates circulating IgE and also prevents attachment onto mast cells and basophils, thereby preventing allergen-induced activation of these immune cells and subsequent release of inflammatory mediators (MacGlashan et al., 1997, Beck et al., 2004). Furthermore, IC is especially prevalent in populations that already have an allergic disorder. The incidence of this has been reported by Yamada (2003), where 80% of the thirty-four young patients investigated had an allergic disorder in addition to IC/BPS.

1.7. Inflammation of the urinary bladder

Inflammation is one of the immune system's defence mechanisms against injury, pathogens, damaged cells or toxic compounds (Medzhitov, 2010). It can be characterised by five symptoms: redness, swelling, heat, pain and loss of tissue function (Takeuchi and Akira, 2010). The symptoms reflect the occurrence of increased vascular permeability, leukocyte recruitment and the release of inflammatory mediators. While this immune defence is essential to respond to injury or pathogens effectively, uncontrolled acute inflammation can become chronic (Isailovic et al., 2015), thereby contributing to a variety of inflammatory diseases (Zhou et al., 2016).

Inflammation plays a central role in the pathogenesis of IC/BPS (Grover et al., 2011) and studies have reported that patients suffering from OAB have shown signs of inflammation in bladder biopsies (Compérat et al., 2006, Loran et al., 2007). Histological investigation of 179 patient biopsies affected by OAB found signs of chronic inflammation in 70% of biopsies, with inflammatory cells having infiltrated the lamina propria in 98% of biopsies and urothelium in 18% (Apostolidis et al., 2008). Similarly, histological samples from patients suffering from neurogenic overactive bladder revealed substantial alternations such as infiltration of inflammatory mediators, oedema and fibrosis of the bladder (Compérat et al., 2006). As confirming inflammation of the bladder wall in patients with OAB using biopsies is invasive, expensive and associated with morbidity (Tyagi et al., 2010), it is seldom performed and therefore accounts for the lack of literature available on this topic.

A useful model for investigating inflammation of the urinary bladder is through local application of ovalbumin (OVA) in ovalbumin-sensitive animals (Ahluwalia et al., 1998). Ovalbumin sensitisation in guinea pig bladders induced release of prostaglandins E_2, D_2 and $F_{2\alpha}$ (in that order) from the urothelium and lamina propria (Saban et al., 1994). The authors noted that the release of prostaglandins was approximately four times greater in U&LP than that released from the detrusor. The authors also noted that histamine was released predominantly from the U&LP, whereas leukotrienes were released from the detrusor. There may be an interaction between these three inflammatory mediators that can potentiate the spasmogenic effect of the target tissue in inflammatory diseases, such as interstitial cystitis. There have been previous reports that damage to the urothelium that occurs in response to ovalbumin challenge resulted in increased release of leukotrienes from the underlying detrusor smooth muscle (Krasnopolsky et al., 1995, Lee et al., 1995). The loss of urothelium may have serious implications, as the cytoprotective effects of PGE_2 will be lost due to the tissue damage (Saban et al., 1994).

1.7.1. Mast cells

Mast cells are a part of the innate immune system and play an essential role in immediate allergic and inflammatory reactions (Billington and Penn, 2003). They are derived from a precursor cell in the bone marrow (Rodewald et al., 1996) and mature under the influence of local microenvironmental factors such as the stem cell factor (Chen et al., 2005). They are present in all vascularised tissues except for the central nervous system and the retina (da Silva et al., 2014). However, they are predominantly located in tissues that are exposed to the external environment such as the skin, respiratory epithelia and the gastrointestinal tract (Benyon, 1989, Metcalfe et al., 1997).

Mast cells populate areas containing connective tissue, particularly underneath the epithelial layer and surrounding nerve fibres, blood vessels and smooth muscle cells (Galli et al., 2005). Within their secretory granules, mast cells store a wide variety of pre-synthesised proinflammatory mediators (Blank and Rivera, 2006). When an antigen contacts a mast cell, crosslinking of two or more FcεRI (IgE receptors) molecules leads to the release of these granules (Kobayashi et al., 2000). The mediators released include histamine, leukotrienes, heparin, proteases and various cytokines and chemokines that act on smooth muscle, connective tissue, mucous glands and other inflammatory cells (Borish and Joseph, 1992). Histamine not only gets released when mast cells encounter an antigen, but is also released when mast cells detect injury, thereby dilating nearby blood vessels and allowing more blood to reach the site of injury (Amin, 2012). The release of these pro-inflammatory mediators increases the sensitivity of sensory neurons in the local area, thereby initiating a positive feedback loop which further activates mast cells and increases the concentration of the released inflammatory mediators (Sant and Theoharides, 1994).

In addition to the release of pre-formed mediators, the activation of mast cells also leads to neoformation of eicosanoids. The eicosanoid synthesis cascade is initiated at the secretory granules, leading to the formation of prostaglandins E_2, D_2 and $F_{2\alpha}$ (Chock and Schmauder-Chock, 1988, Schmauder-Chock and Chock, 1989). The major prostaglandin produced during degranulation is PGD_2 (Tilley et al., 2001) which acts through specific G-protein coupled receptors, DP1 and DP2 (Boie et al., 1995).

There is increasing evidence of the involvement of mast cells in chronic inflammatory disorders, such as rheumatoid arthritis (Woolley, 2003), psoriasis (Ozdamar et al., 1996) and in various neuroinflammatory conditions (Theoharides, 1990). Increased cellular

responsiveness to histamine is a crucial feature of many inflammatory conditions (Bouchelouche and Bouchelouche, 2006). In the urinary bladder mast cells can be found in a urothelium, lamina propria and in the detrusor smooth muscle (Christmas and Rode, 1991, Yamada et al., 2000, Gamper et al., 2015) in close proximity to neurons where they can be activated by sensory neuropeptides such as substance P and by acute stress (Spanos et al., 1997).

The involvement of mast cells has been associated with the pathogenesis of interstitial cystitis due to the occurrence of mastocytosis (accumulation of mast cells) in a significant subset of patients (Sant and Theoharides, 1994, Yamada et al., 2000). Furthermore, symptoms associated with interstitial cystitis tend to overlap with OAB, indicating that similar mechanisms might be involved in the pathogenesis of OAB.

1.7.1.1. Role of MCP-1 in mast cell activation

Monocyte chemoattractant protein-1 (MCP-1) is produced by many different types of cells of the body, including the urothelial cells of the bladder (Luo et al., 2007). Increased numbers of MCP-1 is associated with many inflammatory conditions, including rheumatoid arthritis (Koch et al., 1992), atherosclerosis (Blankenberg et al., 2001) and neuroinflammatory diseases (Conductier et al., 2010). Furthermore, it has been suggested that MCP-1 is involved in inducing mast cell degranulation, thereby contributing to the inflammation in the tissue (Chao et al., 2011). MCP-1 activates the CCR2 receptor, and inhibition of this receptor has been shown to decrease the clinical signs of acute-phase allergic reactions (Tominaga et al., 2009).

In the rat model of IC/BPS, it was found that the upregulation of MCP-1 is one of the possible contributing factors to histamine release from mast cells (Lv et al., 2012). Increased expression of MCP-1 has also been reported in overactive bladder (Tyagi et al., 2010, Ghoniem et al., 2011). Pilot data from Farhan et al. (2017) found that there is a significantly higher concentration of MCP-1 found in the urine of patients with OAB compared to healthy control subjects. Furthermore, the authors concluded that urinary MCP-1 could be used to identify patients with OAB and monitor the progression of this disorder. Therefore, it is plausible that mast cells, which are increased in patients with OAB and IC/BPS, could cause the release of pro-inflammatory mediators due to an increased expression of MCP-1 and thereby cause inflammation of the bladder, affecting overall contractility.

1.8. Inflammatory mediators in the urinary bladder

1.8.1. Histamine

Histamine, a significant component of mast cells granules is released upon degranulation (Riley, 1953). In the target tissue it causes vasodilation, contraction of smooth muscles and increased blood vessel permeability (Lundequist and Pejler, 2011). In addition to direct effects on the tissue, it can also influence physiological functions and regulate aspects of immune response development (Akdis and Blaser, 2003).

Histamine exerts its function by binding to four different G protein-coupled receptors, namely: H1R, H2R, H3R and H4R (Parsons and Ganellin, 2006). Both the H1 and H2 receptors are co-expressed in most tissues, including smooth muscle, epithelial tissue,

neurons and on various white blood cells (Jutel et al., 2009). When histamine binds to the H1 receptor, it couples $G_{q/11}$ which stimulates phospholipase C (PLC), thereby initiating the generation second messengers inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (Hill, 1990). IP_3 then leads to the release of calcium from the sarcoplasmic reticulum within the cell, thereby initiating the process of smooth muscle contraction. However, in order to maintain a contraction, both internal and external sources of calcium are required. Binding to the H2 receptor couples G_s protein triggering adenylyl cyclase (AC) activation and accumulation of cAMP (Hill et al., 1997), leading to calcium decrease in the cell (Morini et al., 1993) and relaxation of the smooth muscle. Both H3 and H4 receptors couple to $G_{i/o}$ that inhibit AC, thereby decreasing cAMP levels. H4 receptors also mediate calcium mobilization in mast cells and the release of calcium from the intracellular calcium stores (Hofstra et al., 2003) which has been suggested as the mechanism responsible for mast cell accumulation in inflamed tissue.

Immunohistochemical analysis of cultured human detrusor cells identified the presence of all four histamine receptor subtypes: H1, H2, H3 and H4 (Neuhaus et al., 2006). Administration of histamine has previously 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) and rabbit bladders (Fredericks, 1975). It has also been shown that in cultured detrusor muscle cells, H1 and H3 receptors are involved in inducing calcium release through the IP_3 pathway or the modulation of N-type calcium channels (Neuhaus et al., 2006). However, 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 and Westfall, 1994), rather than by direct action of histamine.

The mechanisms of H1 receptor activation were further investigated in guinea pig bladder (Rueda et al., 2002), where the authors suggested that the increase in IP_3 was attributed more to calcium release from internal stores rather than by direct activation of PLC. It has also been suggested that leukotriene D4 released from mast cells potentiates the spasmogenic effect of histamine on detrusor tissue (Bouchelouche and Bouchelouche, 2006). The authors noted that low doses of leukotriene D4 (10 nM or less), which do not typically produce an enhancement in smooth muscle contractions, caused an increase in isometric force in response to the addition of 50-200 nM of histamine. The increase in histamine response was attributed to the upregulation of addition H1 receptors or more efficient receptor signalling. The authors concluded that low concentrations of leukotriene D4 induces up-regulation of the H1 receptors in human detrusor smooth muscle cells. The involvement of the H1 receptor has also been noted in the guinea pig prostate (Kerr, 2006), where histamine was shown to potentiate nerve-stimulated twitch contractions in the ventral and dorsal lobes of the prostate. The contractility of the prostate gland in response to histamine appears to be dependant on the IP_3-mediated calcium release (Lam et al., 2011).

Most research on the effects of histamine on bladder contractility was conducted several decades ago and has not been reproduced since. Although previous studies had determined that the H1 receptor was involved in the contractility response, the exact mechanisms and the functional role of each histamine receptor subtype are still not fully understood and require further research. Histamine is likely involved in the pathology of OAB as demonstrated by Christensen et al. (1990), where the release of histamine caused an inflammatory response in guinea pig bladder. However the specific action of histamine, whether direct or mediated by other chemicals released in the bladder, still needs to be established.

1.8.2. Prostaglandins

An inflammatory mediator released from mast cells that can potentially affect the micturition reflex of the urinary bladder are prostaglandins. Additionally, prostaglandins can be produced locally in the bladder in response to mechanical damage or inflammation (Funk, 2001). Therefore, this suggests that the bladder is a potential site of prostaglandin release both locally and from the infiltrated mast cells, and makes investigation on the influence of the different prostaglandins on the contractile activity a crucial area to investigate further. Notably, the involvement of prostaglandins in bladder physiology was first recognised by Gilmore and Vane (1971), when the release of prostaglandins was observed immediately after urinary bladder distension. Stretch-induced release of PGE_2 from the urothelium has been suggested to exert a direct effect of detrusor smooth muscle cells to evoke contraction, or to enhance the release of local ATP via EP1 receptor activation resulting in increased afferent activation (Wang et al., 2008). Furthermore, complex interactions between ATP and NO has been noted to modulate PGE_2 release, thereby initiating a positive feedback process between ATP and PGE_2 (Nile et al., 2010).

Studies involving human detrusor have shown that the release of prostaglandins has a direct influence on the micturition reflex (Bultitude et al., 1976, Andersson et al., 1977). Overproduction of prostaglandins has been observed in several conditions, including bladder outlet obstruction (Masick et al., 2001), bladder overactivity due to spinal cord injury (Masunaga et al., 2006) and in inflammation (Wheeler et al., 2001). Furthermore, contractions of the detrusor smooth muscle in response to acetylcholine and ATP are enhanced by prostaglandins in both rabbit (Anderson, 1982) and guinea-pig animal models (Nile et al., 2010). There are complex interactions between these three molecules, as ATP

enhances PGE_2 release via a P2 purinoreceptor-mediated mechanism (Kasakov and Vlaskovska, 1985) and acetylcholine is a modulator of PGE_2 release (Nile and Gillespie, 2012). The interactions between these three contractile mediators can potentially result in amplification of molecule signalling and therefore in increased contractile activity, further supporting the need to investigate prostaglandin involvement in lower urinary tract dysfunction.

Synthesis of prostaglandins

All prostaglandins are synthesised from arachidonic acid which is a polyunsaturated omega-6 fatty acid that is released from the cell membrane via the hydrolysis of the SN-2 bond by the phospholipase A2 enzyme (PLA2) (Clark et al., 1991). Two cyclooxygenase isoforms COX-1 and COX-2 metabolise arachidonic acid into PGH_2, which is subsequently converted into five primary prostaglandins via their respective synthases: PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 and TXA_2 (Figure 1-4). These signalling molecules exert their function through the stimulation of nine specific G-protein coupled receptors: EP1 – EP4, DP1 and DP2, FP, IP and TP, respectively.

Besides mast cell release, prostaglandins can also be produced locally within the mucosa and smooth muscle layers in the human urinary bladder (Abrams et al., 1979, Jeremy et al., 1987). Both COX-1 and COX-2 are expressed within the bladder wall (de Jongh, 2007, de Jongh, 2009), specifically on the urothelium, lamina propria and the surface of the inner muscle bundles of the detrusor. COX-1 is predominately expressed in the basal and intermediate layers of the urothelium and on the interstitial cells located in the underlying lamina propria, indicating that these cells are capable of being activated by prostaglandins

(de Jongh, 2009). The production of prostaglandins differs between species. For example, in the rat bladder, PGI_2 is the major prostaglandins produced (Jeremy et al., 1984), whereas in the rabbit bladder it is PGE_2 (Leslie et al., 1984). In the human bladder, the main prostaglandins synthesised are PGI_2, followed by PGE_2, $PGF_{2\alpha}$ and TXA_2 (Jeremy et al., 1984, Masunaga et al., 2006). However, there are significant differences in the bladder contractile responses between species for the different prostaglandins receptor subtypes (Root et al., 2015).

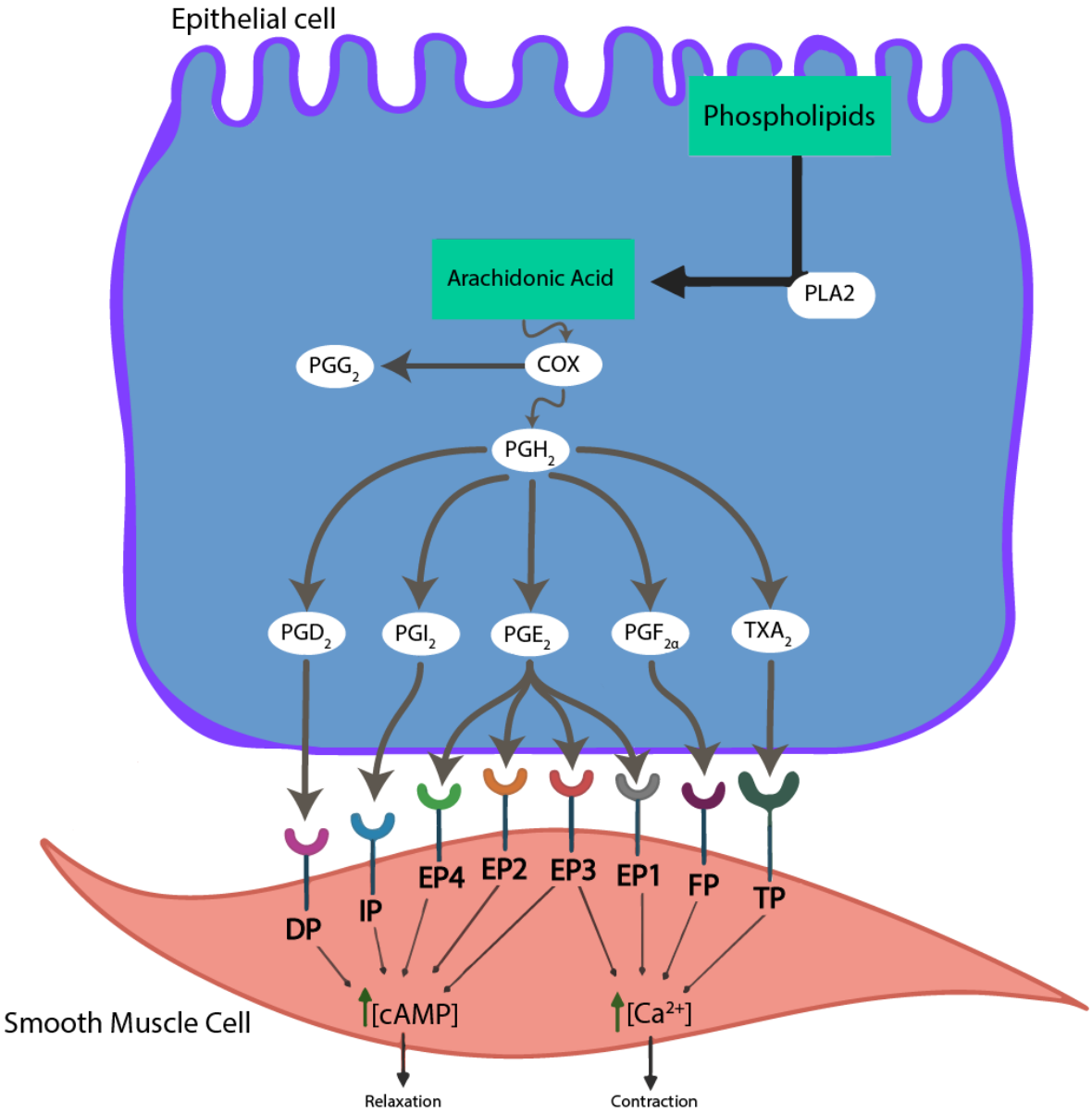

Figure 1-4: Prostaglandin metabolism pathways depicting the conversion from arachidonic acid into PGH_2 and the five primary prostaglandins; adapted from Ruan et al. (2011). This figure was produced under the Creative Commons Attributions license (CC BY 4.0) (https://creativecommons.org/licenses/), under which it was eligible for reuse and distribution under the condition that the original work published was cited.

Prostaglandin E_2

Of the five prostaglandins produced endogenously within the bladder and released from bladder-infiltrated mast cells, PGE_2 is thought to be the most likely contributor to bladder dysfunction. It is involved in the control of the bladder function via afferent signalling (Andersson and 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). This theory was tested by administrating intravesical PGE_2 in human (Schussler, 1990) and rat studies (Ishizuka et al., 1995), causing a strong sense of urgency, bladder instability and reduced bladder capacity. The use of PGE_2 has also been considered as a potential treatment for detrusor underactivity. However, intravesicularadministration has consistently been shown to have limited therapeutic value in the treatment of voiding dysfunction (Delaere et al., 1981, Hindley et al., 2004). Urine samples collected from female patients with OAB revealed a significant increase in PGE_2 and $PGF_{2\alpha}$ levels when compared with the control group (Kim et al., 2006). The excretion of prostaglandins was further investigated in rat bladders, by comparing the concentration of prostaglandins present in the urine collected from the bladder and ureters (Reyes and Klahr, 1990). The authors noted that the excretion of thromboxane B2 (a metabolite of TXA_2), 6-keto PGF1α (a metabolite of PGI_2), and PGE_2 was significantly greater in the urine collected from the bladder than ureters. In another study, a group of patients suffering from detrusor overactivity, interstitial cystitis and increased bladder sensation showed no significant differences in the levels of urine PGE_2 when compared to a healthy control group (Liu et al., 2010). Although prostaglandins might not always accumulate in the urine, past studies have shown a definite involvement of these mediators in bladder dysfunction.

There are four identified prostaglandin E_2 receptor subtypes: EP1, EP2, EP3 and EP4. The presence of the EP1 and EP2 receptor subtypes have been identified in guinea pig urothelium and lamina propria (Rahnama'i et al., 2010). The expression of all four PGE_2 receptor subtypes (EP1-EP4) has been demonstrated in the canine bladder using in situ hybridisation and immunohistochemistry (Ponglowhapan et al., 2010). EP1 and EP3 receptors have been suggested to inflict excitatory effects, whereas EP2 and EP4 are associated with inhibition of smooth muscle contractility (Woodward et al., 2011). Therefore, the involvement of EP1 and EP3 receptors is of particular interest in bladder dysfunction due to their excitatory effect when stimulated.

Rahnama'i (2011) identified the expression of EP1 receptors in the urothelium in the myofibroblasts of lamina propria and detrusor, in addition to smooth muscle cells found in the detrusor layer. Knockout mice studies identified the EP1 receptor as potentially being involved in the development of detrusor overactivity (Schroder et al., 2004). Similarly, in rat models of spinal cord injury, the activation of the EP1 receptors in the spinal cord contributed to detrusor overactivity (Wada et al., 2018), suggesting the EP1 receptor as a target for neurogenic detrusor overactivity as a result of spinal injury. Indeed, EP1 receptors found in the afferent nerve terminals or the urothelium are thought to facilitate afferent nerve activity by eliciting an inflammation-induced micturition reflex (Ikeda et al., 2006). When the EP1 antagonist PF-2907617-02 was administrated to normal rats, it significantly increased bladder capacity, voiding volume and micturition interval, but did not affect other urodynamic parameters (Lee et al., 2007). An EP1 antagonist ONO-8539 was suggested as an alternative treatment approach to OAB, after a study on monkeys found that it decreased both non-voiding contractions and duration of voiding in ATP-induced detrusor overactivity (Okada et al., 2010).

A second-phase clinical trial (Chapple et al., 2014) further investigated the use of ONO-8539, where it was compared to a control and tolterodine (antimuscarinic drug) group. However, this study did not find significant improvement in symptoms experienced by OAB patients when compared to the control group and concluded that the EP1 receptor is not involved in the pathophysiology of OAB. The authors attributed the lack of effect to the mode of action, as antimuscarinic treatment blocks both afferent and efferent signals (Andersson, 2011), whereas prostaglandins modulate only the afferent nerve signals (Andersson and Wein, 2004). Additionally, a study that assessed the contractile prostaglandin receptor subtypes in the bladders of humans and other species concluded that antagonism of the EP1 receptor is unsuitable for clinical treatment of bladder contractile disorders (Root et al., 2015). Even though treatment with an EP1 antagonist had shown great promise in inhibiting detrusor overactivity, the lack of response to this treatment in humans, but not in animal models, could be attributed to the differences in contractile responses between species. Indeed, distribution of contractile EP1 receptors is more widespread in guinea pigs and murine species than in higher species (Coleman et al., 1994), providing evidence as to why human trials were not as successful as animal studies.

EP2 receptors are expressed in guinea pig urothelium and lamina propria; however, their involvement in mediating bladder dysfunction is still unclear. The EP2 and EP3 receptor dual agonist ONO-8055 has been used to treat neurogenic underactive bladder in a rat model, where it decreased post-void residual urine and voiding pressure (Sekido et al., 2016). In a neurogenic underactive bladder model in monkeys it was found that ONO-8055 improved voiding function, making it a potential treatment option for neurogenic UAB (Matsuya et al., 2018). The combined EP1 and EP2 antagonist AH6809 has been shown to decrease the amplitude of detrusor contractions in humans (Palea, 1998). The analysis of EP2 receptor-

induced responses has been hindered due to the lack of selective antagonism, as available studies in the field have used either dual antagonists or agonists of the EP2 receptor. However, PF-04418949 has been recently presented as a highly selective EP2 receptor antagonist (Birrell and Nials, 2011), which would facilitate further investigation of the activity of this receptor.

The EP3 receptor plays a vital role in many physiological and pathological mechanisms. They are found in the central nervous system, where they are involved in the modulation of bladder function and have been proposed as a target for antagonists in detrusor overactivity (Su et al., 2008). The EP3 receptor also activates mast cells via the calcium influx/PI3K pathway, which leads to histamine release and inflammation and swelling of affected tissue (Morimoto et al., 2014). The EP3 receptor has been suggested as a contributor to the pathology of overactive bladder through studies involving EP3 receptor knockout mice (McCafferty et al., 2008). Wild-type mice, when exposed to PGE_2, developed overactive bladder, whereas EP3 receptor knock-out mice did not show a significant response to PGE_2. Administration of EP3 antagonists (CM9 and DG041) in rat studies showed that it enhanced bladder capacity in terms of micturition intervals and larger void volume, whereas agonist administration was associated with reduced bladder capacity (Jugus et al., 2009). Activation of EP3 receptors on the bladder interstitial cells facilitated bladder excitability via hyperpolarisation-activated channels (HCN) (Wu et al., 2017), which are thought to increase calcium concentration in the cell by activating T-type voltage-dependent calcium channels (Bernard et al., 2014). Indeed, EP3 receptors are expressed in the urothelium, sub-epithelial stroma and on the muscle of the bladder neck (Ponglowhapan et al., 2010), upon which PGE_2 can act. There are conflicting results regarding intracellular mechanisms that occur upon activation of the EP3 receptor. Kimple et al. (2013) study found that EP3 activation

decreases intracellular cAMP by binding to the G_i subunit, whereas Tamma et al. (2003) reported the opposite effect. This conflicting evidence is thought to be a result of the EP3 receptor having multiple isoforms, which couple to varying G-proteins leading to different changes in cAMP levels (Kim et al., 2013).

The presence of EP4 receptors in the obstructed bladder detrusor and urothelium has been determined using immunohistochemistry (Beppu, 2011). This study also found that administration of the EP4 agonist ONO-AE1-329 significantly relaxed detrusor muscle, inhibiting contractions and suppressing afferent nerve activity. Even though the EP4 receptor is considered to induce relaxation, the EP4 receptor antagonist MF191 was found to suppress PGE_2 and cyclophosphamide-induced bladder overactivity in rats (Chuang et al., 2012). Therefore, both agonists and antagonists of this receptor subtype have been linked to improvements in detrusor overactivity. Further research into the mechanisms of this receptor is needed in order to establish the role of the EP4 receptor in bladder function confidently.

Prostaglandin D_2, I_2, $F_{2\alpha}$ and thromboxane A_2

While there has been extensive research conducted on the involvement of PGE_2 and its receptor subtypes in bladder physiology, the functional role of other prostaglandins released in the bladder may have a potentially significant role in mediating contractile activity and warrants further investigation. The role of prostaglandin $F_{2\alpha}$, D_2, I_2 and thromboxane A_2 and their therapeutic potential have been investigated to a lesser extent. PGD_2 is the major prostaglandin released by activated mast cells (Lewis et al., 1982). It is also produced locally by cells of the urothelium and lamina propria in normal guinea pig urinary bladders, as confirmed by HPLC and immunohistochemical studies (Guan et al., 2014). Follow-up organ

bath studies from this research group identified that PGD_2 acts upon the DP1 receptor localised in smooth muscle cells in the detrusor layer to cause an inhibitory effect (Guan et al., 2015). The excitatory response observed in this study was found to occur due to the stimulation of TP receptors by PGD_2 when DP1 receptors were blocked by the antagonist BW-A868C. Some studies have reported prostaglandin $F_{2\alpha}$ to have a more potent effect on the contractile response of human detrusor tissue when compared to PGE_2 (Abrams and Feneley, 1975, Andersson et al., 1977, Palea, 1998). However, there remains no literature investigating the involvement of the prostaglandin $F_{2\alpha}$ receptor FP involvement in urinary bladder dysfunction.

A study involving rabbit detrusor has shown that PGI_2 or prostacyclin is synthesised locally in the bladder in response to mechanical trauma, distension and inflammation (Downie and Karmazyn, 1984, Jeremy et al., 1987). The prostacyclin antagonist RO3244019 was found to be effective in treating neurogenic detrusor overactivity after spinal cord injury in rats (Khera et al., 2007). Another study found this antagonist was able to decrease the frequency of bladder contractions and increase micturition threshold in citric acid-induced detrusor overactivity (Cefalu et al., 2007). Additionally, this treatment remained effective even following chronic capsaicin desensitisation. Thromboxane A_2 acting on the TP receptor has been shown to cause a contraction in human detrusor (Palea, 1998). Selective antagonists of the A_2 receptor (ICI-192,605 and SQ-29,548) have been shown to inhibit spontaneous contractions in rabbit smooth muscle (Collins, 2009). There remains a paucity in research investigating the role of IP and TP receptors in mediating contractile activity in the bladder in both health and disease.

Therapeutic potential of prostaglandin antagonists

Even though the use of prostaglandin antagonists in the clinical treatment of bladder and urinary disorders has shown great promise, past studies have shown limited therapeutic translation. The existing prostaglandin antagonists may be limited in their efficacy due to the lack of specificity for the receptor subtypes. It could also be attributed to the mode of action of prostaglandin antagonists when compared to the more effective antimuscarinic treatments. Prostaglandin antagonists target afferent signals, whereas antimuscarinics block both afferent and efferent nerve signals. Other compounds that target afferent signals, such as neurokinin 1 antagonist have shown to have minimal effect on contractile activity when compared with antimuscarinics (Frenkl et al., 2010) and the C fibre blocker resiniferatoxin was identified to be no better than the placebo treatment (Rios et al., 2007). Therefore, it is possible that in order to reduce the contractile activity of the bladder, both afferent and efferent signals need to be blocked. Also, success in identifying contractile responses in animal studies cannot always be translated into studies involving human tissue. It has been confirmed that prostaglandin expression and the contractile response to different prostaglandin subtypes vary significantly between species (Root et al., 2015). Therefore, further research on the functional role of each receptor subtype is required in order to understand the underlying cause of various urinary bladder dysfunctions.

1.8.3. Leukotrienes

Another important lipid inflammatory mediator synthesised from arachidonic acid from various immune cells, such as mast cells, eosinophils, basophils and macrophages are cysteinyl leukotrienes (CysLTs). The synthesis of leukotrienes is initiated by the cleavage

of arachidonic acid from the nuclear membrane of the immune cells by phospholipase A_2 in response to cell activation (Clark et al., 1991, Drazen et al., 1999). The cleaved arachidonic acid is converted into intermediate 5-hydroperoxy-eicosatetraenoic acid (5-HPETE), which is subsequently converted into unstable LTA_4 (Rouzer et al., 1986). In neutrophils, LTA_4 can be converted into LTB_4 by LTA_4 hydrolase (Evans et al., 1985) or to LTC_4 in mast cells, eosinophils and basophils by LTC_4 synthase (Nicholson et al., 1993). Following the conversion, LTC_4 gets exported to the cell surface and converted extracellularly to LTD_4 by a γ-glutamyl transpeptidase (γ-GT) (Anderson et al., 1982) or by a γ-glutamyl leukotrienase (γ-GL) (Carter et al., 1998) and then finally to LTE_4 by a dipeptidase (Lee et al., 1983). LTC_4, LTD_4 and LTE_4 are referred to as the cysteinyl leukotrienes, out of which LTE_4 is the most stable of the CysLTs. It is excreted into urine without further chemical modification and therefore can be used as an indicator of whole-body leukotriene synthesis (Peters-Golden et al., 2006). Previously mentioned LTB_4 produced from the membrane of neutrophils is not considered to be a part of the CysLTs, as it does not contain cysteine. LTB_4 is known as a mediator of leukocyte chemotaxis, whereas CysLTs are potent inflammatory mediators (Dahlen et al., 1980). The actions of CysLTs have been widely studied as a contributor to respiratory diseases, due to their potent spasmogenic effect on the airways (Piacentini and Kaliner, 1991, Hay et al., 1995, McMillan, 2001).

Viggiano et al. (1985) was the first to investigate the effects of leukotrienes on isolated urinary bladders of guinea pig and rats. The authors found that LTB_4 did not have any contractile effect on the isolated tissues, whereas LTC_4 and LTD_4 evoked tonic and phasic contractions in guinea pig bladders, but not in rat detrusor. Out of the two that did influence the contractility of guinea pig bladder, LTC_4 evoked a more significant enhancement in contractile response than LTD_4. Years later, in addition to investigating the effects of

exogenous leukotrienes on the contractility of the urinary bladder, Bjorling et al. (1994) studied the endogenous release of leukotrienes using an experimental model of cystitis. It was demonstrated that LTC_4, LTD_4 and LTE_4 all caused increases in tonic contractions in strips of guinea pig bladder with LTC_4 and LTD_4 being more potent in evoking contractions than LTE_4. The presence of LTD_4 receptors (also known as cysteinyl leukotriene 1 receptors) in human detrusor smooth muscle cells was characterised with immunohistochemical staining and later confirmed using transmission electron microscopy by Bouchelouche et al. (2001), implicating a possible role of LTD_4 in patients with interstitial cystitis and other urinary bladder inflammatory disorders. It was also found that LTD_4 caused an increase in cytosolic free Ca^{2+} in a dose-dependent manner.

Furthermore, it was found that LTD_4 evokes contractions in human detrusor smooth muscle cells that are entirely dependent on the Ca^{2+} release from the intracellular stores (Bouchelouche et al., 2003). Increased synthesis of CysLTs and excretion of LTE_4 have been observed in patients with interstitial cystitis and detrusor mastocytosis, indicating that these mediators play an essential role in the inflammation of the bladder wall (Bouchelouche et al., 2001). To further investigate the potential involvement of leukotrienes in interstitial cystitis, Bouchelouche et al. (2001) treated ten women with IC with montelukast, an antagonist with a high affinity and selectivity for LTD_4 receptor. They found that one month after the treatment, there was a statistically significant decrease in 24-hour urinary frequency, nocturia and pain, which persisted during the 3-month treatment. Case reports of IC patients treated with montelukast (Traut et al., 2011, Wajih Ullah et al., 2018) have suggested that it is an effective treatment option, due to its anti-inflammatory effect and improvement in urinary urgency and pain.

1.9. General aims and hypothesis

The overall aim of this research is to identify the influence of two predominant inflammatory mediators, histamine and prostaglandin, on porcine urothelium with lamina propria and detrusor tissue. Specifically, this thesis aims to:

1. Characterise the responses to histamine and all five prostaglandins in urothelium with lamina propria and detrusor smooth muscle, by measuring changes in tonic contractions and the frequency and amplitude of phasic contractions.

2. Identify each specific receptor subtype involved in modulating the increases in tonic contractions and spontaneous activity.

3. Determine the effects of ageing on contractile activity, receptor subtypes involved in the responses to histamine and prostaglandin E_2.

Hypothesis

It is hypothesised that histamine and the five prostaglandins will influence the contractility of porcine urothelium with lamina propria and detrusor tissues via specific receptor subtypes, in particular the H1, H2, EP1, EP3, and DP receptors. Furthermore, ageing is expected to have variable responses to both histamine and prostaglandin stimulation.

Chapter 2

General materials and methods

2.1. Tissue source

Porcine bladders were chosen in this research, as previous research has shown that the pig bladder is structurally similar to the human bladder and shares similar physiology and pharmacology (Wüst et al., 2002, Templeman et al., 2003, Vahabi et al., 2013, Steiner et al., 2018). Furthermore, common bladder dysfunction such as detrusor overactivity can be exhibited in porcine tissue (Jørgensen et al., 1983, Sibley, 1985, Svalo et al., 2013) making this an appropriate animal model to use in this study.

Bladders of both male and female Large White-Landrace Duroc (*Suf scrofa domestica*) crossbred pigs that weighed between 80 and 100 kg were utilised for this research. Studies that utilised juvenile samples used bladders from pigs aged six months, whereas bladders of two year old pigs were used to adult studies. Sows were used to depict adult model as at two years they had undergone puberty and reproduced. They were obtained from the Highchester Abattoir (Gleneagle, QLD) from pigs killed for provision of food on the morning experiments were conducted.

2.2. Ethics

Tissues were obtained from the local commercial abattoir after slaughter for the routine commercial provision of food. As no animals were bred, harmed, culled, interfered or interacted with as part of this research project, Animal Ethics Approval was not required for bladder use (Queensland Government, 2016). This study complied with the Australian Code

of Practice for the Care and Use of Animals for Scientific Purposes, and the Bond University institutional ethics policy.

2.3. Chemicals and pharmacological agents

Krebs-bicarbonate solution was used in all tissue bath experiments. The purpose of this solution is to maintain osmotic balance, physiological pH and provide carbohydrate (glucose) as an energy source. The solution was made fresh each day using distilled water with chemical components listed in Table 2-1.

Table 2-1: The components, their chemical formula and concentration used to make Krebs-bicarbonate solution.

Chemical name	Formula	Concentration	Supplier
Sodium chloride	NaCl	118.4 mM	Sigma-Aldrich
Glucose	$C_6H_{12}O_6$	11.7 mM	Sigma-Aldrich
Sodium bicarbonate	$NaHCO_3$	24.9 mM	Sigma-Aldrich
Potassium chloride	KCl	4.6 mM	Sigma-Aldrich
Magnesium sulphate	$MgSO_4$	2.41 mM	Sigma-Aldrich
Potassium dihydrogen orthophosphate	KH_2PO_4	1.18 mM	Sigma-Aldrich
Calcium chloride	$CaCl_2$	1.9 mM	Sigma-Aldrich

The chemical compounds used in this study, the concentration used, pharmacological action and supplier information is listed in Table 2-2. Prostaglandin E_2, prostaglandin $F_{2\alpha}$, prostaglandin D_2, prostaglandin I_2, AH6809, cyproheptadine, fexofenadine and indomethacin were dissolved in 100% ethanol and diluted as needed with distilled H_2O. Histamine, amthamine, pyrilamine, cimetidine, thioperamide, atropine, Nω-Nitro-L-arginine and αβ-methylene ATP were dissolved in distilled H_2O and diluted as required. SC19220, L-798196, AH23848, SQ-29548 and AL-8810 were dissolved in DMSO and diluted with distilled H_2O. U-46619 was supplied as a solution in methyl acetate, which was diluted with distilled H_2O. The concentrations selected for receptor antagonists were based on affinity values found in previous literature.

Table 2-2: List of pharmacological agents used in this thesis, concentration used, pharmacological actions and supplier information.

Chemical name	Concentration	Action	Supplier
AH 6809	10 μM	EP_1, EP_2 receptor antagonist	Cayman Chemicals
AH23848	10 μM	EP_4 receptor antagonist	Cayman Chemicals
AL-8810	5 μM	FP receptor antagonist	Cayman Chemicals
Amthamine	100 μM	H_2 receptor agonist	Sigma-Aldrich
Atropine	1 μM	Muscarinic receptor antagonist	Sigma-Aldrich
Cimetidine	1 μM	H_2 receptor antagonist	Sigma-Aldrich
Cyproheptadine	30 nM	H_1 receptor antagonist	Cayman Chemicals
Fexofenadine	1 μM	H_1 receptor antagonist	Cayman Chemicals

Histamine	10 nM - 1 mM	Histamine receptor agonist	Sigma-Aldrich
Indomethacin	5 μM	Cyclooxygenase (COX) inhibitor	Sigma-Aldrich
L-798106	100 nM	EP_3 receptor antagonist	Cayman Chemicals
Nω-Nitro-L-arginine	10 μM	Nitric oxide synthase inhibitor	Sigma-Aldrich
Prostaglandin D_2	1 μM - 10 μM	DP receptor agonist	Cayman Chemicals
Prostaglandin E_2	0.1 μM - 10 μM	EP receptor agonist	Cayman Chemicals
Prostaglandin $F_{2\alpha}$	1 μM - 10 μM	FP receptor agonist	Cayman Chemicals
Prostaglandin I_2	1 μM - 10 μM	IP receptor agonist	Cayman Chemicals
Pyrilamine	30 nM	H_1 receptor antagonist	Sigma-Aldrich
SC-19220	10 μM	EP_1 receptor antagonist	Cayman Chemicals
SQ-29548	100 nM	TP receptor antagonist	Cayman Chemicals
Thioperamide	1 μM	H_3 and H_4 receptor antagonist	Sigma-Aldrich
U-46619	1 μM - 10 μM	TP receptor agonist	Cayman Chemicals
αβ-methylene ATP	10 μM	P2X purinergic receptor agonist	Cayman Chemicals

2.4. Tissue preparation

After slaughter at the abattoir, porcine bladders were removed and immediately placed in cold Krebs-bicarbonate solution (NaCl 118.4 mM, $NaHCO_3$ 24.9 mM, $CaCl_2$ 1.9 mM, $MgSO_4$ 2.41 mM, KCl 4.6 mM, KH_2PO_4 1.18 mM and D-glucose 11.7 mM) until collected by the researcher on the same day. Upon collection, the container with porcine bladders was placed in a portable cooler filled with ice and transported back to Bond University's research laboratory. Tissue preparation included removing the ureters, urethra, serosa, and surrounding arteries on the bladder and the bladder being open longitudinally (Figure 2-1) and rinsed thoroughly with Krebs-bicarbonate solution.

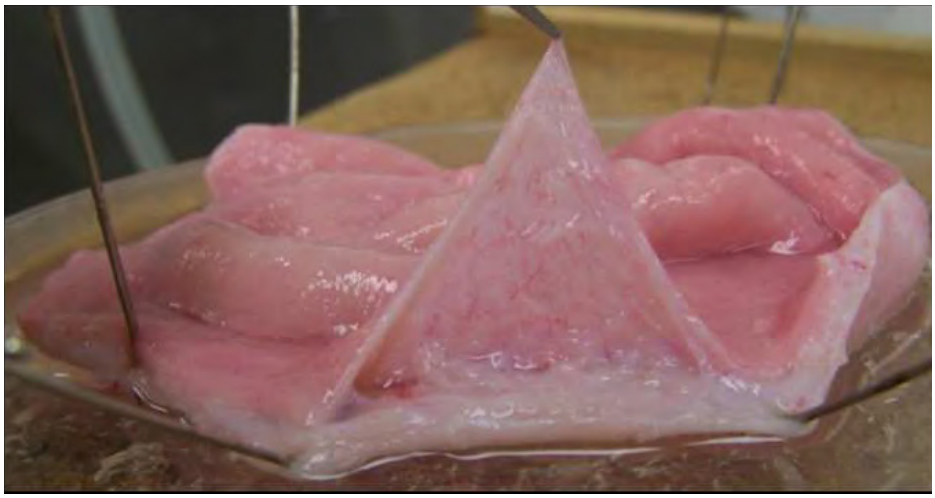

Figure 2-1: Pig urinary bladder demonstrating the separation between the urothelium with lamina propria layer that has been dissected from the underlying detrusor smooth muscle. This figure was produced by Christian Moro.

Transverse strips of the bladder (approximately 4 cm x 0.5 cm) were removed from the anterior wall of the bladder dome region. Urothelium with lamina propria layer was carefully dissected from the underlying detrusor smooth muscle layer consistent with methods carried out in past studies (Moro et al., 2011, Moro et al., 2012, Moro et al., 2013). Throughout the preparation and dissection stage, tissue strips were regularly washed with cold Krebs-bicarbonate solution.

Once tissues were dissected, adjacent strips of urothelium with lamina propria and detrusor (each strip approximately 10 mm x 5 mm) were tied vertically between an isometric force transducer (MCT050/D, ADInstruments, Castle Hill, Australia) and a fixed hook in a 10 mL organ bath (Labglass, Brisbane, Australia), and placed in Krebs-bicarbonate solution and gassed with carbogen (a mixture of 95% oxygen and 5% carbon dioxide) at 37 °C (Figure 2-2). After mounting, tissues were washed three times with warm Krebs-bicarbonate solution and tension adjusted to approximately 2 g. Tissue preparations were then left to equilibrate for 30 min in the absence (control) or presence of a specific receptor subtype antagonist (experimental).

Changes in the tonic contractions and the frequency and amplitude of spontaneous phasic contractions were recorded simultaneously through an isometric force transducer on a Powerlab system using LabChart v7 software (MCT050/D, ADInstruments, Castle Hill, Australia).

The small tissue size that was utilised for these studies allowed for effective diffusion of pharmacological agents and decreased the likelihood of hypoxia while mounted in the tissue baths. During the preparation stage, tissues were washed continuously with cold Krebs-

bicarbonate solution. Once tissue strips were removed, bladders were placed in individual containers filled with cold Krebs and placed in a fridge, where they were stored at 4ºC for no longer than 12 hours.

The viability of the tissue was ensured by adding a single dose of carbamoylcholine chloride (10 μM, Sigma Aldrich, Missouri, USA) to all tissues at the very end of each experiment. U&LP or detrusor strips that did not respond to carbamoylcholine chloride were deemed as non-viable and were not included in the analysis. After each experiment, tissues were removed from each organ bath and measured on a weighing scale to an accuracy of 1 mg (0.001 g).

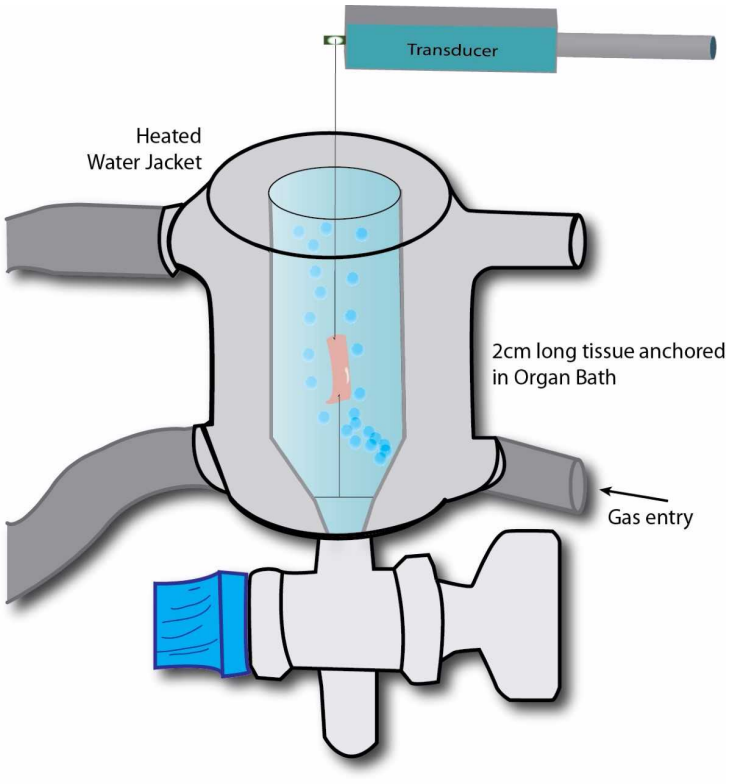

Figure 2-2: Representation of a single isolated tissue bath. It is filled with a Krebs-bicarbonate solution at 37ºC and perfused with a mixture of 95% oxygen and 5% carbon dioxide (carbogen). Strips (10 mm x 5 mm) of urothelium with lamina propria and detrusor are tied between an isometric force transducer and a fixed hook in a 10 mL tissue bath. Tissue strips are kept at 1.5 – 2.0 g resting tension. This figure was produced by Christian Moro.

2.5. Measurements

Measurements of the tonic contractions and the frequency and amplitude of spontaneous phasic contractions were taken before the addition of an agonist, during peak contractile response after the addition of the agonist and 20 minutes after the agonist was added (Figure 2-3). The frequency of spontaneous contractions was measured from the total number of phasic waves occurring over 2-3 minutes, calculated as an average, and expressed as contractions per minute (cpm, Figure 2-4). The amplitude of each contraction was measured as the tension differences between the averaged lowest and highest point of each phasic wave (Figure 2-4). Tonic contractions were measured from the lowest point of each spontaneous phasic contraction before treatment with an agonist and during peak contractile response. Changes in both tonic contractions and the amplitude of spontaneous phasic contractions were expressed as either grams (g) or Newton force per gram tissue weight (mN/g). The results were represented as mean change ± SEM.

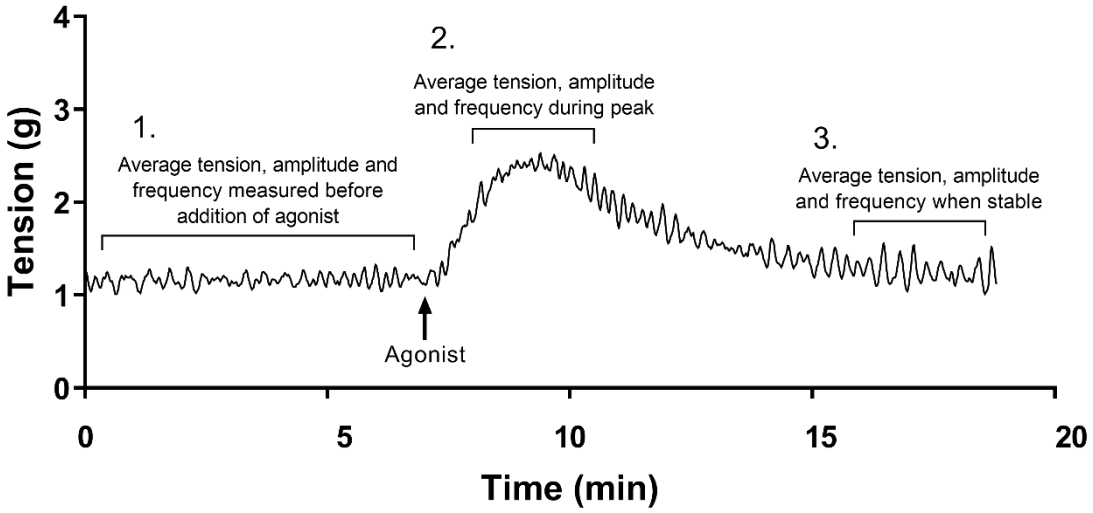

Figure 2-3: Sample trace of urothelium with lamina propria, demonstrating the three time-points at which average measurements of the tonic contractions and the frequency and amplitude of spontaneous phasic contractions were recorded.

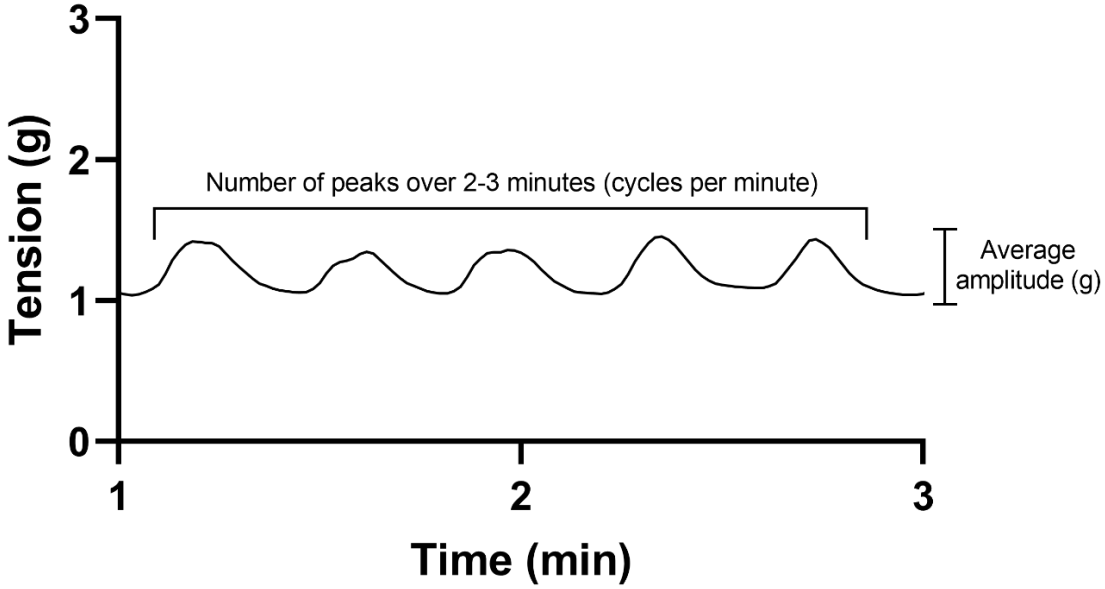

Figure 2-4: Sample trace of urothelium with lamina propria, demonstrating spontaneous phasic contractions which provide an example of how measurements of the frequency and amplitude of the spontaneous contractions were conducted.

2.6. Statistical analysis

The initial analysis and acquisition of raw data were carried out using LabChart v7 software (MCT050/D, ADInstruments, Castle Hill, Australia). Three measurements were taken from a single trace at different time points throughout the experiment: tonic contractions, frequency of spontaneous phasic contractions and amplitude of spontaneous phasic contractions. Changes in the tonic contractions and amplitude were expressed in grams (g) or as Newton force per gram tissue weight (mN/g). Changes in the frequency of spontaneous phasic contractions were expressed as contractions per minute (cpm). Raw data were compiled together and processed using spreadsheets on Microsoft Excel 2020 (Microsoft, Redmond, WA, USA). Analysis and graphing of the results were conducted using GraphPad Prism v8 (GraphPad, San Diego, CA, USA) where changes between control and experimental conditions were compared using a paired Student's two-tailed *t*-test with $p < 0.05$ considered as statistically significant. Data sets that were obtained from two different age groups or from two different bladders were compared using an unpaired Student's two-tailed *t*-test with significance considered at $p < 0.05$.

Chapter 3

Histamine modulation of urinary bladder urothelium, lamina propria and detrusor contractile activity via H1 and H2 receptors

The content in the following chapter is presented as the author's copy of the published document, before being accepted for publication. The final version of this manuscript can be accessed from the relevant peer-reviewed journal. Formatting and terminology changes have been made to maintain consistency throughout this thesis.

All data presented in this manuscript are the sole work of Zane Stromberga.

PUBLISHED MANUSCRIPT

Stromberga, Z., Chess-Williams, R., & Moro, C. (2019). Histamine modulation of urinary bladder urothelium, lamina propria and detrusor contractile activity via H1 and H2 receptors. *Scientific reports*, *9*(1), 3899.

Published abstracts and conference presentations arising from this chapter:

Stromberga, Z., Smith, J., Tan, J., & Moro, C. (2020). Influences of the mast cell degranulates histamine and prostaglandins on urinary bladder contractile activity. *Future Physiology 2020, virtual conference.*

Stromberga, Z., Chess-Williams, R., Moro, C. (2018, October). The involvement of histamine receptors in modulating spontaneous contractile activity in bladder disorders. *Proceedings of the 27th National Conference on Incontinence, Hobart, Australia.*

Stromberga, Z., Chess-Williams, R., Moro, C. (2018, July). Histamine H1 and H2 receptors as regulators of urinary bladder urothelium/lamina propria and detrusor contractile activity. *Proceedings of the 18th World Congress of Basic and Clinical Pharmacology, Kyoto, Japan.*

Stromberga, Z., Chess-Williams, R., Moro, C. (2017, November). The role of histamine in modulating bladder contractile activity. *Proceedings of the 26th National Conference on Incontinence, Sydney, Australia*.

Stromberga, Z., Chess-Williams, R., Moro, C. (2017, November). Histamine as a potential mediator in bladder contractile disorders. *Proceedings of the Gold Coast Health Research Week Conference 2017, Gold Coast, Australia*.

Stromberga, Z., Chess-Williams, R., Moro, C. (2017, December). Is overactive bladder an allergy? The influence of the inflammatory mediators histamine and prostaglandin on contractile activity. *Proceedings of the 9th National Symposium on Advances in Urogenital and Gut Research, Gold Coast, Australia*.

Stromberga, Z., Chess-Williams, R., Moro, C. (2017, December). Histamine receptors as regulators of urothelial and detrusor contractile activity. *Proceedings of the APSA-ASCEPT joint scientific meeting, Brisbane, Australia*.

3.1. Abstract

Introduction: The mechanisms underlying bladder contractile disorders, such as overactive bladder, are not fully understood and there is limited understanding of the receptor systems modulating spontaneous bladder contractions. This study aimed to investigate the potential for histamine to have a role in mediating contractility of the urothelium with lamina propria (U&LP) or detrusor via the H1-H4 histamine receptor subtypes.

Methods: Isolated strips of porcine U&LP or detrusor smooth muscle were mounted in gassed Krebs-bicarbonate solution and responses to histamine obtained in the absence and presence of selective receptor antagonists.

Results: The presence of histamine increases the frequency of U&LP spontaneous phasic contractions and tonic contractions. In response to histamine, H1-antagonists pyrilamine, fexofenadine and cyproheptadine were effective at inhibiting contractile responses. Cimetidine enhanced the increases in tonic contractions in response to histamine, whereas amthamine induced relaxation. Although thioperamide increased tonic contractions in response to histamine, selective H1/H2-receptor antagonism revealed no influence of these receptors. In detrusor preparations, pyrilamine, fexofenadine and cyproheptadine were effective at inhibiting increases in tonic contractions in response to histamine.

Conclusions: These findings provide evidence that histamine produces both contractile responses in the U&LP and detrusor via the H1-receptor, and this response is significantly inhibited by activation of the H2-receptor in the U&LP, but not the detrusor.

3.2. Introduction

The underlying mechanisms of various lower urinary tract disorders are unclear. However, it is known that detrusor smooth muscle provides the leading force for contractions during voiding. In recent years, interest has been building on the role of the urothelium and lamina propria (U&LP) for regulating and modulating overall bladder contractile activity. Among other functions, U&LP is capable of releasing various mediators when activated by chemical or mechanical stimuli such as acetylcholine, which can influence detrusor contractions (Moro et al., 2011). It is also capable of responding to external stimuli such as noradrenaline (Moro et al., 2013) or nitric oxide (Moro et al., 2012) and can release agents such as ATP, which may contribute to diseases such as overactive bladder (Andersson et al., 2018). Research also suggests that U&LP is capable of developing spontaneous phasic contractile activity in the absence of any external stimulation (Moro et al., 2011), and this activity can potentially influence the bladder as a whole.

Bladder contractions are mainly controlled by parasympathetic nerves (de Groat et al., 1981), which release acetylcholine to activate M3 muscarinic receptors on the detrusor smooth muscle (Chess-Williams, 2002). Anti-muscarinic treatment has been used as first-line pharmaco-therapeutic intervention for many years. 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). One alternative target involved in the pathogenesis of contractile dysfunctions, such as OAB and interstitial cystitis may be the inflammatory mediators released from mast cells at sites of inflammation.

Mast cells play an essential role in immediate allergic reactions and during inflammation (Billington and Penn, 2003). Upon activation, they release and synthesise potent inflammatory mediators including histamine, prostaglandins, proteases and cytokines that act on smooth muscle, connective tissue, mucous glands and other inflammatory cells (Amin, 2012). In the urinary bladder, mast cells can be found in the urothelium, lamina propria and smooth muscle layers of the bladder wall (Christmas and Rode, 1991, Yamada et al., 2000, Gamper et al., 2015) and have previously been associated with the pathogenesis of interstitial cystitis (Sant and Theoharides, 1994, Yamada et al., 2000) and OAB (Liu et al., 2012).

Furthermore, upregulation of chemoattractant protein-1 (MCP-1), which causes degranulation of mast cells has been observed in patients with OAB (Tyagi et al., 2010). A histological investigation of 179 patient biopsies from OAB sufferers found signs of chronic inflammation in 70% of biopsies. Inflammatory cells had infiltrated the lamina propria in 98% of those biopsies and the urothelium in 18% (Apostolidis et al., 2008). Therefore, the involvement of histamine is of great interest, as it is the predominant mediator involved in the mediation of acute allergy and inflammation.

Histamine exerts its effects by binding to four different G protein-coupled receptors, H1, H2, H3 and H4 (Parsons and Ganellin, 2006). Both H1 and H2 receptors are co-expressed in most tissues and cell types, including smooth muscle, epithelial tissue, neurons and various white blood cells (Jutel et al., 2009). Western blot analysis of cultured human detrusor cells has revealed the presence of all four histamine receptors subtypes: H1, H2, H3 and H4, although functional roles of these receptor subtypes are still not fully understood. There is also evidence that histamine is involved in the modulation of bladder contractile activity via

the activation of H1 receptors in the guinea pig (Khanna et al., 1977, Kondo et al., 1985, Poli et al., 1988) and rabbit detrusor muscle (Fredericks, 1975). It has also been suggested that responses to histamine may stimulate acetylcholine release from sites proximal to the muscle (Rubinstein et al., 1987), or influence purinergic neurotransmission on nearby nerves (Patra and Westfall, 1994). This study aims to investigate the effect of histamine on the contractility of U&LP and detrusor smooth muscle and identify the histamine receptor subtypes responsible for mediating contractile responses.

3.3. Materials and methods

3.3.1. Tissue preparation

The urinary bladder from juvenile Large White-Landrace pigs (aged approximately six months) was separated from the urethra and the ureters. As no animals were bred, harmed, culled, interfered, or interacted with as part of this research project, Animal Ethics Approval was not required for offal use (Queensland Government, 2016). Isolated tissue strips of U&LP and detrusor were prepared and set up as described previously in Chapter 2.

Tissue strips were washed three times, and tension readjusted to approximately 2 g. A single dose of agonist was added to the tissue both in the presence and absence of antagonists. Tissue strips that were exposed to antagonists and tissues treated with vehicle control were incubated for 30 minutes to allow full equilibration with the receptor. Tonic contractions and the frequency and amplitude of spontaneous phasic contractions were recorded simultaneously using isometric force transducers (MCT050/D, ADInstruments, Castle Hill,

Australia) on a Powerlab system using LabChart v7 software (ADInstruments). Changes in tension were measured in grams, the frequency was expressed as the number of spontaneous phasic contractions per minute (cycles/min), and amplitude was expressed in grams (the lowest point of spontaneous phasic contraction to peak). Porcine tissue was acquired from the local commercial abattoir after the slaughter for the routine provision of food. Data were graphed and analysed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla California USA) and results shown as the mean change ± SEM. Responses between control and experimental tissues were compared using Student's two-tailed *t*-test with $p < 0.05$ considered significant.

3.3.2. Histamine agonist effects on tonic contractions, amplitude and spontaneous phasic contractions

A single dose of histamine (100 μM, Sigma Aldrich, Missouri, USA) or amthamine (100 μM, H2 agonist, Sigma Aldrich, Missouri, USA) was applied to the tissue after a 30-minute equilibration period. Tonic contractions, amplitude and spontaneous phasic contractions of U&LP were measured before the application of the agonist and 2 minutes after. The tonic contractions for detrusor were measured at the same time-points as used for U&LP.

A single concentration of 100 μM histamine was chosen as the first few experiments involved concentration-response-curves (CRCs), which showed no changes in contractions. It was later determined that desensitisation of the histamine receptors had occurred. After CRC experiments, a single concentration of 0.1 μM, 1 μM, 10 μM, 100 μM and 1 mM were tried. It was determined that 100 μM produced 80% of the maximal contraction.

3.3.3. The effects of histamine on tonic contractions, amplitude and spontaneous phasic contractions in the presence of histamine receptor antagonists

The H1 receptor antagonists pyrilamine (30 nM, Cayman Chemicals, Michigan, USA), fexofenadine (1 μM, Cayman Chemicals, Michigan, USA) and cyproheptadine (30 nM, Cayman Chemicals, Michigan, USA), the H2 receptor antagonist cimetidine (1 μM, Sigma Aldrich, Missouri, USA) and the dual H3&H4 receptor antagonist thioperamide (1 μM, Sigma Aldrich, Missouri, USA) were separately applied to tissues.

After 30-minute incubation with antagonists, a single dose of histamine (100 μM) was applied to the tissues. In another group of experiments, a combination of antagonists was used. Histamine responses to pyrilamine (30 nM) treated tissues were compared with a combination of pyrilamine (30 nM) and cimetidine (1 μM) treated tissues; cimetidine (1 μM) treated tissues were compared with a combination of pyrilamine (30 nM) and cimetidine (1 μM) treated tissues; a combination of pyrilamine (30 nM) and cimetidine (1 μM) treated tissues were compared with a combination of pyrilamine (30 nM), cimetidine (1 μM) and thioperamide (1 μM) treated tissues.

3.4. Results

3.4.1. Effect of histamine receptor agonists in U&LP and detrusor preparations

In the absence of histamine or any antagonists, strips of urothelium with lamina propria (U&LP) developed spontaneous phasic contractions at a frequency of 3.56 ± 0.13 cycles per

min^{-1} (cpm), with an amplitude of 0.46 ± 0.03 g (n = 48). When histamine (100 μM) was added to the tissues, U&LP tonic contractions increased by 0.98 ± 0.13 g ($p < 0.001$, n = 48, Figure 3-1). Additionally, frequency of spontaneous phasic contractions increased by 1.32 ± 0.25 cpm ($p < 0.001$, n = 48) and amplitude decreased by 22.9 ± 5.8 % ($p < 0.001$, n = 48) during the peak response to histamine (100 μM). Tonic contractions, frequency and amplitude of spontaneous phasic contractions in response to histamine were not affected by the presence of the muscarinic receptor antagonist atropine (1 μM). When H2 agonist amthamine (1 μM) was added to strips of U&LP that were left to equilibrate at a tension of approximately 2 g in the absence of any agonists and antagonists, resting tension decreased by 0.13 ± 0.02 g ($p < 0.01$, n = 12) in the first 5 minutes after the addition of agonist and by 0.22 ± 0.04 g after 20 minutes ($p < 0.01$, n = 12).

In detrusor preparations, an increase in the tonic contractions of 0.99 ± 0.27 g ($p < 0.001$, n = 48, Figure 3-2) was observed in response to histamine (100 μM). Spontaneous phasic contractions in the absence of any stimulation with receptor agonists or antagonists developed in 15% of all preparations, with an average frequency of 2.12 ± 0.28 cpm (n = 8) and amplitude of 0.56 ± 0.18 g (n = 8). After the treatment with histamine (100 μM), these preparations exhibited an average frequency of 3.96 ± 0.68 cpm (n = 8) and amplitude of 0.84 ± 0.26 g (n = 8).

In those detrusor preparations that did not exhibit spontaneous activity, the addition of histamine (100 μM) initiated spontaneous contractions in 54% of preparations, with a frequency of 2.91 ± 0.20 cpm (n = 23) and amplitude of 0.53 ± 0.19 g (n = 23). The remaining 38% (n = 18) of detrusor preparations did not show any spontaneous activity before or after

the treatment with histamine (100 μM). Addition of the H2 agonist amthamine (1 μM) or muscarinic receptor antagonist atropine (1 μM) did not influence the tonic contractions or the spontaneous contractile activity in detrusor preparations.

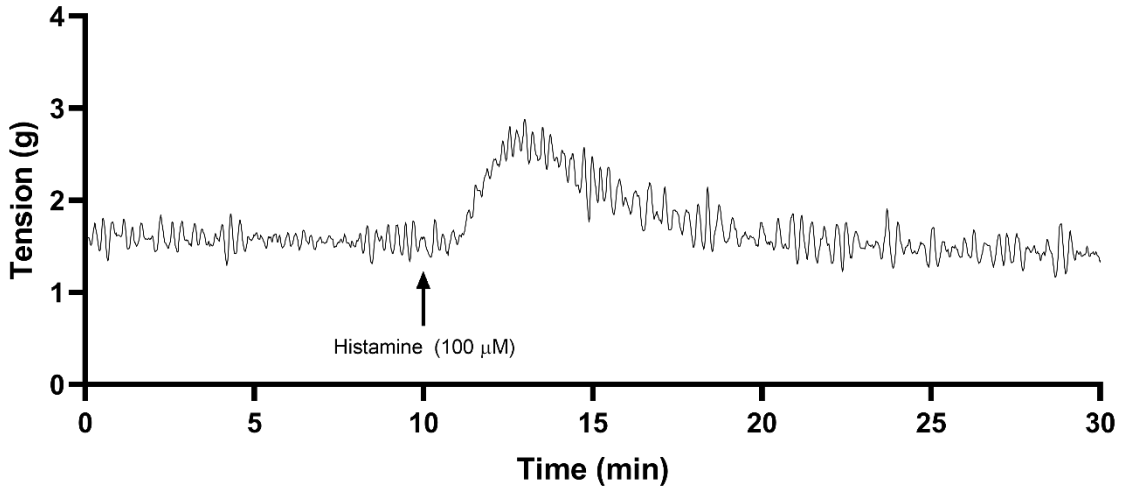

Figure 3-1: Sample trace of U&LP after the addition of histamine (100 μM) demonstrating increases in the tonic contractions and the frequency of spontaneous phasic contractions.

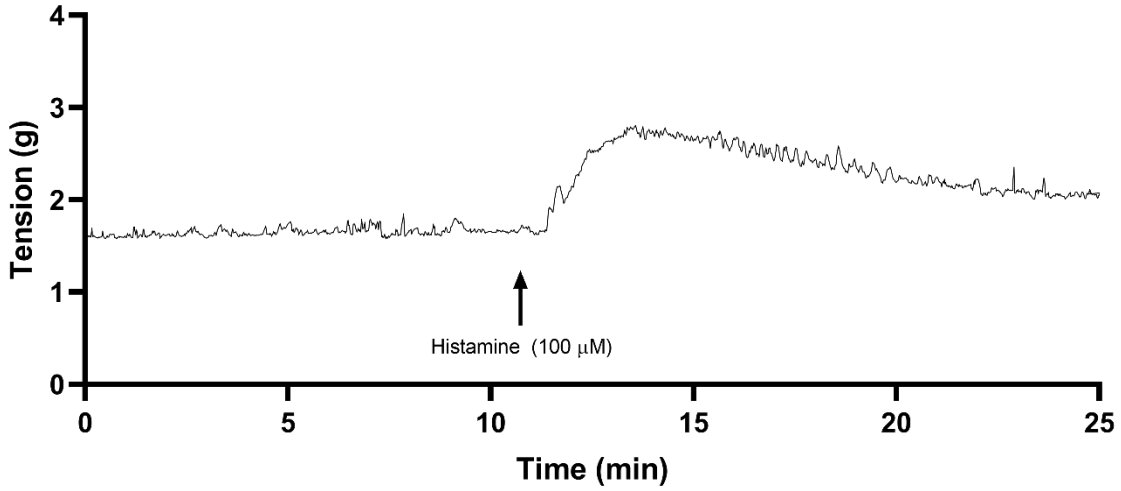

Figure 3-2: Sample trace of detrusor after the addition of histamine (100 μM) demonstrating increases in the tonic contractions and the frequency of spontaneous phasic contractions.

3.4.2. Selective histamine receptor antagonists in U&LP

The responses to histamine were observed in the presence of five selective histamine receptor antagonists: pyrilamine, cyproheptadine, fexofenadine, cimetidine and thioperamide. In the presence of pyrilamine (H1 antagonist, 30 nM), increases in tonic contractions and frequency of spontaneous phasic contractions in response to histamine (100 μM) were significantly inhibited ($n = 8$, $p < 0.05$ for both). Histamine (100 μM) in the presence of pyrilamine caused an increase of tonic contractions by 0.17 ± 0.11 g (Table 3-1, Figure 3-3) and phasic contraction frequency by 0.15 ± 0.25 cpm (Table 3-2).

Furthermore, when the alternative H1-selective antagonist cyproheptadine (30 nM, $n = 8$, Figure 3-4) and fexofenadine (1 μM, $n = 8$, Figure 3-5) were added to the U&LP tissue strips, inhibition of tonic contractions (Table 3-1, $p < 0.01$) and frequency of spontaneous phasic contractions in response to histamine (100 μM) were also observed (Table 3-2, $p < 0.01$).

Cimetidine (H2 antagonist, 1 μM) caused a significant increase of 0.81 ± 0.25 g above the baseline tension in response to histamine (100 μM) ($n = 8$, $p < 0.05$, Table 3-1, Figure 3-6) and an increase in frequency of spontaneous phasic contractions of 1.41 ± 0.94 cpm (Table 3-2). The addition of thioperamide (H3 and H4 antagonist, 1μM) resulted in the tissue achieving significantly greater contractions to histamine (100 μM, $n = 6$) than in control samples, resulting in a 1.75 ± 0.29 g increase in tonic contractions ($p < 0.05$, Table 3-1, Figure 3-7), with no influence on the frequency of spontaneous contractions (Table 3-2).

Decreases in the amplitude of spontaneous phasic contractions in response to histamine (100 μM) were observed in both control and experimental conditions. Treatment with selective histamine receptor antagonists did not have any significant effects on the amplitude of these contractions (Table 3-3).

Table 3-1 – Tonic contractions: U&LP changes in tonic contractions in response to histamine (100 μM) in the presence of histamine receptor antagonists (mean change ± SEM).

			ΔTension (g)		
Antagonist	Receptor	Conc.	Absence	Presence	n
Pyrilamine	H1	30 nM	0.97 ± 0.25	0.17 ± 0.11*	8
Cyproheptadine	H1	30 nM	1.19 ± 0.38	0.42 ± 0.16*	8
Fexofenadine	H1	1 μM	0.89 ± 0.24	-0.03 ± 0.02**	8
Cimetidine	H2	1 μM	0.81 ± 0.25	1.28 ± 0.38*	8
Thioperamide	H3/H4	1 μM	1.20 ± 0.29	1.75 ± 0.29*	6

*$p < 0.05$, **$p < 0.01$. Paired Student's two-tailed *t*-test.

Table 3-2 – Frequency of phasic contractions. U&LP changes in frequency responses to histamine (100 μM) in the presence of histamine receptor antagonists (mean change ± SEM).

			ΔFrequency (cpm)		
Antagonist	Receptor	Conc.	Absence	Presence	n
Pyrilamine	H1	30 nM	1.67 ± 0.26	0.24 ± 0.22**	8
Cyproheptadine	H1	30 nM	1.87 ± 0.78	0.56 ± 0.28*	8
Fexofenadine	H1	1 μM	1.11 ± 0.28	-0.09 ± 0.14*	8
Cimetidine	H2	1 μM	1.20 ± 0.65	1.27 ± 1.08	8
Thioperamide	H3/H4	1 μM	1.49 ± 0.63	1.76 ± 0.55	6

*p < 0.05, **p < 0.01. Paired Student's two-tailed *t*-test.

Table 3-3 – Amplitude of phasic contractions. U&LP amplitude responses to histamine (100 μM) in the presence of histamine receptor antagonists (mean change ± SEM). None of the antagonists had statistically significant effects using a paired two-tailed Student's *t*-test.

			ΔAmplitude (g)		
Antagonist	Receptor	Conc.	Absence	Presence	n
Pyrilamine	H1	30 nM	-0.14 ± 0.06	-0.11 ± 0.10	8
Cyproheptadine	H1	30 nM	-0.11 ± 0.04	-0.09 ± 0.07	8
Fexofenadine	H1	1 μM	-0.06 ± 0.11	-0.07 ± 0.06	8
Cimetidine	H2	1 μM	-0.08 ± 0.03	-0.09 ± 0.03	8
Thioperamide	H3/H4	1 μM	-0.17 ± 0.08	-0.19 ± 0.12	6

U&LP

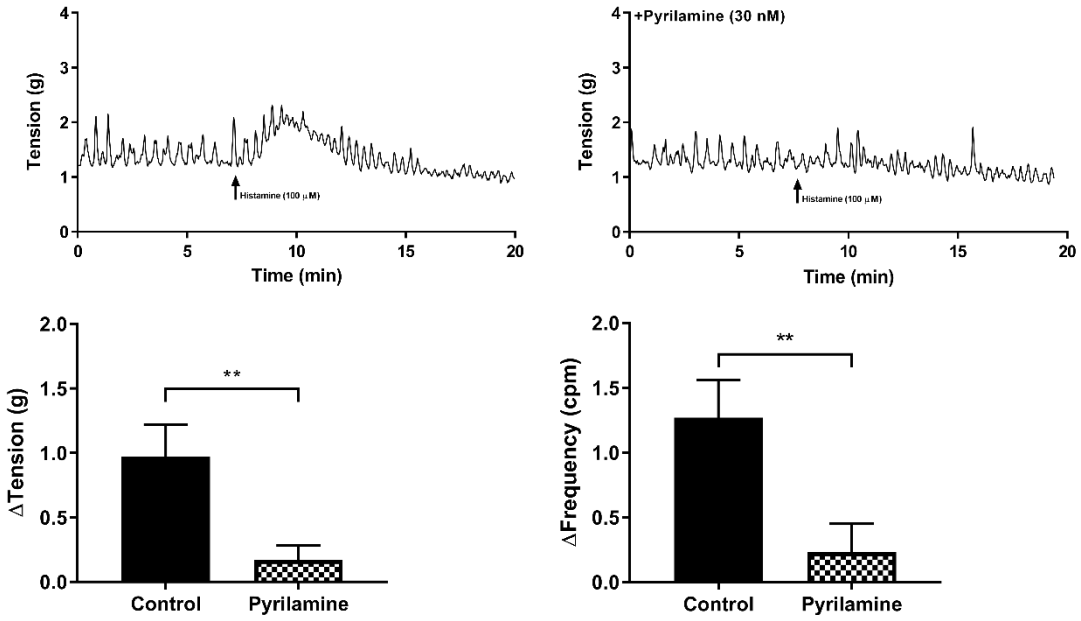

Figure 3-3: U&LP tonic contractions in response to histamine (100 μM, *top row*) as control (*left*) and in the presence of histamine 1 (H1) receptor antagonist pyrilamine (30 nM, *right*). Changes in tonic contractions (*bottom left*) and in the frequency of phasic activity (*bottom right*) in response to histamine are represented as mean change ± SEM. Changes in tonic contractions between control and experimental conditions were evaluated using a paired Student's two-tailed *t*-test, where **$p < 0.01$

U&LP

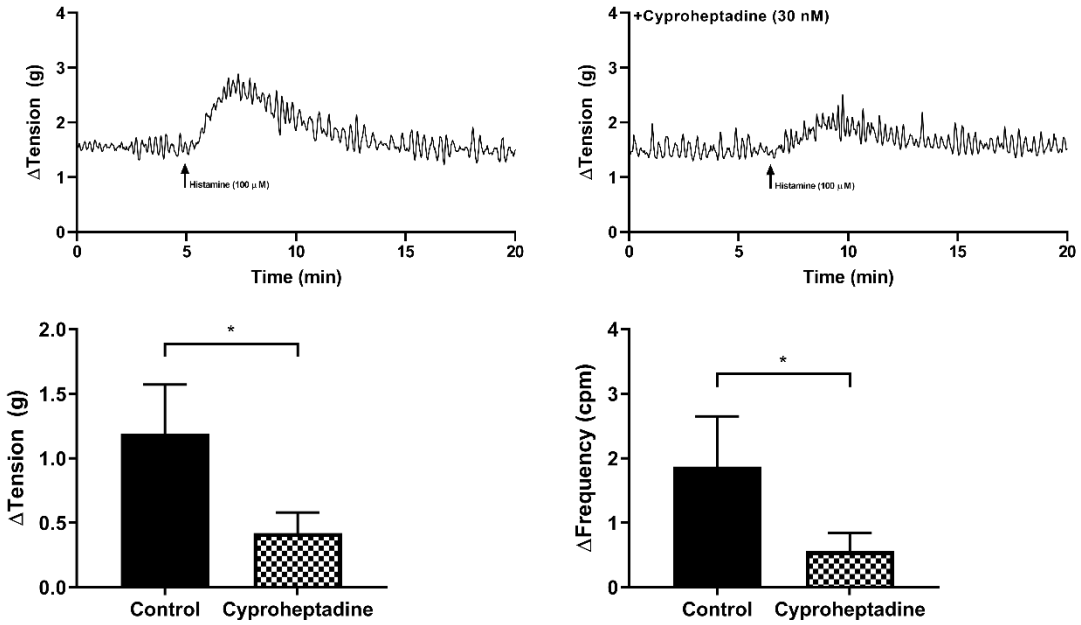

Figure 3-4: U&LP tonic contractions in response to histamine (100 μM, *top row*) as control (*left*) and in the presence of histamine 1 (H1) receptor antagonist cyproheptadine (30 nM, *right*). Changes in tonic contractions (*bottom left*) and in the frequency of phasic activity (*bottom right*) in response to histamine are represented as mean change ± SEM. Changes in tonic contractions between control and experimental conditions were evaluated using a paired Student's two-tailed *t*-test, where *p < 0.05

U&LP

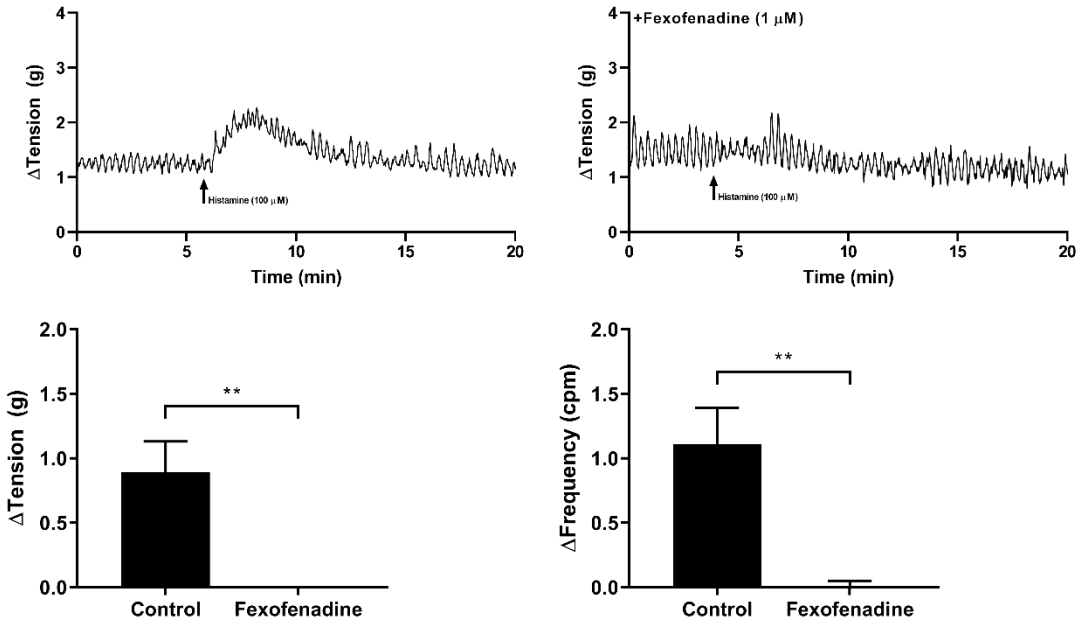

Figure 3-5: U&LP tonic contractions in response to histamine (100 μM, *top row*) as control (*left*) and in the presence of histamine 1 (H1) receptor antagonist fexofenadine (1 μM, *right*). Changes in tonic contractions (*bottom left*) and in the frequency of phasic activity (*bottom right*) in response to histamine are represented as mean change ± SEM. Changes in tonic contractions between control and experimental conditions were evaluated using a paired Student's two-tailed *t*-test, where **$p < 0.01$

U&LP

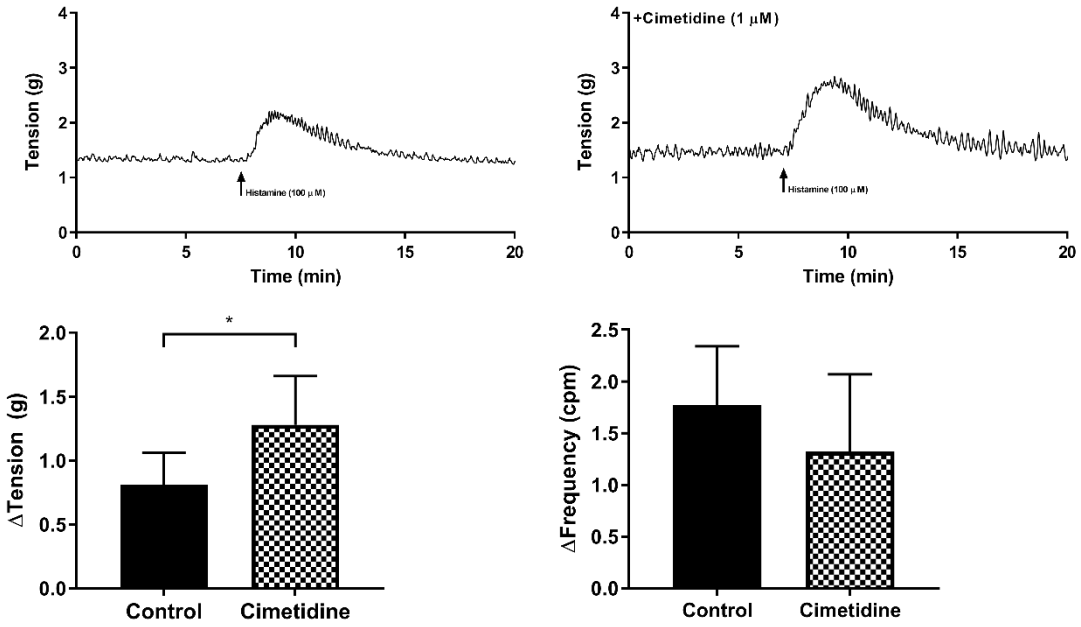

Figure 3-6: U&LP tonic contractions in response to histamine (100 μM, *top row*) as control (*left*) and in the presence of histamine 2 (H2) receptor antagonist cimetidine (1 μM, *right*). Changes in tonic contractions (*bottom left*) and in the frequency of phasic activity (*bottom right*) in response to histamine are represented as mean change ± SEM. Changes in tonic contractions between control and experimental conditions were evaluated using a paired Student's two-tailed *t*-test, where *$p < 0.05$

U&LP

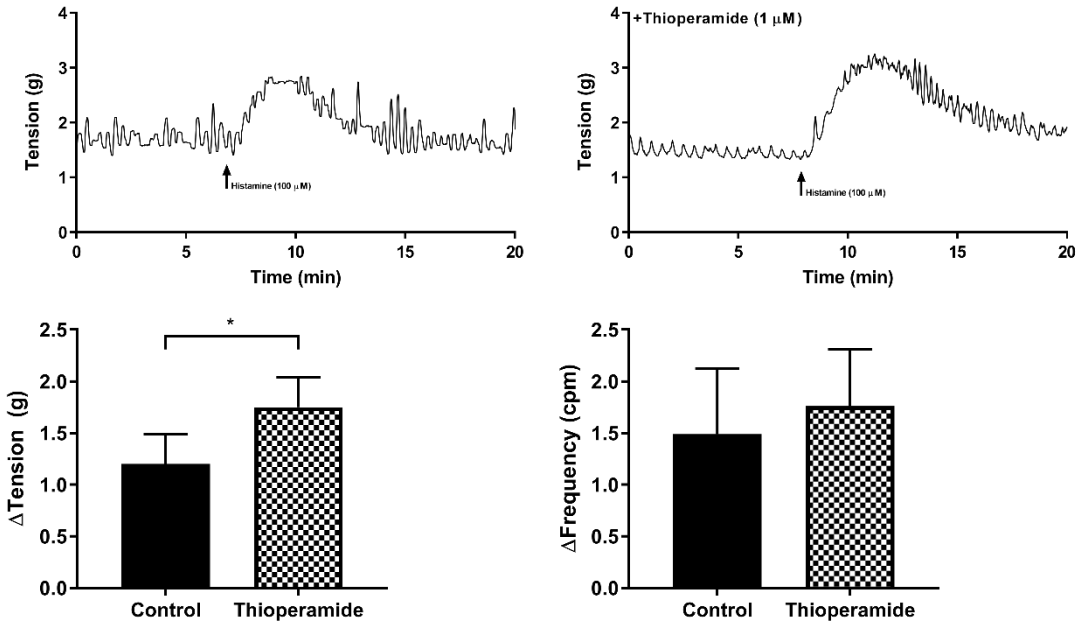

Figure 3-7: U&LP tonic contractions in response to histamine (100 μM, *top row*) as control (*left*) and in the presence of histamine 3 and 4 (H3/H4) receptor antagonist thioperamide (1 μM, *right*). Changes in tonic contractions (*bottom left*) and in the frequency of phasic activity (*bottom right*) in response to histamine are represented as mean change ± SEM. Changes in tonic contractions between control and experimental conditions were evaluated using a paired Student's two-tailed *t*-test, where *p < 0.05

3.4.4. Combination of selective histamine receptor antagonists in U&LP

In experiments where combinations of antagonists were used, the ability of cimetidine (1 μM) to enhance contraction to histamine (100 μM) was abolished when tissues were also treated with pyrilamine (30 nM, $n = 8$, $p < 0.001$, Figure 3-8). Alternatively, the effectiveness of pyrilamine (30 nM) inhibiting histamine response (100 μM) was equally effective in baths containing a combination of pyrilamine (30 nM) and cimetidine (1 μM, $n = 8$, Figure 3-9).

Thioperamide (1 μM) was added to tissues pre-treated with pyrilamine (30 nM) and cimetidine (1 μM, $n = 8$) to isolate the potential influence of H3 and H4 receptors. Responses to histamine (100 μM) in the presence of thioperamide (1 μM) exhibited a small but observable increase in tonic contractions. However, this increase was not significant (Figure 3-10).

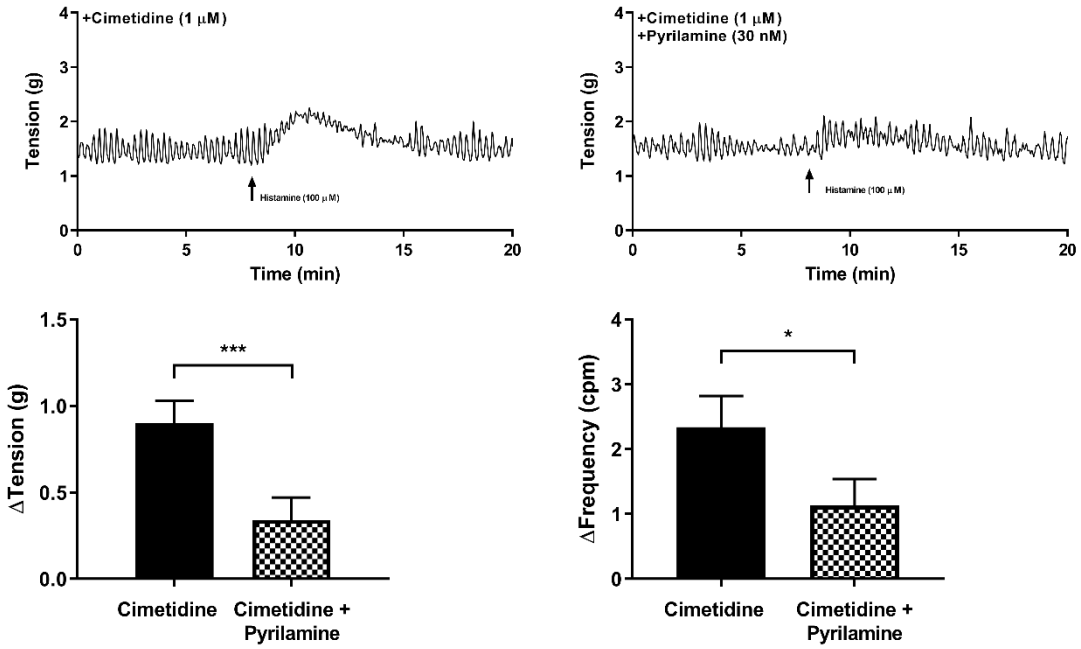

Figure 3-8: Comparison of U&LP tonic contractions in response to histamine (100 μM, *top row*) in the presence of H2 receptor antagonist cimetidine (1 μM, *left*) and in the presence of the combination of H1 and H2 receptor antagonists cimetidine (1 μM) and pyrilamine (30 nM, *right*). Changes in tonic contractions (*bottom left*) and in the frequency of phasic activity (*bottom right*) in response to histamine are represented as mean change ± SEM. Changes in tonic contractions between control and experimental conditions were evaluated using a paired Student's two-tailed *t*-test, where *$p < 0.05$, ***$p < 0.001$

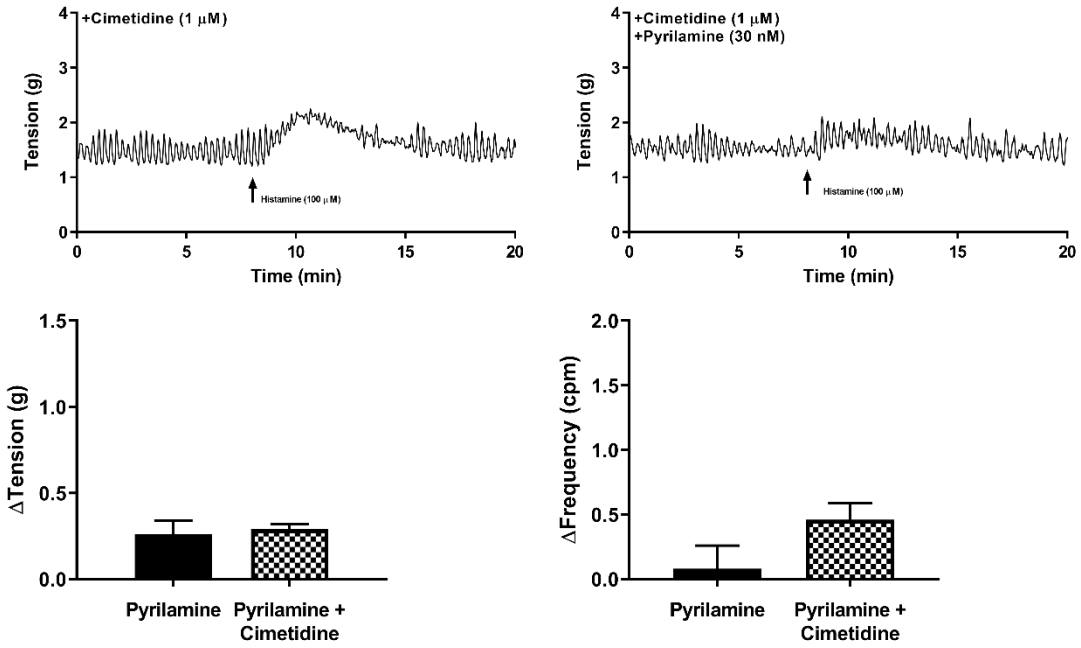

Figure 3-9: Comparison of U&LP tonic contractions in response to histamine (100 µM, *top row*) in the presence of H1 receptor antagonist pyrilamine (30 nM, *left*) and in the presence of the combination of H1 and H2 receptor antagonists pyrilamine (30 nM) and cimetidine (1 µM, *right*). Changes in tonic contractions (*bottom left*) and in the frequency of phasic activity (*bottom right*) in response to histamine are represented as mean change ± SEM. Changes in tonic contractions between control and experimental conditions were evaluated using a paired Student's two-tailed *t*-test, NSD.

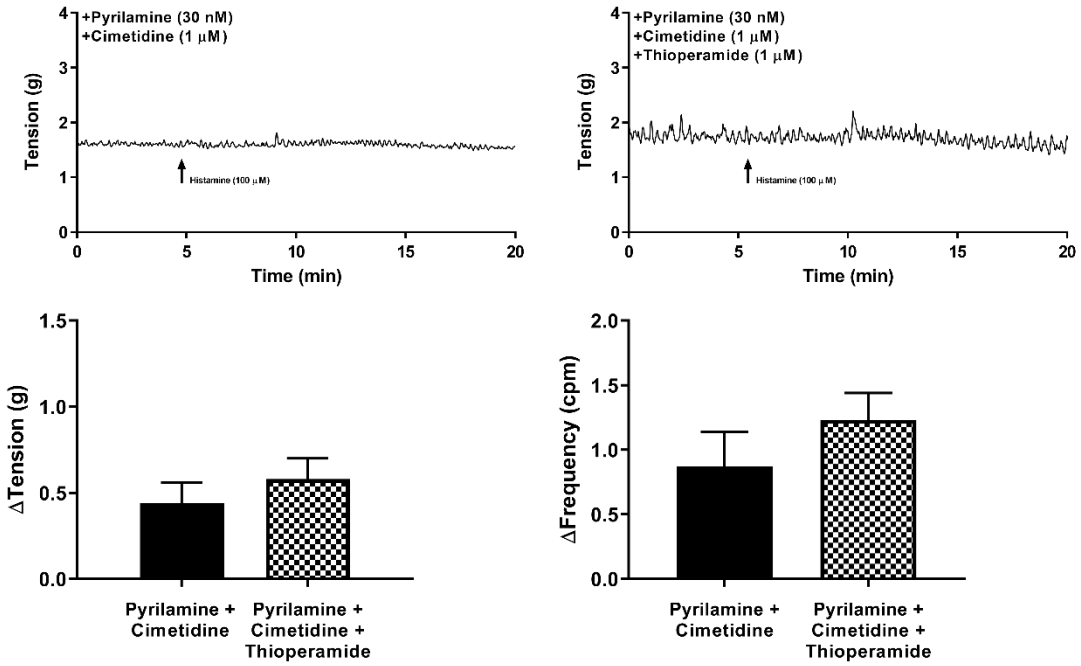

Figure 3-10: Comparison of U&LP tonic contractions in response to histamine (100 μM, *top row*) in the presence of the combination of H1 and H2 receptor antagonist pyrilamine (30 nM) and cimetidine (1 μM, *left*) and in the presence of all histamine receptor (H1-4) antagonists pyrilamine (30 nM), cimetidine (1 μM) and thioperamide (1 μM, *right*). Changes in tonic contractions (*bottom left*) and in the frequency of phasic activity (*bottom right*) in response to histamine are represented as mean change ± SEM. Changes in tonic contractions between control and experimental conditions were evaluated using a paired Student's two-tailed *t*-test, NSD.

3.4.5. Selective histamine receptor antagonists on the detrusor

In the presence of 30 nM pyrilamine (H1 antagonist), responses to histamine (100 μM) were significantly inhibited in comparison to the control tissues ($n = 8$, $p < 0.05$, Table 3-4, Figure 3-11). Treatment with histamine (100 μM) in the presence of alternative H1 antagonist cyproheptadine (30 nM, $n = 8$, $p < 0.01$, Figure 3-12) fexofenadine (1 μM, $n = 8$, $p < 0.05$, Figure 3-13) also showed significantly inhibited increases in the tonic contractions ($p < 0.05$, Table 3-4). In the presence of 1 μM cimetidine (H2 antagonist, $n = 8$, Figure 3-14) and 1 μM thioperamide (H3 and H4 antagonist, $n = 4$, Figure 3-15) no significant differences between control and experimental tissues were observed (Table 3-4).

Table 3-4 – Tonic contractions: Detrusor tonic contractions in response to histamine (100 μM) in the presence of histamine receptor antagonists (mean change ± SEM).

			ΔTension (g)		
Antagonist	Receptor	Conc.	Absence	Presence	n
Pyrilamine	H1	30 nM	0.98 ± 0.30	0.27 ± 0.12*	8
Cyproheptadine	H1	30 nM	0.62 ± 0.21	0.07 ± 0.03**	8
Fexofenadine	H1	1 μM	0.47 ± 0.05	0.08 ± 0.04*	8
Cimetidine	H2	1 μM	0.63 ± 0.17	0.29 ± 0.08	8
Thioperamide	H3/H4	1 μM	0.38 ± 0.12	0.27 ± 0.07	4

*p < 0.05, **p < 0.01. Paired Student's two-tailed *t*-test.

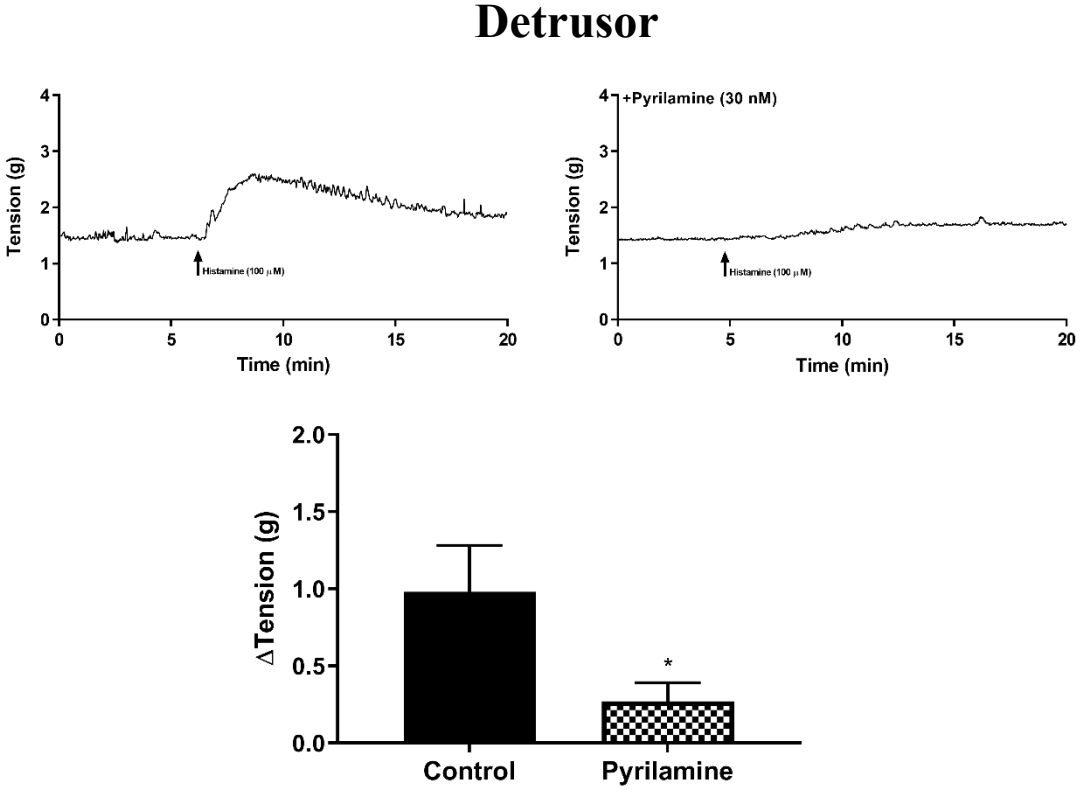

Figure 3-11: Detrusor tonic contractions in response to histamine (100 μM, *top row*) as control (*left*) and in the presence of histamine 1 (H1) receptor antagonist pyrilamine (30 nM, *right*). Increases in tonic contractions after treatment with histamine are represented as mean change ± SEM (*bottom*). Changes in tonic contractions between control and experimental conditions were evaluated using a paired Student's two-tailed *t*-test, where *$p < 0.05$

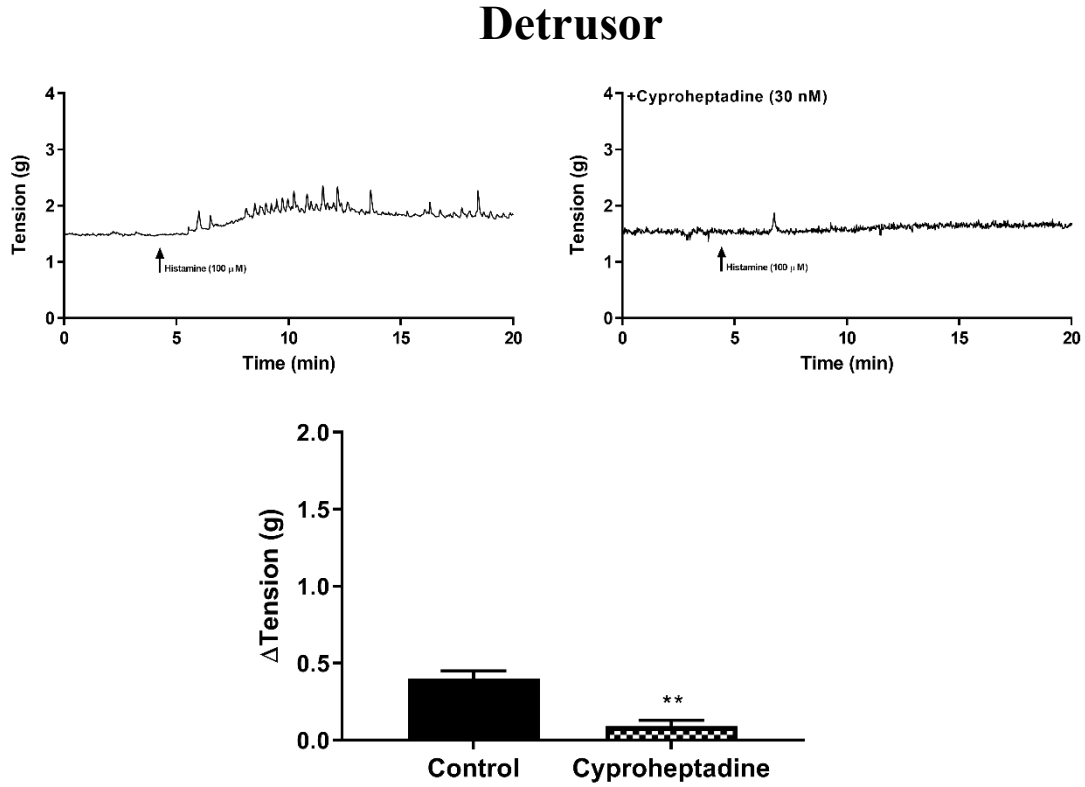

Figure 3-12: Detrusor tonic contractions in response to histamine (100 μM, *top row*) as control (*left*) and in the presence of histamine 1 (H1) receptor antagonist cyproheptadine (30 nM, *right*). Increases in tonic contractions after treatment with histamine are represented as mean change ± SEM (*bottom*). Changes in tonic contractions between control and experimental conditions were evaluated using a paired Student's two-tailed *t*-test, where **p < 0.01

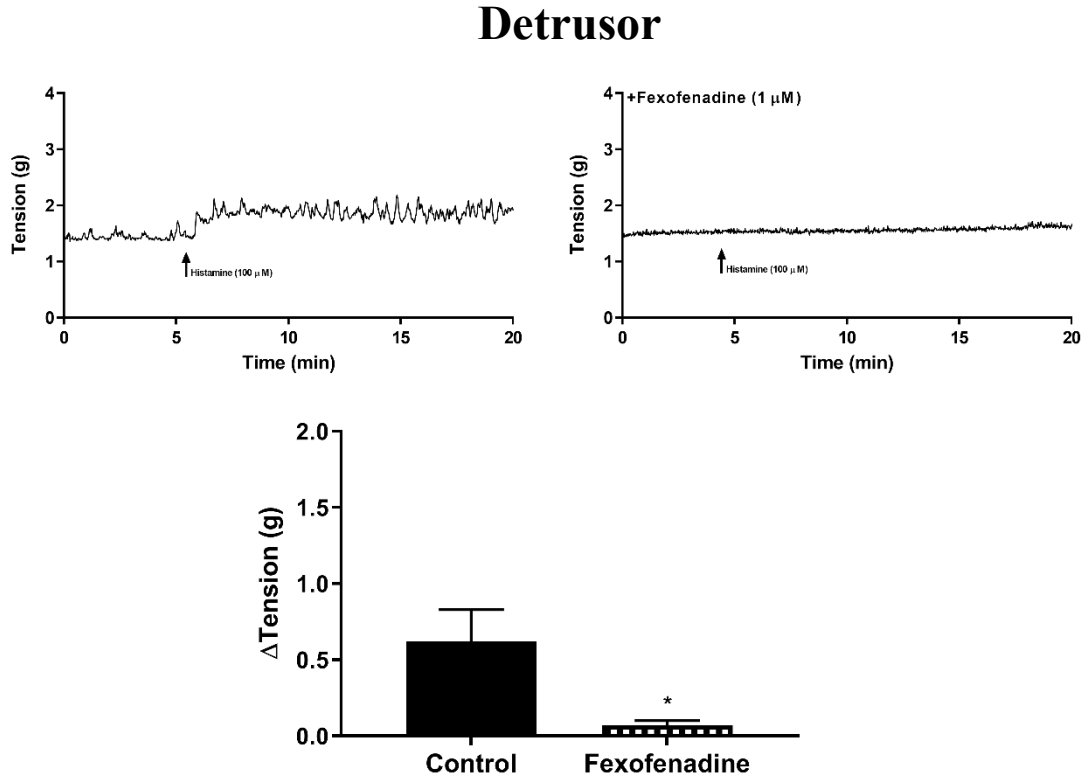

Figure 3-13: Detrusor tonic contractions in response to histamine (100 μM, *top row*) as control (*left*) and in the presence of histamine 1 (H1) receptor antagonist fexofenadine (1 μM, *right*). Increases in tonic contractions after treatment with histamine are represented as mean change ± SEM (*bottom*). Changes in tonic contractions between control and experimental conditions were evaluated using a paired Student's two-tailed *t*-test, where *p < 0.05

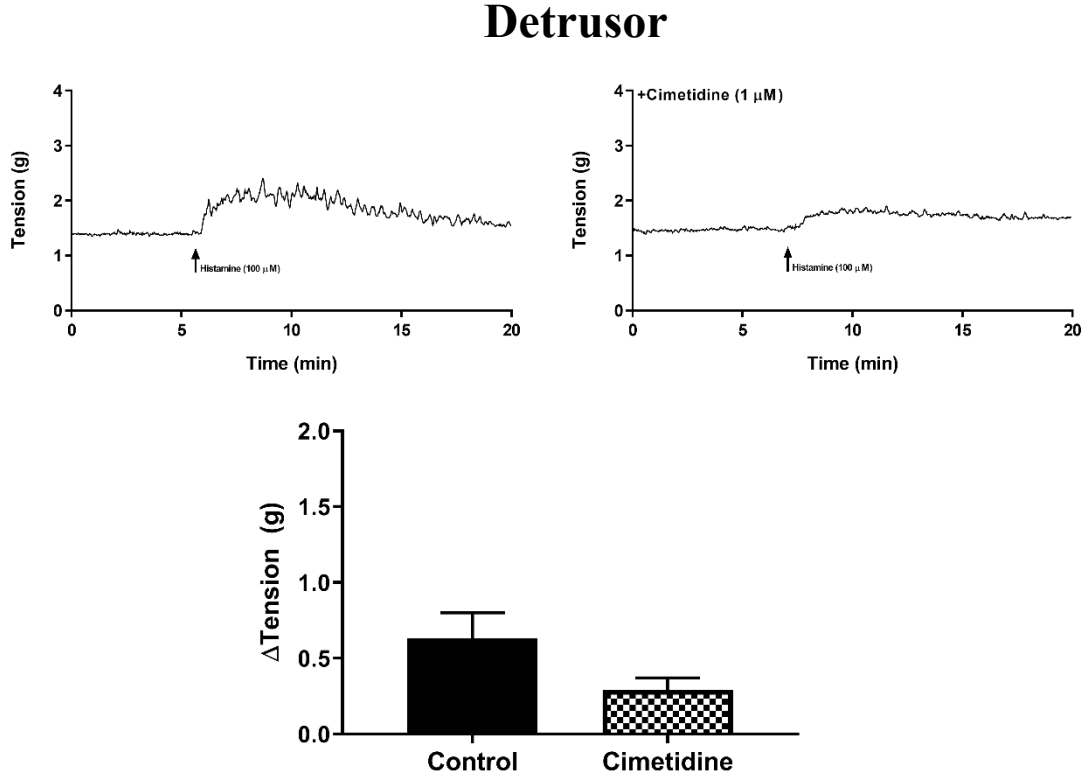

Figure 3-14: Detrusor tonic contractions in response to histamine (100 μM, *top row*) as control (*left*) and in the presence of histamine 2 (H2) receptor antagonist cimetidine (1 μM, *right*). Increases in tonic contractions after treatment with histamine are represented as mean change ± SEM (*bottom*). Significant changes in tonic contractions between control and experimental conditions were evaluated using a paired Student's two-tailed *t*-test, NSD.

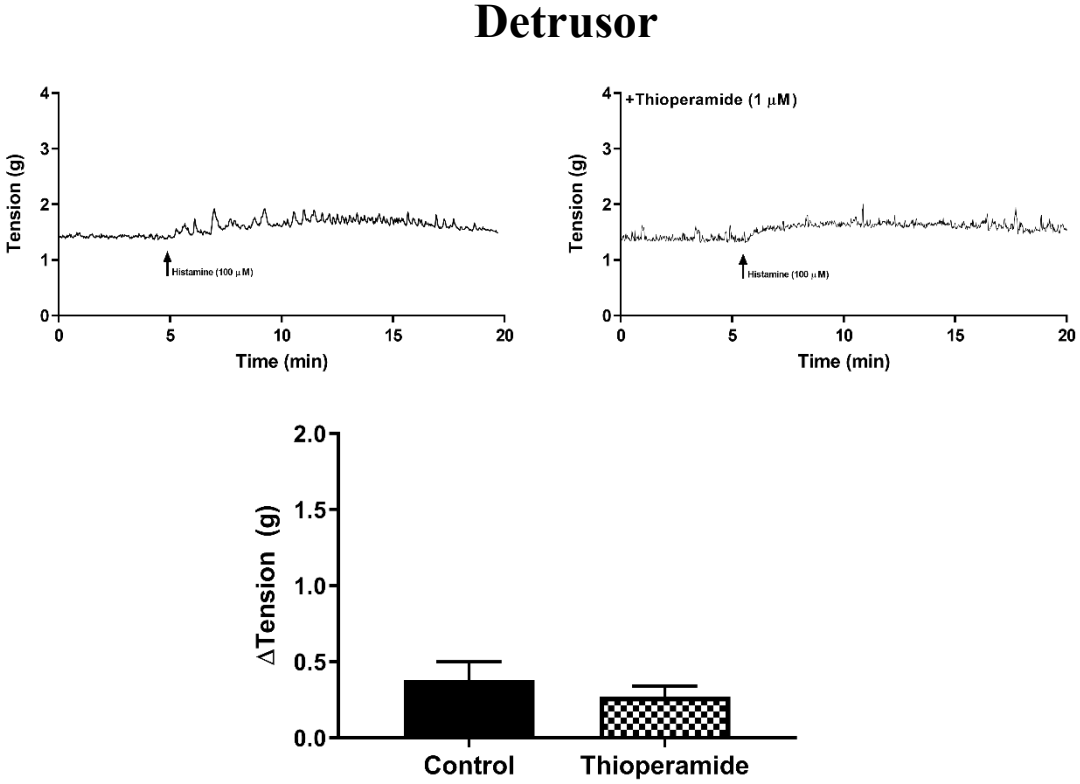

Figure 3-15: Detrusor tonic contractions in response to histamine (100 μM, *top row*) as control (*left*) and in the presence of histamine 3 and 4 (H3/H4) receptor antagonist thioperamide (1 μM, *right*). Increases in tonic contractions after treatment with histamine are represented as mean change ± SEM (*bottom*). Changes in tonic contractions between control and experimental conditions were evaluated using a paired Student's t-test, two-tailed NSD.

3.5. Discussion

Data obtained in this study have provided several novel findings to aid in the understanding of histamine's influence on bladder contractility. There are four main findings. 1) Histamine causes an increase in tonic contractions in both the U&LP and detrusor layers. 2) In U&LP, histamine not only increases tonic contractions but also influences the frequency and amplitude of spontaneous phasic contractions observed in the U&LP tissue. 3) Activation of muscarinic receptors, as suggested in previous literature (Rubinstein et al., 1987), is not involved in the contractile response to histamine. 4) H2 receptor activation stimulates relaxation of U&LP in response to histamine.

This study focused on the role of histamine receptors on the U&LP and detrusor, two distinct layers of the bladder. Previous research has identified increased mast cells within the lamina propria layers of those suffering from overactive bladder and interstitial cystitis/bladder pain syndrome (Liu et al., 2012), It is possible that degranulates from these infiltrating mast cells could be causing the observed inflammation via the release of inflammatory mediators such as histamine and prostaglandins (Borish and Joseph, 1992). In addition to mast cells, other immune cells of the body are also capable of releasing histamine, such as basophils and neutrophils at sites of inflammation.

Furthermore, biopsies of patients suffering from OAB have demonstrated clear histological evidence of chronic inflammation (Andersson and Wein, 2004, Apostolidis et al., 2008). Previously, functional studies have shown that histamine is capable of causing contractions in human detrusor strips (Palea et al., 1993), guinea pig bladder (Khanna et al., 1977, Kondo

et al., 1985, Poli et al., 1988) and rabbit detrusor (Fredericks, 1975). However, this study is the first to identify and compare the responses to histamine in two distinct layers of the bladder that differ in structure and function: U&LP and detrusor. U&LP is comprised of several layers of epithelial cells (urothelium) that line the lumen of the bladder and the underlying connective tissue layer (lamina propria), whereas detrusor is made up of smooth muscle cells.

It was determined that in the presence of the H1 antagonist pyrilamine, increases in tonic contractions in response to histamine were significantly inhibited in both U&LP and detrusor preparations. These findings are consistent with past studies showing pyrilamine's effectiveness in inhibiting contractions to histamine in rabbit detrusor tissue (Van Buren and Anderson, 1979) and cultured human detrusor cells (Neuhaus et al., 2006). It is known that U&LP is capable of developing spontaneous phasic contractions in the absence of any stimulation (Moro et al., 2011). Significant increases in phasic contractions were observed when tissues were treated with a single dose of histamine in the absence of any antagonists. However, when the H1 receptor was blocked, the frequency of spontaneous phasic contractions did not increase. That indicates that the H1 receptor not only is responsible for mediating the changes in tonic contractions but also in the frequency of spontaneous phasic contractions in response to histamine.

To further isolate the role of H1 in mediating contractions, combinations of antagonists were used. Whenever an H1 antagonist was present in the antagonist combination, increases in tonic contractions in response to histamine were significantly inhibited. These findings ascertain the involvement of the H1 receptor in mediating contractions in the presence of histamine and further the conclusions from previous studies that present the involvement of

H1 receptor in the mediation of bladder contractile responses (Khanna et al., 1977, Kondo et al., 1985).

The role of the H2 receptor was established through agonist and antagonist studies. When tissues were treated with amthamine, which is a potent and highly selective H2 agonist, a relaxation in tonic contractions was observed in U&LP samples, but not in detrusor preparations. The addition of an H2 antagonist, cimetidine, caused increases in tonic contractions in the U&LP but did not affect the frequency of spontaneous phasic contractions. The lack of responses to either the H2 antagonist cimetidine or agonist amthamine in detrusor preparation indicates that the H2 receptor subtype, even though present in the tissue, is not involved in detrusor contractions. The physiological role of the contrasting effects from activation of the H1 and H2 receptor systems remains unclear. Previous research identified that the H2-receptor located on non-cholinergic excitatory neurons may be involved in the inhibitory action produced by histamine in guinea pig bladder (Taniyama et al., 1984). This leads to a potential for some of the role of these systems to be neurogenic in origin, given that nerve varicosities are located in the lamina propria.

When the combined H3 and H4 receptor antagonist thioperamide was added to the U&LP, increases in tonic contractions in response to histamine were observed. This increase in tonic contractions most likely occurred due to the antagonist having an affinity for the other receptor subtypes present in the tissue. These findings were supported by experiments involving several antagonists. When U&LP was treated with all four receptor antagonists, the same small increase in tonic contractions was observed, indicating that H3 and H4 receptors had no role in mediating the increase in tension.

Previous literature suggests that increases in tonic contractions to histamine may be due to a subsequent release of acetylcholine, acting on muscarinic receptors (Rubinstein et al., 1987) rather than an influence of histamine itself. However, the lack of any inhibition from atropine to the histamine response or receptor antagonism in this study suggests no involvement of muscarinic receptors. Any small increases in tonic contractions observed in the presence of the H1 antagonist pyrilamine are likely due to incomplete antagonism, as the concentrations were relatively low.

There were several differences between U&LP and detrusor tissue in response to histamine. It was determined that H1 and H2 receptors were only functional in U&LP tissue. Even though western blot analysis has shown all four histamine receptor subtypes present in the detrusor (Neuhaus et al., 2006), solely H1 was functional and contractions were mediated by H1 receptors in both tissues. As epithelial cells of the urothelium have no contractile properties, the cells involved in the contraction are likely the myofibroblasts or muscularis mucosa cells found in the lamina propria. Given the size and distribution of these cells within the connective tissue, they stimulate a strong contraction.

3.6. Conclusions

Histamine influences the contractile activity of both U&LP and detrusor of the urinary bladder. It was determined that stimulation of the H1 receptor results in contractions in both U&LP and detrusor tissue samples. Activation of the H2 receptor inhibits the H1-mediated contractions in the urothelium/lamina propria but not the detrusor, with H3 and H4 receptors having no functional role in bladder contractility. This study presents the H1 and H2

receptors as a potential target for future treatments for overactive bladder and other bladder contractile diseases.

Chapter 4

The influence of five major prostaglandins on stimulating contractions and spontaneous activity of the urinary bladder urothelium, lamina propria and detrusor

The content in the following chapter is presented as the author's copy of the published document before the reviewer and publisher's requested alterations. The final version of this manuscript can be accessed from the relevant peer-reviewed journal. Formatting and terminology changes have been made to maintain consistency throughout this thesis.

All data presented in this manuscript are the sole work of Zane Stromberga.

PUBLISHED MANUSCRIPT

Stromberga, Z., Chess-Williams, R., & Moro, C. (2020). The influence of five major prostaglandins on stimulating contractions and spontaneous activity of the urinary bladder urothelium, lamina propria and detrusor. *BMC Urology, 20(1). https://doi.org/10.1186/s12894-020-00619-0*

Published abstracts and conference presentations arising from this chapter:

Stromberga, Z., Chess-Williams, R., Moro, C. (2019, November). The role of prostaglandin E2 in mediating urinary bladder contractions. *Proceedings of the ASCEPT-PAGANZ joint scientific meeting, Queenstown, New Zealand.*

4.1. Abstract

Background: Inflammation is often associated with several bladder dysfunction, including overactive bladder (OAB) and interstitial cystitis/bladder pain syndrome (IC/PBS). As such, bladder inflammation and the actions of inflammatory mediators may contribute to the development of the urinary symptoms. This study assessed the actions of PGE_2, PGF_2, PGD_2, TXA_2, and PGI_2 on urinary bladder urothelium with lamina propria (U&LP), as well as the detrusor smooth muscle.

Method: Studies were carried out using isolated tissue baths, where strips porcine bladder U&LP or detrusor were exposed to varying concentrations of prostaglandin agonists (1 μM and 10 μM).

Results: All assessed prostaglandin agonists contracted both the U&LP and detrusor smooth muscle, with the rank order of contractile response effectiveness as $PGE_2 > PGF_{2\alpha} > TXA_2 > PGD_2 > PGI_2$. In U&LP, treatment with PGE_2 (10 μM) increased tonic contractions by 1.36 ± 0.09 g ($n = 42$, $p < 0.001$) and phasic contractions by 40.4 ± 9.6% ($n = 42$, $p < 0.001$). In response to $PGF_{2\alpha}$ (10 μM), U&LP tonic contractions increased by 0.79 ± 0.06 g ($n = 14$, $p < 0.001$) and phasic activity by 13.3% ± 5.3% ($n = 15$, $p < 0.05$). In detrusor preparations, PGE_2 (10 μM) increased tonic contractions by 1.32 ± 0.13 g ($n = 38$, $p < 0.001$) and $PGF_{2\alpha}$ (10 μM) by 0.97 ± 0.14 g ($n = 12$, $p < 0.001$). Only 34% ($n = 48$) of all detrusor preparations exhibited spontaneous activity before the addition of an agonist at a frequency of 2.03 ± 0.12 cpm. In preparations that did not exhibit initial phasic activity, all of the prostaglandin agonists were capable of commencing the phasic activity.

Conclusions: The urinary bladder U&LP and detrusor respond to a variety of prostaglandin agonists, with their activation resulting in direct contractions, as well as increases to spontaneous contractile activity. This study presents the prostaglandin receptor system as a potential therapeutic target for lower urinary tract dysfunction.

4.2. Introduction

Urinary bladder inflammation has been observed in various lower urinary tract dysfunctions, including interstitial cystitis/painful bladder syndrome (IC/BPS) (Grover et al., 2011) and overactive bladder (OAB) (Compérat et al., 2006, Loran et al., 2007). It is also widely reported that there is an increase in the presence of inflammatory mediators within the bladder wall (Kastrup et al., 1983, Liu et al., 2010) and urine (El-Mansoury et al., 1994, Jacobs et al., 2010, Furuta et al., 2018) of patients suffering from these conditions. Mediators include histamine, nerve growth factor, proteases and chemokines released from nearby mast cells (Theoharides et al., 2007, Amin, 2012), serotonin (Matsumoto-Miyai et al., 2016) and prostaglandins synthesised in the bladder wall (Rahnama'i et al., 2013).

Furthermore, significantly increased expression of histamine receptors has been noted in patients with BPS/IC (Shan et al., 2019). The actions of inflammatory mediators not only can cause urinary bladder contractions (Saban et al., 2007, Moro et al., 2016, Stromberga et al., 2019) but also are known to sensitise afferent nerve endings, resulting in an increased spinal cord neuronal activation (Davidson et al., 2014, Grundy et al., 2019). Therefore, inflammation and the actions of these pro-inflammatory mediators may contribute to the

development of urinary frequency and urgency symptoms observed in OAB, and pain in IC/BPS.

The involvement of prostaglandins in bladder physiology was first recognised from their release during, or immediately after urinary bladder distension or inflammatory injury of the urothelium (Gilmore and Vane, 1971, Rahnama'i et al., 2012). An increase of prostaglandins in the urine of patients suffering from OAB has been well-reported previously (Reyes and Klahr, 1990, Kim, 2005, Kim et al., 2006, Tanaka et al., 2010), suggesting the prostaglandin system as a potential future therapeutic target in various bladder disorders. The exact role and mechanism of endogenous prostaglandins in the urinary bladder are not well understood. However, previous studies utilising exogenous prostaglandins have shown that these chemicals can alter contractility and the micturition reflex in human bladders (Andersson et al., 1977).

Prostaglandin production is generally low in healthy tissue, but can increase immediately following acute inflammation (Ricciotti and FitzGerald, 2011). They are synthesised in the bladder by cyclooxygenase (COX) and then subsequently converted into five primary prostanoids via their respective synthases: PGE_2, PGD_2, $PGF_{2\alpha}$, prostacyclin (PGI_2) and thromboxane (TXA_2) (Khan et al., 1998). Prostaglandins are synthesised in both the bladder urothelium with lamina propria (U&LP) and in detrusor smooth muscle in response to stretch, nerve stimulation, U&LP damage or other inflammatory mediators (Andersson, 2002, Rahnama'i et al., 2013). The production of prostaglandins is determined by the cells present at sites of inflammation capable of synthesising prostaglandins and the activity of the two cyclooxygenase isoenzymes, namely COX-1 and COX-2. For example, macrophages predominantly generate PGE_2 and TXA_2, whereas mast cells produce PGD_2

(Tilley et al., 2001). COX-1 is present in most cells, whereas the expression of COX-2 is generally low in cells, but can increase dramatically upon stimulation by immune cells (Smith and Dewitt, 1996). Prostaglandin I_2 is the main prostaglandin synthesised in the human bladder, followed by PGE_2, $PGF_{2\alpha}$ and PGA_2 (Jeremy et al., 1987, Masunaga et al., 2006).

These five prostaglandins exert their function by activating eight different G-protein-coupled receptors. These receptors include EP1, EP2, EP3 and EP4 subtypes of prostaglandin E_2 receptor; FP receptor for $PGF_{2\alpha}$; TP receptor for thromboxane; DP receptor for PGD_2 and IP receptor for PGI_2 (Woodward et al., 2011). The majority of studies conducted on the effects of prostaglandins in the urinary bladder have focused specifically on PGE_2-mediated contractions. Generally, the stimulation EP1 and EP3 receptors are thought to cause bladder contractions, whereas EP2 and EP4 receptors induce bladder relaxation (Coleman et al., 1994). Indeed, the EP1 receptor is involved in initiating micturition in both humans and animals and has shown to play a role in bladder overactivity in an animal model of bladder obstruction (Lee et al., 2007).

In a guinea pig model, application of PGE_2 has produced increases in amplitude of urinary bladder phasic contractions without affecting frequency (Rahnama'i et al., 2013). Furthermore, the 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 stimulation of EP1 receptors resulting in an increased afferent activation (Wang et al., 2008). The role of other prostaglandins in the urinary bladder has also been explored, albeit to a lesser extent. PGD_2, the major prostaglandin released from mast cells at sites of inflammation, has been shown to cause inhibitory effects on detrusor

smooth muscle cells (Guan et al., 2014, Guan et al., 2015). The use of PGI_2 antagonists has been shown to decrease neurogenic detrusor overactivity (Khera et al., 2007) and the frequency of bladder contractions in citric-acid induced detrusor overactivity (Cefalu et al., 2007) of rat models, but the actions of the agonist on the layers of the bladder are unclear. Thromboxane and $PGF_{2\alpha}$ have been shown to induce direct contractions of the isolated human detrusor (Palea, 1998); however, it is unclear how these mediators affect the urothelium with lamina propria.

Although past studies have explored the effects of the different prostaglandins on the urinary bladder with a large focus on the actions of PGE_2, a complete understanding of the contractile effects of the other four prostaglandins on the urinary bladder remain unclear. Specifically of interest is to determine how the actions of the prostaglandins affect urothelium with lamina propria that is separated from the detrusor smooth muscle. Therefore, this study aimed to determine the influence of PGE_2, $PGF_{2\alpha}$, PGD_2, TXA_2 PGI_2 on urinary bladder urothelium with lamina propria and detrusor smooth muscle contractions and spontaneous activity.

4.3. Materials and methods

4.3.1. Tissue preparation

Urinary bladders were obtained from Large White-Landrace pigs (approximately six months old, weighing between 80 and 100 kg) from the local abattoir after slaughter for the routine commercial provision of food. As no animals were bred, harmed, culled, interfered, or

interacted with as part of this research project, Animal Ethics Approval was not required for offal use (Queensland Government, 2016). Tissue strips of U&LP and detrusor were prepared and set up as described previously in Chapter 2.

Adjacent strips of U&LP and detrusor (10 mm x 5 mm) were tied vertically between an isometric force transducer (MCT050/D, ADInstruments, Castle Hill, Australia) and a fixed hook in 10 mL organ baths (Labglass, Brisbane, Australia), and superfused with Krebs-bicarbonate solution (NaCl 118.4 mM, $NaHCO_3$ 24.9 mM, $CaCl_2$ 1.9 mM, $MgSO_4$ 2.41 mM, KCl 4.6 mM, KH_2PO_4 1.18 mM and D-glucose 11.7 mM) and carbogen gas (95% oxygen and 5% carbon dioxide) at 37°C. After tissue mounting, strips of U&LP and detrusor were washed three times, tension adjusted to 1.5 – 2.0 g and tissues left to equilibrate for 30 min. After the equilibration period, a single dose of a prostaglandin receptor agonist was added to the tissue strip. A set of adjanced strips (control with experimental strip) was exposed one prostaglandin over the course of the experiment.

4.3.2. Pharmacological agents

The following compounds were used in this study: prostaglandin E_2, prostaglandin $F_{2\alpha}$, prostaglandin D_2, prostaglandin I_2 and thromboxane A_2 (U-46619, Cayman Chemicals, Michigan, USA). Prostaglandin E_2, prostaglandin $F_{2\alpha}$, prostaglandin D_2, and prostaglandin I_2 were dissolved in 100% ethanol and diluted with distilled H_2O. U-46619 was supplied as a solution in methyl acetate, which was diluted with distilled H_2O. Two concentrations of each prostaglandin receptor agonists were selected; 1 μM and 10 μM.

4.3.3. Data analysis

Data were graphed and analysed using GraphPad Prism version 8.3 for Windows (GraphPad Software, La Jolla, California, USA). Statistical analysis was conducted using either a paired or unpaired Student's two-tailed *t*-test depending on the sample used, where $p < 0.05$ was considered as significant. All values were reported as mean change ± SEM. *N* equates to the number of individual bladder strips used in this study.

4.4. Results

4.4.1. Effect of prostaglandin agonists on the frequency of U&LP phasic contractions

Strips of U&LP exhibited spontaneous phasic contractions in the absence of any stimulation at a mean frequency of 3.26 ± 0.07 cycles per minute (cpm, n = 146) and an amplitude of 0.57 ± 0.02 g (n = 146). Treatment with PGE_2 caused the most prominent increases to U&LP spontaneous contractile activity. When PGE_2 (1 μM) was added to isolated tissues, spontaneous activity increased by 39.2% ± 6.7% (n = 38, $p < 0.001$, Figure 4-1). A greater concentration of PGE_2 (10 μM) showed similar increases of 40.4% ± 9.6% to the U&LP spontaneous activity (n = 42, $p < 0.001$). Treatment with $PGF_{2\alpha}$ showed smaller increases of 10.5% ± 4.6% to spontaneous activity when treated with 1 μM (n = 10, $p < 0.05$) and 13.3% ± 5.3% when treated with 10 μM (n = 14, $p < 0.05$). The addition of PGI_2 (10 μM) increased spontaneous activity by 6.2% ± 1.6% (n = 8, $p < 0.01$) but had no effect at a lower concentration (1 μM, n = 8). The frequency was not significantly affected by PGD_2 (1 - 10 μM, n = 12) or TXA_2 (1 - 10 μM, n = 16).

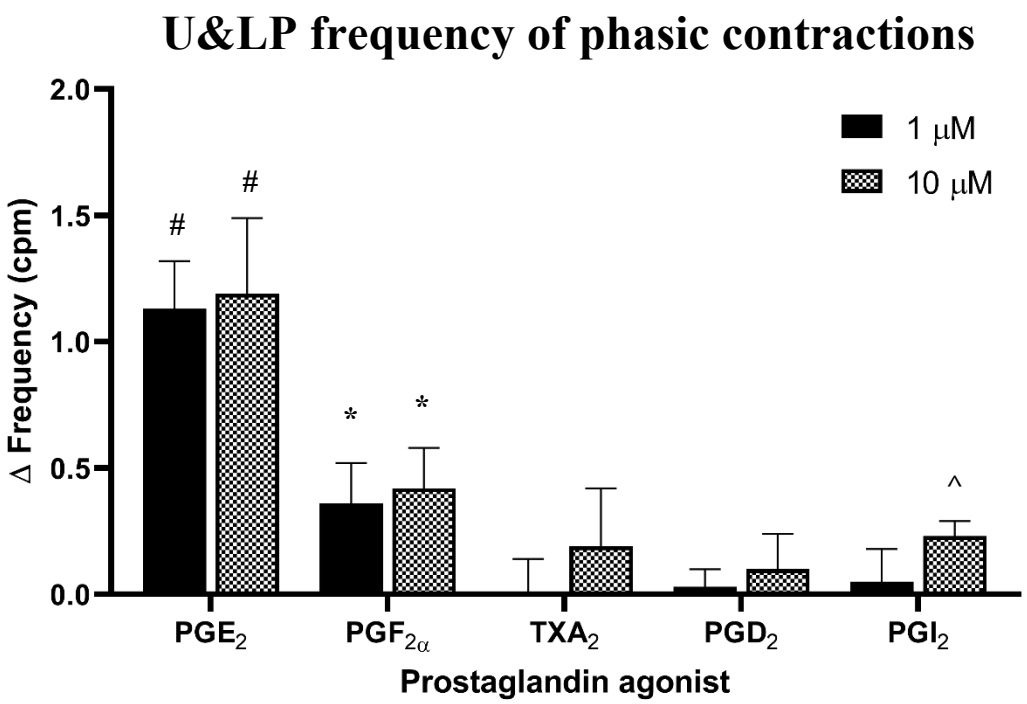

Figure 4-1 – Frequency of phasic contractions: U&LP changes in the frequency of spontaneous phasic contractions after the treatment with 1 μM and 10 μM of each specific prostaglandin agonists E_2 (1 μM: n = 38, 10 μM: n = 42), $F_{2\alpha}$ (1 μM: n = 10, 10 μM: n = 14), TXA_2 (1 μM: n = 8, 10 μM: n = 6), D_2 (1 μM: n = 4, 10 μM: n = 8), and I_2 (1 μM: n = 8, 10 μM: n = 8). There were no statistically significant differences in frequency changes between the 1 μM and 10 μM concentrations for any of the agonists (unpaired Student's two-tailed *t*-test). Changes in the frequency between control and experimental preparations for each dose were evaluated using a paired Student's two-tailed t-test, where $^p < 0.05$, $*p < 0.01$, $\#p < 0.001$

4.4.2. Effect of prostaglandin agonists on the amplitude of U&LP spontaneous phasic contractions

Decreases in the amplitude of spontaneous contractions were observed in response to treatment with PGE_2. The addition of 1 μM PGE_2 caused an amplitude decrease of 0.14 ± 0.04 g ($n = 38$, $p < 0.001$, Table 4-1). Similar decreases of 0.16 ± 0.03 g were also observed in response to a higher PGE_2 concentration (10 μM, $n = 42$, $p < 0.01$). Treatment with TXA_2 (1 μM) showed a significant decrease in the amplitude by 0.28 ± 0.06 g ($n = 8$, $p < 0.01$), which was not observed at a higher concentration (10 μM, $n = 6$). The addition of PGI_2 (10 μM) decreased amplitude of spontaneous activity by 0.14 ± 0.05 ($n = 8$, $p < 0.05$) but had no effect at a lower concentration (1 μM, $n = 8$). The amplitude of spontaneous contractions was not altered by the addition of either $PGF_{2\alpha}$ (1-10 μM, $n = 24$) or PGD_2 (1-10 μM, $n = 12$, Table 4-1). None of the decreases in the amplitude of spontaneous phasic contractions in the U&LP was significantly affected by the two different prostaglandin receptor agonist concentrations (1 μM and 10 μM).

Table 4-1 – Amplitude of phasic contractions: U&LP changes in amplitude of spontaneous phasic contractions in response to varying concentrations of prostaglandin receptor agonists PGE_2, $PGF_{2\alpha}$, TXA_2, PGD_2, and PGI_2 (1 μM and 10 μM).

	1 μM of agonist			10 μM of agonist		
Agonist	Absence	Presence	n	Absence	Presence	n
PGE_2	0.53 ± 0.05	0.40 ± 0.03***	38	0.53 ± 0.04	0.37 ± 0.03**	42
$PGF_{2\alpha}$	0.30 ± 0.03	0.29 ± 0.01	10	0.51 ± 0.06	0.46 ± 0.08	14
TXA_2	0.90 ± 0.16	0.62 ± 0.14**	8	0.75 ± 0.16	0.71 ± 0.25	6
PGD_2	0.59 ± 0.10	0.46 ± 0.04	4	0.55 ± 0.08	0.43 ± 0.06	8
PGI_2	0.64 ± 0.07	0.56 ± 0.07	8	0.57 ± 0.09	0.43 ± 0.06*	8

*p < 0.05, **p < 0.01, ***p < 0.001. Paired Student's two-tailed *t*-test.

4.4.3. Effect of prostaglandin agonists on U&LP tonic contractions

The addition of PGE_2 (1 μM) to isolated U&LP induced tissue contractions, with increases of 1.01 ± 0.08 g ($n = 38$, $p < 0.001$) to tonic contractions. When a greater concentration of PGE_2 (10 μM) was selected, increases of 1.36 ± 0.09 g ($n = 42$, $p < 0.001$, Figure 4-2) were observed. Treatment with 1 μM $PGF_{2\alpha}$ showed a small increase to tonic contractions of 0.15 ± 0.04 g ($n = 10$, $p < 0.01$) when compared to a higher concentration of 10 μM, which exhibited increases of 0.79 ± 0.06 g ($n = 14$, $p < 0.001$, Figure 4-3). The addition of two concentrations of TXA_2 induced similar contractions, where tonic contractions increased by 0.70 ± 0.07 g when treated with 1 μM ($n = 8$, $p < 0.001$), and by 0.65 ± 0.12 g after treatment with 10 μM ($n = 6$, $p < 0.001$, Figure 4-4). When PGD_2 (1 μM) was added to the U&LP tissue preparations, tonic contractions increased by 0.19 ± 0.04 g ($n = 4$, $p < 0.05$). Treatment with a higher concentration of PGD_2 (10 μM) exhibited increases of 0.63 ± 0.09 g ($n = 8$, $p < 0.001$, Figure 4-5). The addition of PGI_2 showed small increases in tonic contractions of 0.11 ± 0.02 g in response to 1 μM PGI_2 ($n = 8$, $p < 0.001$), and 0.22 ± 0.03 g in response to 10 μM PGI_2 ($n = 8$, $p < 0.001$, Figure 4-6).

U&LP

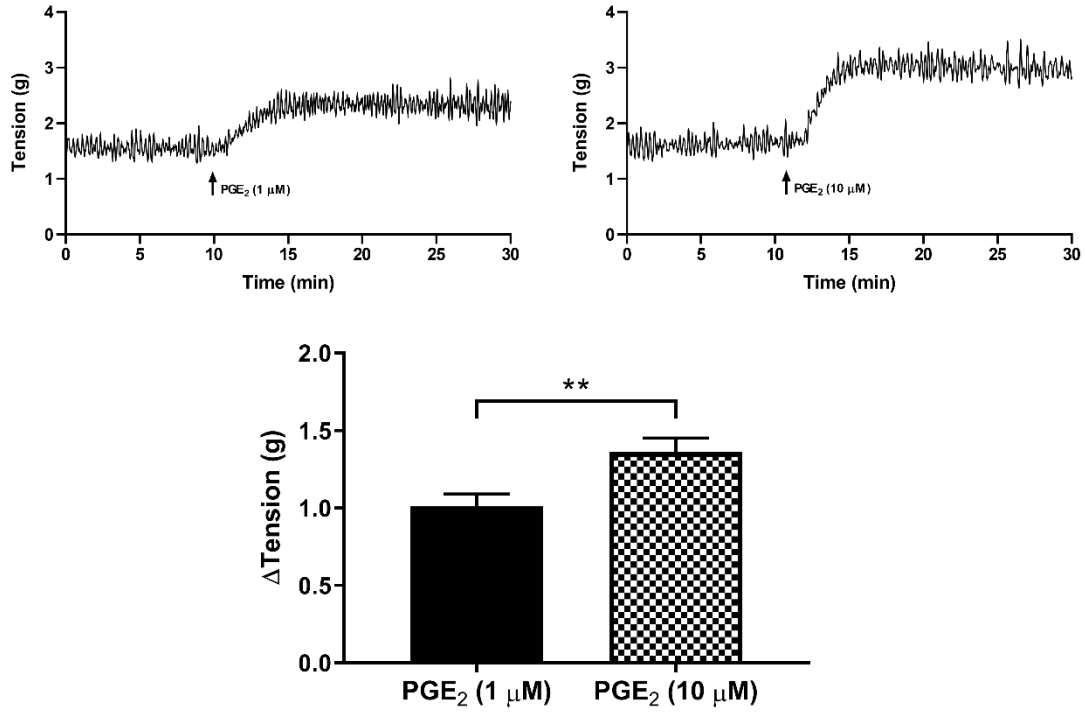

Figure 4-2: U&LP changes in tonic contractions after the treatment with 1 μM (n = 38) and 10 μM (n = 42) prostaglandin E_2. Sample traces of the responses observed to two concentrations of prostaglandin agonist (*top row*). Increases in tonic contractions after treatment with each agonist are represented as mean change ± SEM (*bottom*). Changes in the tonic contractions between 1 μM and 10 μM were evaluated using an unpaired Student's two-tailed *t*-test, where **$p < 0.01$

U&LP

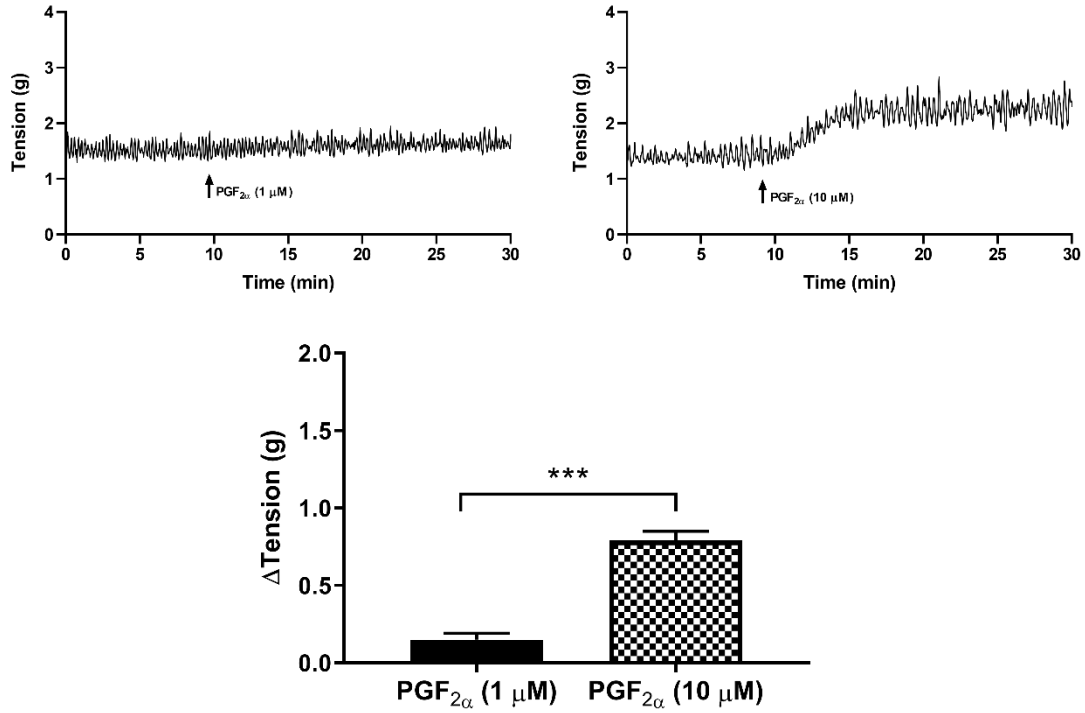

Figure 4-3: U&LP changes in tonic contractions after treatment with 1 μM (n = 10) and 10 μM (n = 14) prostaglandin $F_{2\alpha}$. Sample traces of the responses observed to two concentrations of prostaglandin agonist (*top row*). Increases in tonic contractions after treatment with each agonist are represented as mean change ± SEM (*bottom*). Changes in tonic contractions between 1 μM and 10 μM were evaluated using an unpaired Student's two-tailed *t*-test, where ***$p < 0.001$

U&LP

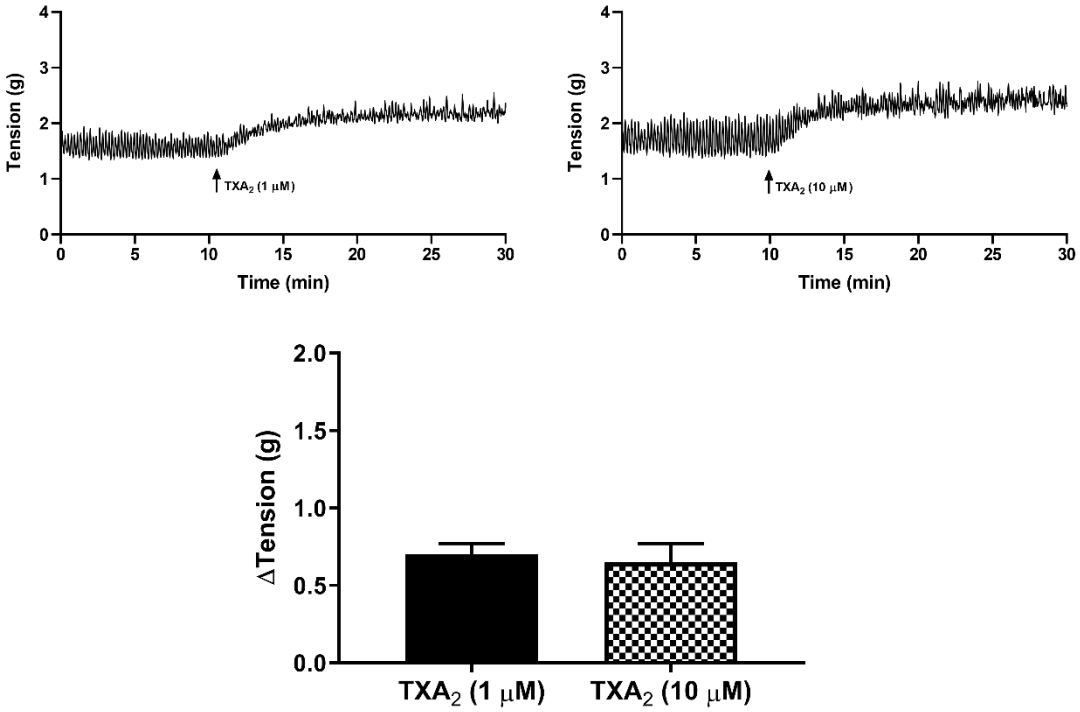

Figure 4-4: U&LP changes in tonic contractions after treatment with 1 μM (n = 8) and 10 μM (n = 6) Thromboxane A_2. Sample traces of the responses observed to two concentrations of prostaglandin agonist (*top row*). Increases in tonic contractions after treatment with each agonist are represented as mean change ± SEM (*bottom*). Changes in tonic contractions between 1 μM and 10 μM were evaluated using an unpaired Student's two-tailed *t*-test, NSD.

U&LP

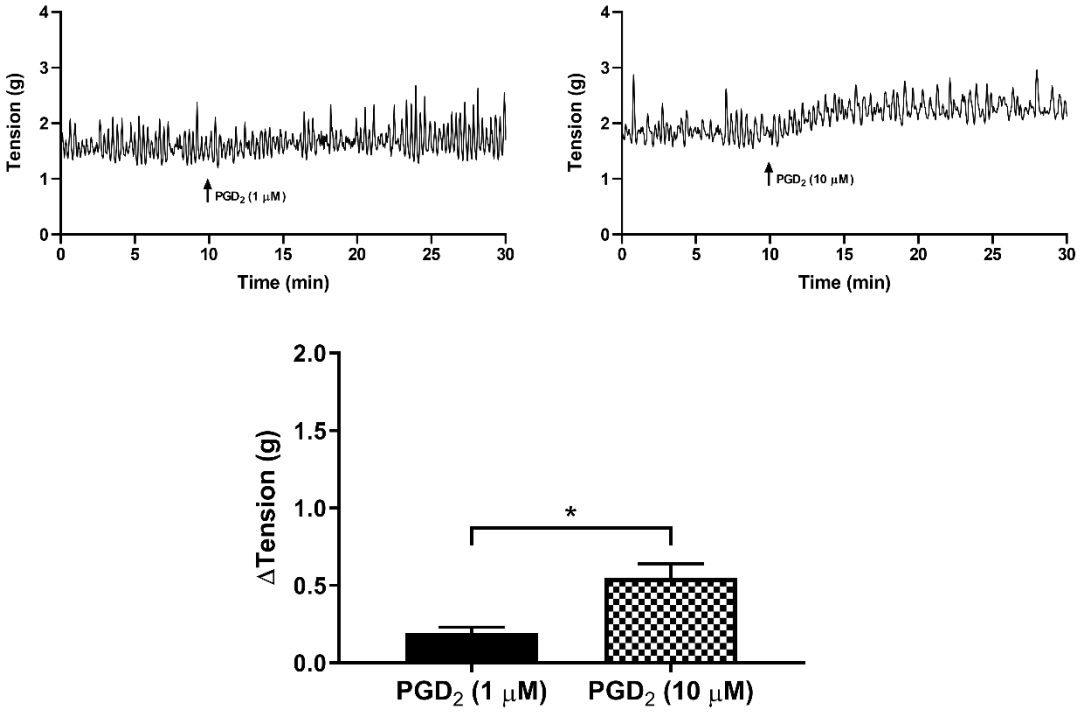

Figure 4-5: U&LP changes in tonic contractions after treatment with 1 μM (n = 4) and 10 μM (n = 8) Prostaglandin D_2. Sample traces of the responses observed to two concentrations of prostaglandin agonist (*top row*). Increases in tonic contractions after treatment with each agonist are represented as mean change ± SEM (*bottom*). Changes in tonic contractions between 1 μM and 10 μM were evaluated using an unpaired Student's two-tailed *t*-test, where *$p < 0.05$

U&LP

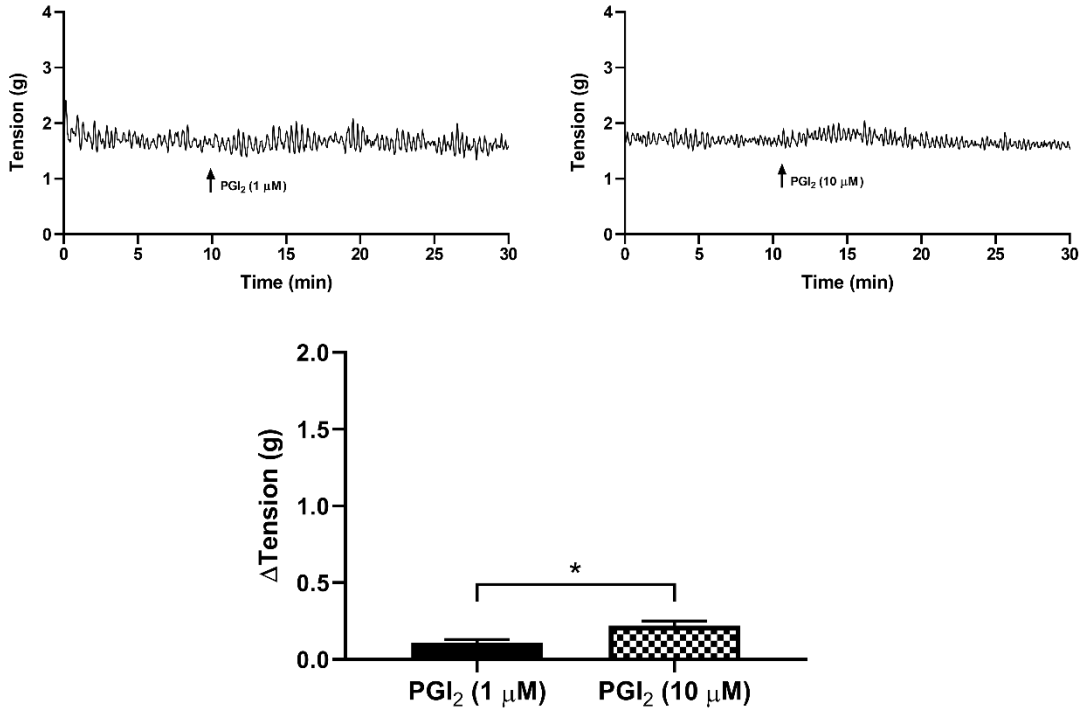

Figure 4-6: U&LP changes in tonic contractions after treatment with 1 μM (n = 8) and 10 μM (n = 8) Prostaglandin I_2. Sample traces of the responses observed to two concentrations of prostaglandin agonist (*top row*). Increases in tonic contractions after treatment with each agonist are represented as mean change ± SEM (*bottom*). Changes in tonic contractions between 1 μM and 10 μM were evaluated using an unpaired Student's two-tailed *t*-test, where *$p < 0.05$

4.4.4. Prostaglandin agonists for stimulating detrusor spontaneous activity

A total of 34% (n = 48) of the detrusor preparations that were set up in the organ baths exhibited spontaneous activity before the addition of any agonists. These contractions occurred at an average frequency of 2.03 ± 0.12 cpm with an average amplitude of 0.26 ± 0.02 g.

The addition of PGE_2 (1 μM, n = 8, $p < 0.01$) and PGI_2 (1 μM, n = 8, $p < 0.01$) significantly enhanced spontaneous phasic contractions in detrusor preparations (Figure 4-2). However, this increase in the phasic activity was not observed when a larger concentration (10 μM) of PGE_2 and PGI_2 was used. Phasic contractions during baseline were not observed in any of the preparations before the addition of PGD_2 (1 μM and 10 μM). The amplitude of spontaneous phasic contractions was enhanced in response to treatment with $PGF_{2\alpha}$ (1 μM, n = 8, $p < 0.001$) and TXA_2 (1 μM, n = 6, $p < 0.05$).

Table 4-2 – Frequency of phasic contractions: Detrusor changes in the frequency of spontaneous phasic contractions in response to varying concentrations of prostaglandin receptor agonists PGE_2, $PGF_{2\alpha}$, TXA_2, PGD_2, and PGI_2 (1 μM and 10 μM) reported only in detrusor preparations that developed phasic contractions in the absence of any stimulation.

	1 μM of agonist			10 μM of agonist		
Agonist	Absence	Presence	n	Absence	Presence	n
PGE_2	1.49 ± 0.05	2.34 ± 0.34**	8	2.74 ± 0.48	3.40 ± 0.69	8
$PGF_{2\alpha}$	2.09 ± 0.17	2.24 ± 0.19	8	1.96 ± 0.23	2.08 ± 0.36	3
TXA_2	2.39 ± 0.37	2.46 ± 0.46	6	1.63 ± 0.30	1.87 ± 0.07	3
PGD_2	Not observed	Not observed		Not observed	Not observed	
PGI_2	1.95 ± 0.07	2.33 ± 0.08**	8	1.58 ± 0.37	1.94 ± 0.26	4

**p < 0.01. Paired Student's two-tailed *t*-test.

Table 4-3 – Amplitude of phasic contractions: Detrusor changes in the amplitude of spontaneous phasic contractions in response to varying concentrations of prostaglandin receptor agonists PGE_2, $PGF_{2\alpha}$, TXA_2, PGD_2, and PGI_2 (1 μM and 10 μM) reported only in detrusor preparations that developed phasic contractions in the absence of any stimulation.

	1 μM of agonist			10 μM of agonist		
Agonist	Absence	Presence	n	Absence	Presence	n
PGE_2	0.34 ± 0.06	0.87 ± 0.31	8	0.30 ± 0.06	0.58 ± 0.18	8
$PGF_{2\alpha}$	0.27 ± 0.07	0.78 ± 0.14***	8	0.12 ± 0.03	0.20 ± 0.07	3
TXA_2	0.16 ± 0.05	0.31 ± 0.05*	6	0.24 ± 0.04	0.41 ± 0.05	6
PGD_2	Not observed	Not observed		Not observed	Not observed	
PGI_2	0.33 ± 0.05	0.37 ± 0.06	8	0.17 ± 0.02	0.24 ± 0.05	4

*p < 0.05, ***p < 0.001. Paired Student's two-tailed *t*-test.

The majority of the detrusor preparations that were otherwise quiescent during the baseline period developed phasic contractions after the addition of a prostaglandin agonist (as can be seen in Figure 4-7).

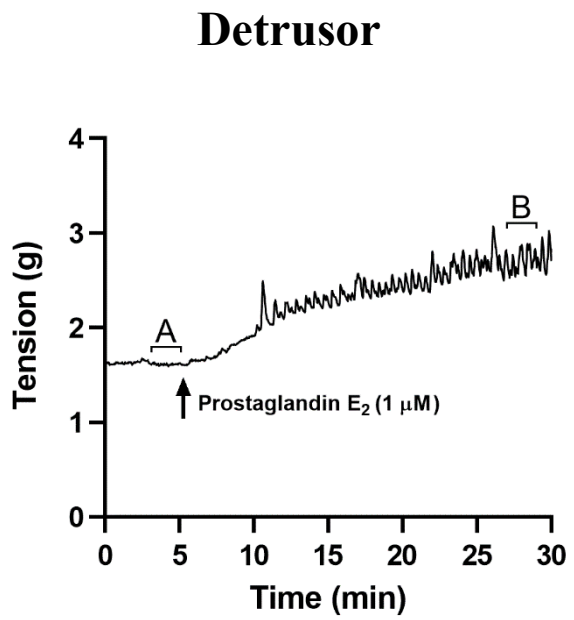

Figure 4-7: Example of how the addition of prostaglandin E_2 (1 μM) initiated phasic contractions in detrusor preparations. In this particular instance, the frequency was initiated at a rate of 1.99 cpm. This initiation of phasic contractions in otherwise quiescent detrusor samples was also observed in most tissues after addition of PGE_2 (10 μM), $PGF_{2\alpha}$ (1 – 10 μM), TXA_2 (1 – 10 μM), PGD_2 (1 – 10 μM) and PGI_2 (1 – 10 μM).

Of those detrusor preparations that did not exhibit initial phasic activity during baseline: PGE_2 (1 μM) sparked contractions in 68% of preparations (n = 19) and PGE_2 (10 μM) in 69% (n = 22); $PGF_{2\alpha}$ (1 μM) initiated contractions in in 56% (n = 5) and $PGF_{2\alpha}$ (10 μM) in 88% (n = 7); TXA_2 (1 μM) initiated contractions in 63% (n = 5) and TXA_2 (10 μM) in 80% (n = 4); PGD_2 (1 μM) initiated phasic activity in 50% (n = 2) and PGD_2 (10 μM) in 75% (n

= 6); and lastly PGI_2 (10 μM) initiated contractions in 40% (n = 2) of preparations. This demonstrates the ability of prostaglandin agonists to induce spontaneous activity in otherwise quiescent detrusor tissue strips.

4.4.5. Prostaglandin agonists for stimulating detrusor contractions

In detrusor preparations, PGE_2 (1 μM) increased tonic contractions by 0.73 ± 0.09 g (n = 38, $p < 0.001$), whereas PGE_2 (10 μM) nearly doubled the response, producing an average increase of 1.32 ± 0.13 g (n = 34, $p < 0.001$, Figure 4-8). Treatment with 1 μM $PGF_{2\alpha}$ showed a small increase of 0.20 ± 0.05 g (n = 10, $p < 0.01$), whereas 10 μM of $PGF_{2\alpha}$ increased the tonic contractions by 0.97 ± 0.14 g (n = 14, $p < 0.001$, Figure 4-9). When TXA_2 was added, tonic contractions increased by 0.47 ± 0.12 g when treated with 1 μM (n = 8, $p < 0.001$) and by 1.03 ± 0.14 g (n = 6, $p < 0.001$, Figure 4-10) when treated with 1 μM TXA_2. PGD_2 showed a small increase in the tonic contractions of 0.12 ± 0.04 g when 1 μM was added (n = 4, $p < 0.05$) and an increase of 0.36 ± 0.06 g when 10 μM PGD_2 was added (n = 6, $p < 0.01$, Figure 4-11). PGI_2 showed small increases in tonic contractions at both concentrations, showing an increase of 0.16 ± 0.02 g when treated with 1 μM (n = 8, $p < 0.001$) and 0.13 ± 0.03 g when treated with 10 μM PGI_2 (n = 8, $p < 0.001$, Figure 4-12).

The effects of prostaglandin agonists on tonic contractions of detrusor smooth muscle were significantly different between the two concentrations (1 μM and 10 μM) for PGE_2 ($p < 0.001$), $PGF_{2\alpha}$ ($p < 0.001$) and PGD_2 ($p < 0.05$). Treatment with a higher concentration of agonist (10 μM) produced significantly enhanced increases in tonic contractions when compared to a lower concentration of the agonist (1 μM).

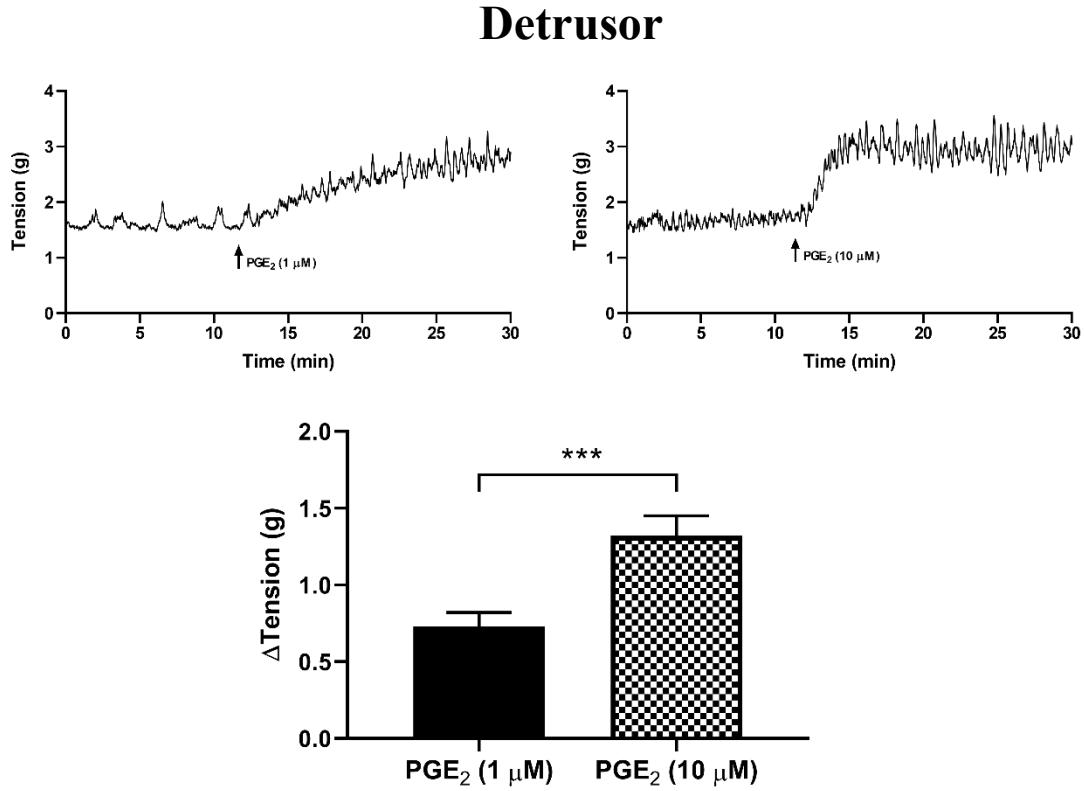

Figure 4-8: Detrusor changes in tonic contractions after treatment with 1 μM (n = 38) and 10 μM (n = 34) of prostaglandin E_2. Sample traces of responses observed to two concentrations of prostaglandin agonist (*top row*). Increases in tonic contractions after treatment with each agonist are represented as mean change ± SEM (*bottom*). Changes in tonic contractions between 1 μM and 10 μM were evaluated using an unpaired Student's two-tailed *t*-test, ***$p < 0.001$.

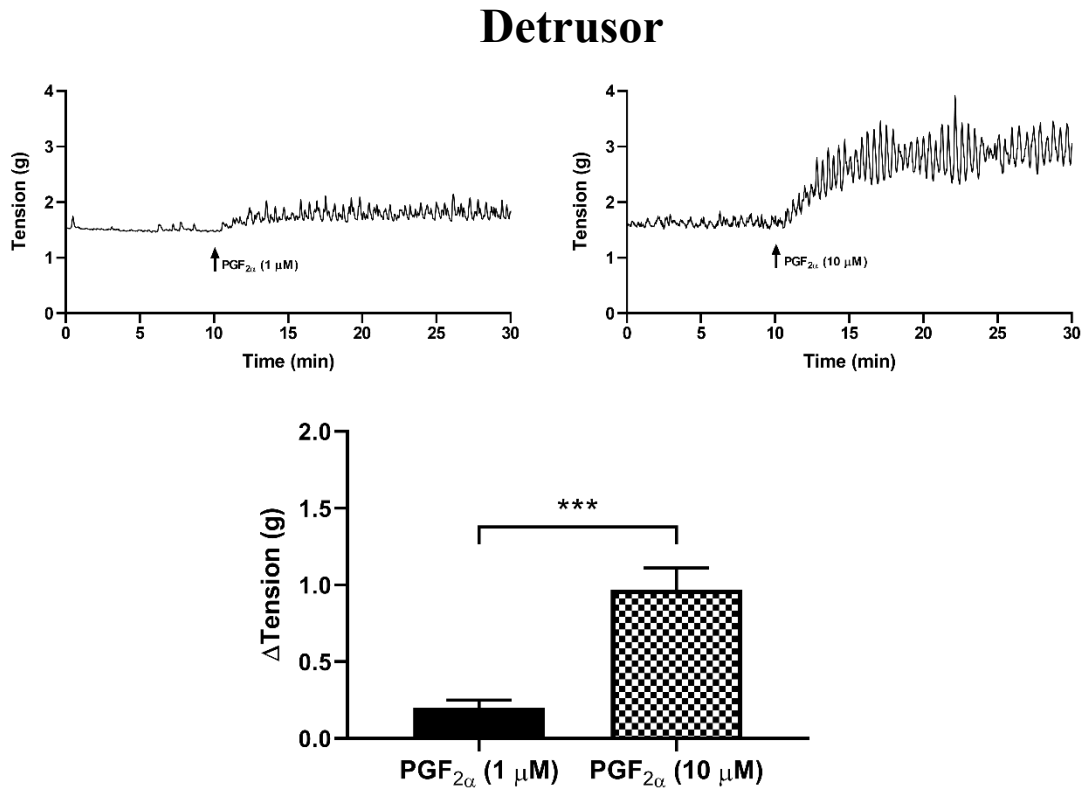

Figure 4-9: Detrusor changes in tonic contractions after treatment with 1 μM (n = 10) and 10 μM (n = 14) of Prostaglandin $F_{2\alpha}$. Sample traces of responses observed to two concentrations of prostaglandin agonist (*top row*). Increases in tonic contractions after treatment with each agonist are represented as mean change ± SEM (*bottom*). Changes in tonic contractions between 1 μM and 10 μM were evaluated using an unpaired Student's two-tailed *t*-test, where ***$p < 0.001$.

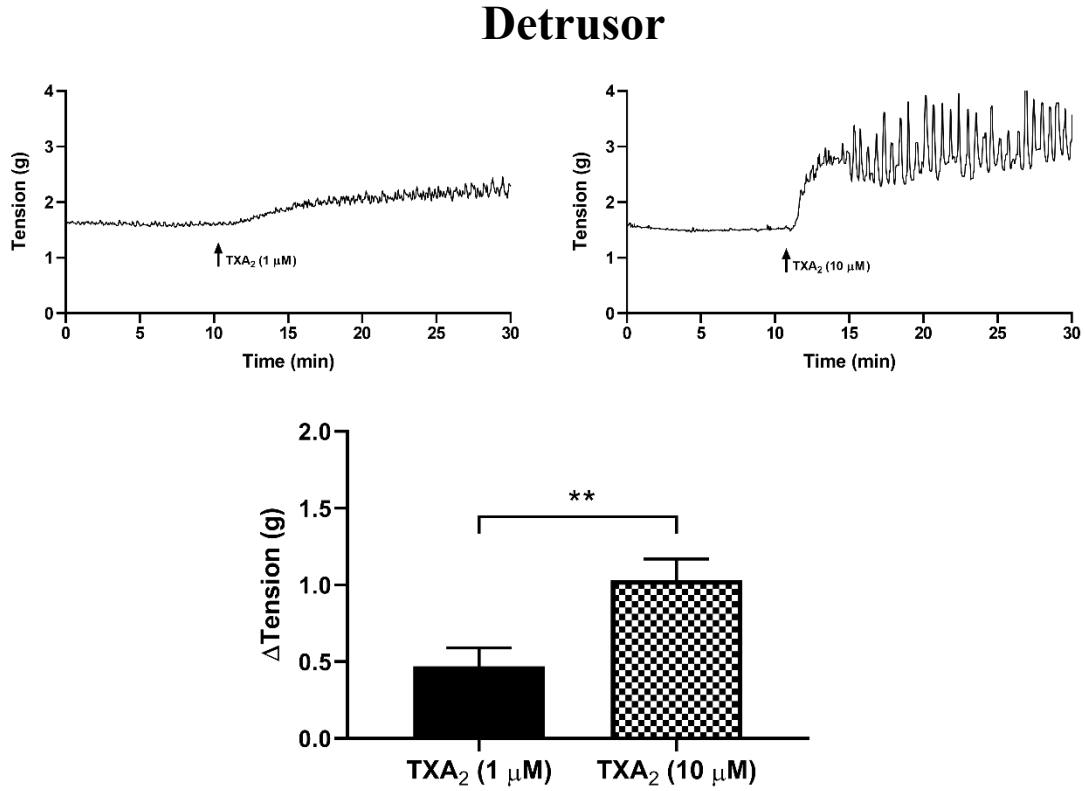

Figure 4-10: Detrusor changes in tonic contractions after treatment with 1 μM (n = 8) and 10 μM (n = 6) of Thromboxane A_2. Sample traces of responses observed to two concentrations of prostaglandin agonist (*top row*). Increases in tonic contractions after treatment with each agonist are represented as mean change ± SEM (*bottom*). Changes in the contractions between 1 μM and 10 μM were evaluated using an unpaired Student's two-tailed *t*-test, where **$p < 0.01$.

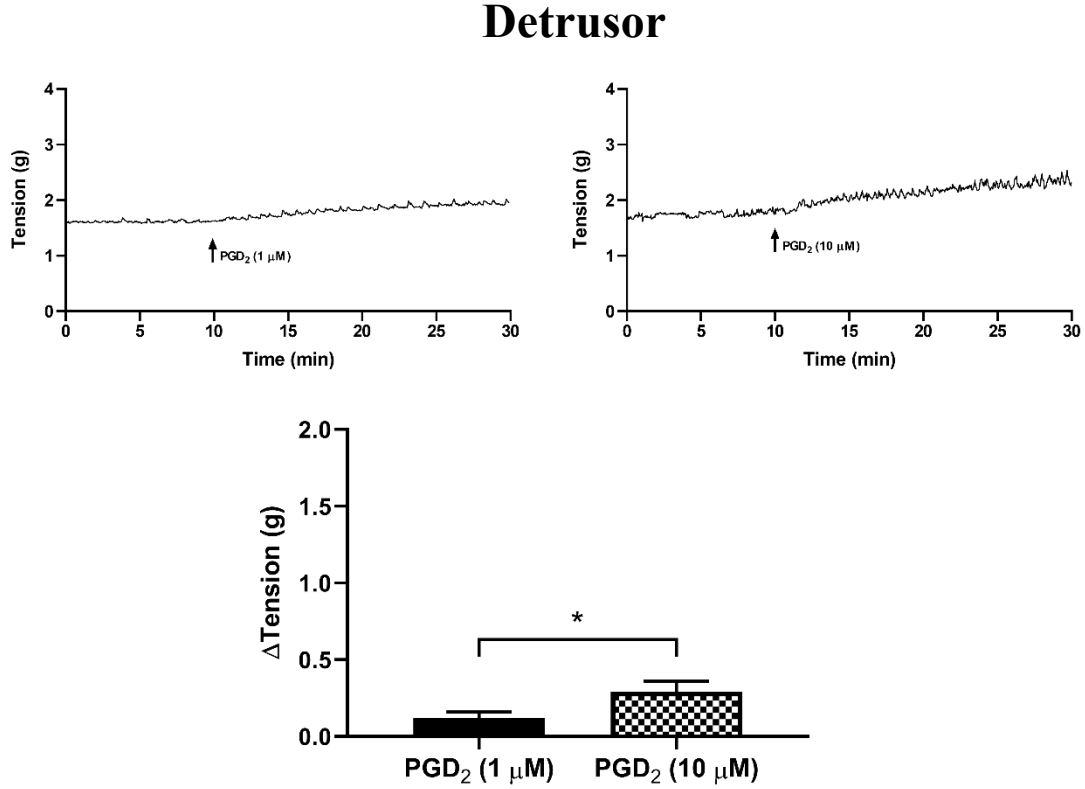

Figure 4-11: Detrusor changes in tonic contractions after treatment with 1 μM (n = 4) and 10 μM (n = 6) of Prostaglandin D_2. Sample traces of responses observed to two concentrations of prostaglandin agonist (*top row*). Increases in tonic contractions after treatment with each agonist are represented as mean change ± SEM (*bottom*). Changes in tonic contractions between 1 μM and 10 μM were evaluated using an unpaired Student's two-tailed *t*-test, where *$p < 0.05$.

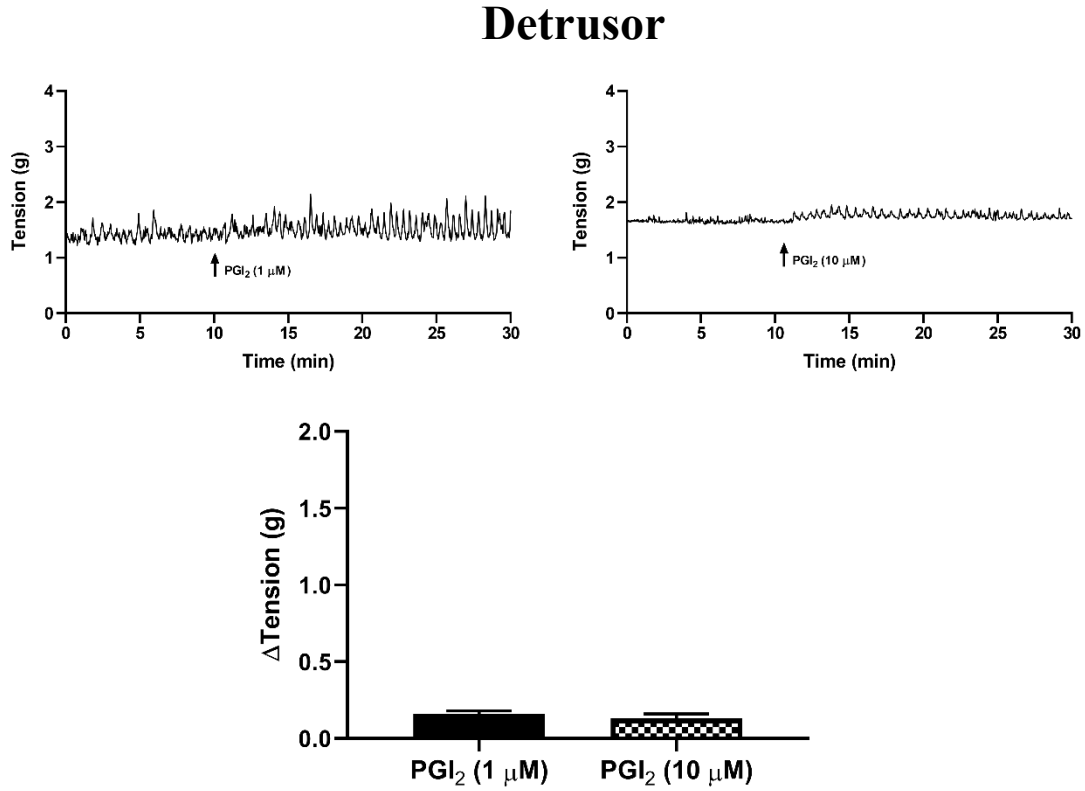

Figure 4-12: Detrusor changes in tonic contractions after treatment with 1 μM (n = 8) and 10 μM (n = 8) of Prostaglandin I_2. Sample traces of responses observed to two concentrations of prostaglandin agonist (*top row*). Increases in tonic contractions after treatment with each agonist are represented as mean change ± SEM (*bottom*). Changes in tonic contractions between 1 μM and 10 μM were evaluated using an unpaired Student's two-tailed *t*-test, NSD.

4.5. Discussion

Urinary bladder inflammation is observed in various lower urinary tract disorders, including IC/BPS (Grover et al., 2011) and OAB (Compérat et al., 2006). The inflammation can be mediated by immune cells, such as mast cells capable of releasing a variety of pro-inflammatory mediators, including histamine and prostaglandins (Theoharides et al., 2007). It has previously been thought that prostaglandins may play an important role in the function of the lower urinary tract in health and disease. This study determined the influence of the five major prostaglandins on urinary bladder U&LP and detrusor smooth muscle contractility and spontaneous activity, and demonstrated the relative differences in the agonist-evoked contractions.

U&LP strips are known to exhibit spontaneous phasic contractions in the absence of any stimulation (Moro et al., 2011). These spontaneous contractions that are thought to be propagated by the muscularis mucosae present within the U&LP (Fry and Vahabi, 2016, Drake et al., 2018, Mitsui et al., 2019) and which can be invoked by prostaglandin agonists observed in this study may have a modulatory role in the bladder function.

Immunohistochemical analysis has demonstrated that this muscularis mucosae layer is distinct from its adjacent detrusor smooth muscle layers and has been observed in pig, human and guinea pig bladders (Mitsui et al., 2019). These observations are also further reinforced with consistent findings where U&LP preparations still developed large spontaneous contractions when the apical urothelial layer and larger blood vessels were removed (Heppner et al., 1997, Lee et al., 2016). Interestingly, both rat and mouse bladders lack

muscularis mucosae, and this may be the underlying reason as to why the spontaneous contractions developed remain very small (Mitsui et al., 2019). Nonetheless, these spontaneous contractions occurring in rat U&LP arise from noradrenaline stimulation of the vasculature (Shimizu et al., 2014), which is similar to that observed in pig tissue (Moro et al., 2013).

Previous research has shown that stimulation of the M3 muscarinic receptor in U&LP causes immediate contractions, as well as increases in the frequency of spontaneous phasic contractions and reductions in their amplitude (Moro et al., 2011). These contractile changes may be one of the actions of muscarinic receptors in disease, and one of the mechanisms underlying antimuscarinic therapy as the first-line pharmaceutical treatment in people suffering from overactive bladder (Chapple et al., 2008). In our study, the prostaglandin agonists have shown similar contractile responses to both tonic contractions and spontaneous activity, thereby associating the actions of prostaglandins with many of the bladder contractile disorders, such as OAB and IC/BPS.

This spontaneous contractile activity has been suggested to occur as a means to prevent the stretching of the microvasculature upon bladder distension (Lee et al., 2016). Muscularis mucosae specifically appears to be the main contractile element present in the U&LP, capable of generating ten times more contractile strength when normalised to a cross-sectional area (Lee et al., 2016). The effects of prostaglandins on U&LP contractility and their ability to increase spontaneous phasic contractions are of special interest, as there is increasing evidence that this system can modulate the underlaying detrusor smooth muscle contractions (Chakrabarty et al., 2019).

All five prostaglandin agonists had varying effects on the frequency of spontaneous phasic contractions, with PGE_2 causing the greatest contractile response as well as the most substantial increases in the frequency of spontaneous contractions. $PGF_{2\alpha}$ also induced spontaneous activity while stimulating the tissues to contract, although at a smaller response compared to the same concentration of PGE_2. After addition of PGE_2 the amplitude of spontaneous contractions was significantly smaller when compared to baseline activity; however, this reduction was not reproduced in response to $PGF_{2\alpha}$. Our result is not replicated in all species, with Rahnama'i et al. (2013) reporting that treatment with PGE_2 reduced the amplitude of phasic contractions in intact guinea pig bladders. Finally, PGD_2, $PGTXA_2$ and PGI_2 did not affect the spontaneous activity exhibited by U&LP.

The ability to contract tissue varied between the different prostaglandin agonists. The rank order of agonist response in stimulating contractions in U&LP and detrusor was: PGE_2 > $PGF_{2\alpha}$ > TXA_2 > PGD_2 > PGI_2. These findings contrast those of Palea et al. (1993), where agonist potency in contracting detrusor muscle was: $PGF_{2\alpha}$ > TXA_2 > PGE_2. In this study, prostaglandin E_2 had the most substantial effect on increasing the tonic contractions when compared to the other agonists in both U&LP and detrusor. These findings are consistent with previous research that reported the involvement of PGE_2 in the initiation of micturition in both humans and animals (Lee et al., 2007).

Treatment with $PGF_{2\alpha}$ showed minimal increases at a concentration of 1 μM, yet responses were significantly enhanced in both U&LP and detrusor when increased to 10 μM. At the smaller concentration of 1 μM, treatment with TXA_2 reached maximal contractile responses, and as such, was not enhanced at the higher agonist concentration of 10 μM. These findings were not observed in detrusor preparations, wherein the higher concentration of TXA_2 (10

μM) resulted in significantly enhanced contractions. The responses observed in porcine tissue in response to $PGF_{2\alpha}$ and TXA_2 are consistent with the Palea (1998) findings. Additionally, our study has established that U&LP isolated tissue is also capable of responding and producing definitive increases in tonic contractions in response to these prostaglandin agonists.

Of the five prostaglandins, PGD_2 and PGI_2 had the smallest effect on both tonic contractions and spontaneous activity. This lack of increase to tonic contractions or spontaneous contractile frequency may be explained by PGD_2 having potential inhibitory actions via the stimulation of the DP receptor (Guan et al., 2015). These authors also noted that the excitatory effect was stimulated via the TP receptor system when PGD_2 concentrations were increased. While there is no previous literature investigating the effects of PGI_2 agonists on urinary bladder contractility, studies involving PGI_2 antagonists have shown decreases in the frequency of bladder contractions and increased micturition threshold in rat models (Cefalu et al., 2007, Khera et al., 2007), suggesting their potential in treating detrusor overactivity. An explanation for the small contractile effects observed in our study in response to PGI_2, the main prostaglandin synthesised in the human bladder (Jeremy et al., 1987, Masunaga et al., 2006), is that the aqueous solutions of PGI_2 are extremely chemically unstable with a relatively short half-life, depending on the buffer concentration (Stehle, 1982, Moncada, 1983). As such, future studies utilising more chemically stable PGI_2 agonist analogues might provide further insights into the actions of this inflammatory mediator on the urinary bladder.

The actions of each prostaglandin agonist were varied, with different responses exhibited depending on the concentrations used. At this stage, it is unclear which receptor subtype is activated for the observed changes in tonic contractions and spontaneous activity to occur.

There is the potential for agonists to activate other receptors, or even for the prostaglandin agonists to convert into other metabolites upon contact with the tissue (Canete Soler et al., 1987, Abadir and Siragy, 2015). Therefore, additional studies that can utilise selective antagonism of each prostaglandin receptor subtype in response to prostaglandin agonists, as well as explore potential receptor systems capable of modulating the effects of prostaglandins, would be beneficial. Additionally, future studies utilising immunohistochemical or radioligand binding assessments to determine the location, density, and prevalence of the prostaglandin receptors would provide further insights into this response. Lastly, it would be beneficial to further investigate endogenous constitutive prostaglandin production in the healthy porcine bladder using COX-1 inhibition, and from there investigating inducible COX-2 expression using selective COX-2 inhibitors in inflammatory models. Overall, this is the first study to show actions of all five prostaglandin agonists on the two separate layers on the urinary bladder, U&LP and detrusor, presenting an interesting potential therapeutic target for the management of bladder contractile disorders.

4.6. Conclusions

The urinary bladder is capable, to some extent, of responding to all five major prostaglandins produced in the urinary bladder. However, the exact underlying cellular mechanisms and receptor subtypes involved in the observed responses are unknown. Out of the five prostaglandins, PGE_2 and $PGF_{2\alpha}$ had the most significant impact on both contraction and increases to the spontaneous contractile frequency in the U&LP.

All five prostaglandin receptor agonists were also capable of inducing spontaneous phasic contractions in otherwise quiescent detrusor tissue strips. Although PGI_2 is thought to be the primary prostaglandin synthesised in the human bladder, its effects on inducing contractions or spontaneous phasic activity were minimal. Based on the responses observed in both U&LP and detrusor, the specific involvement of EP1 to EP4, FP and TP receptors in urinary bladder function should be further explored. In addition, the mechanism of action for these prostaglandin responses may represent an additional therapeutic target in the treatment of bladder overactivity or interstitial cystitis/bladder pain syndrome.

Chapter 5

Prostaglandin E2 and F2α mediated contractions of the urinary bladder urothelium, lamina propria and detrusor

The content in the following chapter is presented as the author's copy of the published document, before the reviewer and publisher's requested alterations. The final version of this manuscript can be accessed from the relevant peer-reviewed journal. Formatting and terminology changes have been made to maintain consistency throughout this thesis.

All data presented in this manuscript are the sole work of Zane Stromberga.

PUBLISHED MANUSCRIPT

Stromberga, Z., Chess-Williams, R., & Moro, C. (2020). Prostaglandin E2 and F2alpha modulate urinary bladder urothelium, lamina propria and detrusor contractility via the FP receptor. *Frontiers in Physiology*, 11, 705. doi:10.3389/fphys.2020.00705

Published abstracts and conference presentations arising from this chapter:

Stromberga, Z., Smith, J., Tan, J., & Moro, C. (2020, July). Influences of the mast cell degranulates histamine and prostaglandins on urinary bladder contractile activity. *Future Physiology 2020, virtual conference.*

Stromberga, Z., Chess-Williams, R., Moro, C. (2019, November). The role of prostaglandin E2 in mediating urinary bladder contractions. *Proceedings of the ASCEPT-PAGANZ joint scientific meeting, Queenstown, New Zealand.*

Stromberga, Z., Chess-Williams, R., Moro, C. (2017, December). Is overactive bladder an allergy? The influence of the inflammatory mediators histamine and prostaglandin on contractile activity. *Proceedings of the 9th National Symposium on Advances in Urogenital and Gut Research, Gold Coast, Australia.*

5.1. Abstract

Introduction: Current pharmacological treatment options for many bladder contractile disorders are not suitable for all patients, thereby bringing interest to the investigation of therapies that target a combination of receptors. This study aimed to compare responses of PGE_2 on the urinary bladder urothelium with lamina propria (U&LP, also called the bladder mucosa) and detrusor smooth muscle, and attempt to identify the receptor subtypes involved in mediating PGE_2 contractile responses in these tissues.

Methods: In the presence of selective EP1 – 4 receptor antagonists, varying concentrations of PGE_2 were applied to isolated strips of porcine U&LP and detrusor that were mounted in organ baths filled with Krebs-bicarbonate solution and gassed with carbogen.

Results: The addition of PGE_2 (1 μM and 10 μM) and $PGF_{2\alpha}$ (10 μM) to U&LP preparations caused significant increases in the tonic contractions and in the spontaneous phasic contractile frequency. In detrusor preparations, significant increases in the tonic contractions were observed in response to PGE_2 (1 μM and 10 μM) and $PGF_{2\alpha}$ (10 μM), and phasic contractions were initiated in 83% of preparations. None of the selective PGE_2 receptor antagonists inhibited the increases in tonic contractions in both U&LP and detrusor. However, the antagonism of $PGF_{2\alpha}$ receptor showed significantly inhibited contractile responses in both layers of the bladder.

Conclusions: This study presents prostaglandin receptor systems as a potential regulator of urinary bladder contractility. The main contractile effects of PGE_2 in both U&LP and

detrusor are mediated via the FP receptor, with no observed contribution from any of the four EP receptors.

5.2. Introduction

Bladder contractile disorders, such as the overactive bladder (OAB) and underactive bladder (UAB), are common lower urinary tract disorders that reduce overall quality of life (Haylen et al., 2010, D'Ancona et al., 2019), heighten the likelihood of developing depression and anxiety, as well as increasing healthcare usage (Milsom et al., 2012). Despite extensive research investigating the mechanisms that potentially mediate bladder contractions, the cause and development of many lower urinary tract disorders are poorly understood. Bladder contractions are under the control of the parasympathetic nervous system (de Groat et al., 1981, Panicker, 2019) and mediated by the release of acetylcholine that acts upon the M2 and M3 muscarinic receptors found in the detrusor smooth muscle (Chess-Williams, 2002, Fowler et al., 2008).

It has been recognized that the urothelium which lines the lumen of the bladder is capable of responding to mechanical, chemical and thermal stimuli (Birder, 2006, Stromberga et al., 2019). Furthermore, it is capable of releasing several signalling molecules in response to stretch, including acetylcholine (Moro et al., 2011), ATP and nitric oxide (Ferguson et al., 1997, Fry and Vahabi, 2016) that are involved in the modulation of the micturition reflex. Myofibroblasts (also referred to as interstitial cells) found in the underlying lamina propria are thought to be involved in modulating bladder behaviour by amplifying sensory responses to stretch occurring during the filling phase (Fry et al., 2007). Therefore, the involvement of

the urothelium and the underlying lamina propria should be considered as potential contributors to bladder contractile pathology.

Prostaglandins have been suggested to be involved in the modulation of bladder function for decades. They are synthesised from arachidonic acid that is released from the cell membrane via the hydrolysis of the SN-2 bond by the phospholipase A2 enzyme (Clark et al., 1991). Prostaglandins can also be produced locally within the U&LP and smooth muscle layers in the human urinary bladder (Abrams et al., 1979, Jeremy et al., 1987). Two cyclooxygenase isoforms COX-1 and COX-2 metabolise arachidonic acid into PGH_2, which is subsequently converted into five primary prostaglandins via their respective synthases: PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 and TXA_2 (Woodward et al., 2011). These signalling molecules exert their function through the stimulation of nine specific G-protein coupled receptors: EP1 – EP4, DP1 and DP2, FP2, IP and TP, respectively.

Both COX-1 and COX-2 are expressed within the bladder wall (de Jongh, 2007, de Jongh, 2009), specifically on the urothelium, lamina propria and on the surface of the inner muscle bundles of the detrusor. COX-1 is predominately expressed in the basal and intermediate layers of the urothelium and on the interstitial cells located in the lamina propria, indicating that these cells are capable of responding to prostaglandins (de Jongh, 2009). The production of prostaglandins differs between species. For example, in the rat bladder, PGI_2 is the major prostaglandin produced (Jeremy et al., 1984), whereas in the rabbit bladder it is PGE_2 (Leslie et al., 1984). 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). However, there are significant differences in the bladder contractile responses between species for the different prostaglandin receptor subtypes (Root et al., 2015).

Studies involving human detrusor have shown that the release of prostaglandins has direct influences on the micturition reflex (Bultitude et al., 1976, Andersson et al., 1977). Overproduction of prostaglandins have been observed in several conditions, including bladder outlet obstruction (Masick et al., 2001), bladder overactivity due to spinal cord injury (Masunaga et al., 2006) and in inflammation (Wheeler et al., 2001). Furthermore, contractions of the detrusor smooth muscle in response to acetylcholine and ATP are enhanced by prostaglandins in both rabbit (Anderson, 1982) and guinea-pig animal models (Nile et al., 2010). There are complex interactions between the three chemicals, as ATP enhances PGE_2 release via a P2-purinoreceptor-mediated mechanism (Kasakov and Vlaskovska, 1985), and acetylcholine is a modulator of PGE_2 release (Nile and Gillespie, 2012).

The interactions between these mediators can potentially result in amplification of molecule signalling and therefore in increased contractile activity, further supporting the interest in investigating prostaglandin involvement in contractile disorders such as OAB. Other research has also shown interest in prostaglandin release from the lower urinary tract. For example, the presence of PGE_2 in urine has been linked to bladder dysfunction and proposed as a potential future biomarker (Reyes and Klahr, 1990). In support of this, urine samples collected from female patients with overactive bladder showed that there was a significant increase in PGE_2 and $PGF_{2\alpha}$ levels when compared with the control group (Kim et al., 2006).

Prostaglandin E_2, in particular, is thought to be the most likely contributor to bladder disorders, as it can modulate bladder function via afferent signalling (Andersson, 2010) and potentially detrusor overactivity by sensitising capsaicin-sensitive afferent nerve endings (Maggi, 1988, Park et al., 1999). It is released upon degranulation of mast cells (Schmauder-

Chock and Chock, 1989) together with histamine, which is also capable of impacting bladder contractility (Stromberga et al., 2019). Moreover, PGE_2 is produced locally in the bladder in response to distension and inflammation (Funk, 2001). Notably, the excitatory effects exerted on the bladder wall via the stimulation of PGE_2 receptors has previously been studied in rat (Chuang et al., 2012) and mouse (Schroder et al., 2004, McCafferty et al., 2008) animal models. There are four identified prostaglandin E_2 receptor subtypes: EP1, EP2, EP3 and EP4. The presence of the EP1 and EP2 receptor subtypes has been shown in guinea pig urothelium and lamina propria (Rahnama'i et al., 2010), and the expression of all four PGE_2 receptor subtypes (EP1-EP4) has been identified in the canine bladder using *in situ* hybridisation and immunohistochemistry (Ponglowhapan et al., 2010). Generally, activation of EP1 and EP3 receptors is associated with an excitatory response, whereas EP2 and EP4 receptors are associated with inhibition of smooth muscle contractility (Woodward et al., 2011). Furthermore, clinical uses of the prostaglandin E_2 agonist dinoprostone include the stimulation of uterine smooth muscle contractions during labor (Stephenson and Wing, 2015), with the prostaglandin $F_{2\alpha}$ agonist carboprost being a viable alternative to oxytocin during the management of labor (Sunil Kumar et al., 2016). Therefore, the involvement of EP1, EP3 and FP receptors is of particular interest in the modulation of lower urinary tract contractility due to their excitatory effects when stimulated.

Alterations in bladder physiology in disease states have been thought to primarily occur through the regulation of detrusor smooth muscle contractility. However, it is increasingly recognised that the bladder urothelium with lamina propria (U&LP, also called the bladder mucosa) plays an important role in the overall contractility of the bladder and its intracellular signalling. As such, recent research has focused on the effects of pharmacological mediators on the physiology of the U&LP and the detrusor as separate layers. This study aimed to

compare responses of the urothelium with lamina propria to PGE_2 with those of the detrusor and identify the receptor subtypes responsible for the PGE_2-mediated contractions in these tissues.

5.3. Methods

5.3.1. Tissue preparation

Isolated tissue strips of U&LP and detrusor were prepared and set up as described previously in Chapter 2. Two adjacent strips were taken from each animal's bladder and paired together as control-experimental tissues, with each paired strip preparation expressed as "*n*" where comparisons or changes are recorded. *n* equates to the number of individual bladders used in this study.

The preparations were washed three times with Krebs-bicarbonate solution and tension adjusted to approximately 2 g, which became the tissue baseline. A single dose of agonist was added to the U&LP and detrusor preparations after an incubation period with an antagonist or vehicle control. The concentrations specific for each receptor subtype were determined using affinity values appearing in the literature utilising similar methodologies and isolated tissue bath experiments. Tissue strips exposed to antagonists or vehicle controls were incubated for 30 minutes to allow full equilibration with the receptor. Each experiment was completed within 20 minutes after the addition of the agonist.

Tonic contractions, frequency and amplitude of spontaneous phasic contractions were recorded simultaneously using isometric force transducers (MCT050/D, ADInstruments, Castle Hill, Australia) on a PowerLab system using LabChart v7 software (ADInstruments). Porcine urinary bladders are a well-recognised animal model for bladder research (Cheng et al., 2011, Parsons et al., 2012, Mitsui et al., 2019, Stromberga et al., 2019) as it has similar physiology and pharmacology to human bladders. As no animals were bred, harmed, culled, interfered, or interacted with as part of this research project, Animal Ethics Approval was not required for bladder use (Queensland Government, 2016).

5.3.2. Pharmacological agents

Prostaglandin E_2, $F_{2\alpha}$, EP1 receptor antagonist SC-19220, EP1 and EP2 receptor antagonist AH6809, EP3 receptor antagonist L-798106, EP4 receptor antagonist AH23848, FP receptor antagonist AL-8810 and ATP receptor agonist αβ-methylene ATP were obtained from Cayman Chemicals, Ann Arbor, MI, United States. The muscarinic receptor antagonist atropine, cyclooxygenase (COX) inhibitor indomethacin and the nitric oxide synthase inhibitor N_ω-Nitro-L-arginine were obtained from Sigma Aldrich, St. Loius, MO, United States. Prostaglandin E_2, prostaglandin $F_{2\alpha}$, AH6809 and indomethacin were dissolved in 100% ethanol and diluted as needed with distilled H_2O. Atropine, N_ω-Nitro-L-arginine and αβ-methylene ATP were dissolved in distilled H_2O and diluted as required. SC19220, L-798196, AH23848 and AL-8810 were dissolved in DMSO and diluted with distilled H_2O.

5.3.3. Experimental procedure

A single dose of prostaglandin E_2 (1 μM or 10 μM) or prostaglandin $F_{2\alpha}$ (10 μM) was applied to an isolated strip of U&LP or detrusor after a 30-minute equilibration period with a selective receptor antagonist. Tonic contractions and the amplitude and frequency of spontaneous phasic contractions of U&LP were measured before the application of the agonist and 5 minutes after addition. Tonic contractions and frequency and amplitude of phasic contractions in detrusor were measured before agonist application and then at 10 minutes after and 20 minutes after.

5.3.4. Measurements and statistical analysis

The changes in the tonic contractions were measured before the addition of an agonist and at the peak contractile response. The frequency of spontaneous contractions was measured over 5 minutes and expressed as the number of spontaneous phasic contraction cycles per minute (cpm). The average amplitude was measured from base to peak of each spontaneous contraction. Frequency and amplitude measurements were taken before any agonist was added and during the peak contractile response to the agonist. Data were graphed and analyzed using GraphPad Prism version 8.1.1 for Windows (GraphPad Software, La Jolla, California USA) and results shown as the mean change ± SEM. Responses between control and experimental tissues were compared using a Student's *t*-test with $p < 0.05$ considered significant.

5.4. Results

5.4.1. Prostaglandin E_2 for stimulating U&LP contractions

In the absence of prostaglandin E_2 or any receptor antagonists, strips of urothelium with lamina propria (U&LP) exhibited spontaneous phasic contractions at frequencies of 3.15 ± 0.08 cycles per min^{-1} (cpm, n = 80) and amplitudes of 0.52 ± 0.03 g (n = 80). When a small concentration of prostaglandin E_2 (100 nM) was added to the tissues, U&LP tonic contractions increased by 24 ± 9 % (n = 4, Figure 5-1), frequency of phasic contractions increased by 19 ± 8 % (n = 4, NSD) and amplitude decreased by 29 ± 19% (n = 4, NSD). with no change in the frequency or amplitude of the spontaneous phasic contractions (Table 5-1). In response to a higher concentration of prostaglandin E_2 (1 μM), U&LP tonic contractions increased by 91 ± 9 % (n = 38, $p < 0.001$), the frequency of spontaneous phasic contractions increased by 39 ± 7 % (n = 38, $p < 0.001$) and amplitudes decreased by 18 ± 5 % (n = 38, $p < 0.001$) during the peak response (Table 5-1, Figure 5-1). The addition of an even higher concentration of prostaglandin E_2 (10 μM) caused increases in the U&LP tonic contractions by 106 ± 9 % (n = 42, $p < 0.001$), frequencies of spontaneous phasic contractions by 40 ± 10 % (n = 42, $p < 0.01$) and amplitude decreases by 28 ± 6 % (n = 22, $p < 0.001$) during peak response (Table 5-1, Figure 5-1).

Table 5-1: U&LP responses to prostaglandin E_2 (100 nM, 1 μM and 10 μM) on tonic contractions and the frequency and amplitude of spontaneous phasic contractions (mean change ± SEM).

PGE_2 conc.	Δ Tension (g)	Δ Frequency (cpm)	Δ Amplitude (g)	n
100 nM	0.33 ± 0.12	0.64 ± 0.21	-0.12 ± 0.08	4
1 μM	1.01 ± 0.08***	1.13 ± 0.19***	-0.14 ± 0.04***	38
10 μM	1.36 ± 0.09***	1.36 ± 0.36**	-0.17 ± 0.04**	34

**p < 0.01, ***p < 0.001. Paired Student's two-tailed *t*-test.

U&LP

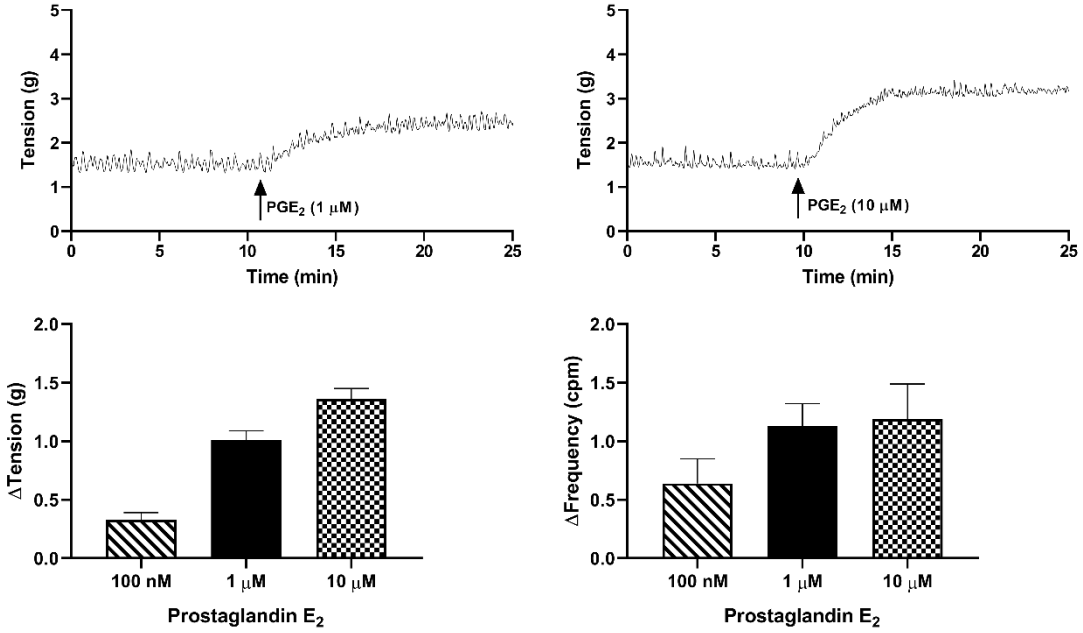

Figure 5-1: U&LP traces (*top row*) showing changes to tonic contractions in response to PGE_2 at concentrations of 1 μM (*left*) and 10 μM (*right*) over 15 minutes. *Bottom:* Changes in tonic contractions (*left*) and in the frequency of spontaneous phasic contractions (*right*) after the addition of three concentrations of PGE_2 (100 nM, 1 μM and 10 μM).

5.4.2. Prostaglandin E_2 for stimulating detrusor contractions

In detrusor preparations, PGE_2 caused concentration-dependent increases in tonic contractions over increasing concentrations (100 nM – 10 μM, Figure 5-2). Detrusor preparations did not reach a visible peak in response to PGE_2 within the experimental timeframe, so each tension recording was completed at set intervals of 10 and 20 minutes. A small concentration of prostaglandin E_2 (100 nM) increased the tonic contractions of detrusor by 5 ± 2 % (n = 4). In response to prostaglandin E_2 (1 μM, Figure 5-2, Table 5-2), an increase of 80 ± 13 % (n = 38, $p < 0.001$) was observed 10 min after the addition of the agonist, and in response to prostaglandin E_2 (10 μM, Figure 5-2, Table 5-2) an increase of 155 ± 21 % (n = 34, $p < 0.001$) was observed 10 minutes after the addition of agonist. Twenty minutes after the addition of prostaglandin E_2 (1 μM, Table 5-2), tonic contractions had increased by 107 ± 13 % (n = 38, $p < 0.001$), whereas 20 minutes after the treatment with prostaglandin E_2 (10 μM, Table 5-2), contractions had increased by 175 ± 21% (n = 34, $p < 0.001$).

Table 5-2: Detrusor changes in the tonic contractions in response to prostaglandin E_2 (1 μM and 10 μM) measured 10 min and 20 min after the treatment with the agonist (mean change ± SEM).

PGE_2 conc.	ΔTension (g) at 10 min	ΔTension (g) at 20 min	n
100 nM	0.07 ± 0.03	Not recorded	4
1 μM	0.77 ± 0.09***	1.06 ± 0.09***	38
10 μM	1.34 ± 0.14***	1.46 ± 0.11***	34

***$p < 0.001$. Paired Student's two-tailed *t*-test.

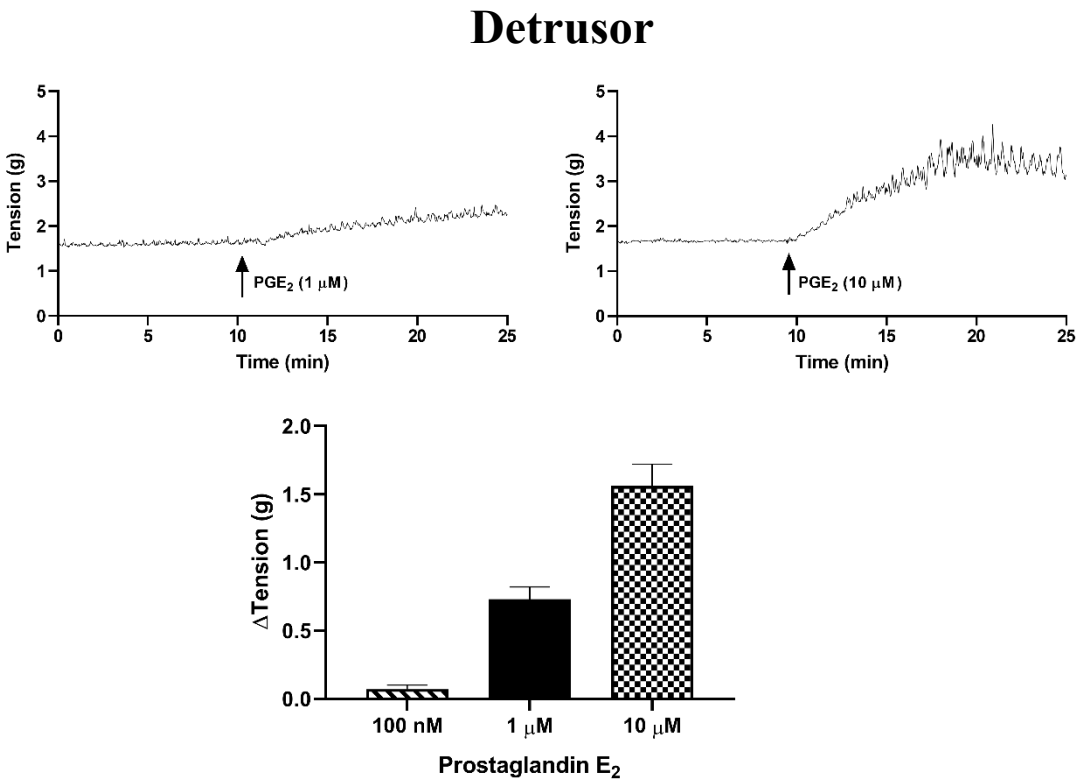

Figure 5-2: Detrusor traces (*top row*) showing changes to tonic contractions in response to PGE_2 at concentrations of 1 μM (*left*) and 10 μM (*right*) over 15 minutes. *Bottom:* Maximal contractile responses after the addition of three concentrations of PGE_2 (100 nM, 1 μM and 10 μM).

5.4.3. Prostaglandin E_2 for stimulating detrusor spontaneous activity

In experiments where a single dose of 1 μM PGE_2 was applied to the tissue strip, spontaneous phasic contractions during baseline were present in 21% of all preparations (n = 6). At baseline prior to any stimulation, these spontaneous contractions occurred at 1.48 ± 0.29 cpm (n = 8) with an amplitude of 0.34 ± 0.06 g (n = 8). After the treatment with PGE_2 (1 μM), spontaneous contractions were significantly enhanced to 2.34 ± 0.34 cpm (n = 8, $p < 0.01$) with an amplitude of 0.87 ± 0.31 g (n = 8, NSD). Out of the remaining detrusor preparations that did not exhibit spontaneous activity at baseline, these contractions were initiated in 77% of preparations (n = 23) after the addition of PGE_2 (1 μM). The spontaneous contractions occurred at 1.94 ± 0.15 cpm (n = 23) with an amplitude of 0.48 ± 0.13 g (n = 22).

In experiments involving PGE_2 (10 μM), spontaneous phasic activity was present at baseline in 24% of preparations (n = 8). At baseline, these contractions occurred at 2.74 ± 0.48 cpm (n = 8) with an amplitude of 0.30 ± 0.06 g (n = 8). After the treatment with PGE_2 (10 μM), the frequency of spontaneous contractions increased to 3.40 ± 0.48 cpm (n = 8, NSD) with an amplitude of 0.58 ± 0.18 g (n = 8, NSD). In preparations that did not exhibit spontaneous activity at baseline, phasic contractions were initiated in 81% of preparations (n = 21) with a frequency of 1.82 ± 0.10 cpm (n = 21) and an amplitude of 0.48 ± 0.07 g (n = 21).

5.4.4. Source of calcium for contractions to PGE_2 in U&LP and detrusor

The source of calcium for contractions in response to PGE_2 (10 μM) was investigated using Krebs-bicarbonate solution with and without added calcium chloride. Tissues that were kept in Krebs-bicarbonate solution with calcium chloride showed significantly greater increases in tonic contractions of 0.72 ± 0.08 g (n = 8) when compared to 0.50 ± 0.09 g that developed in calcium-free Krebs-bicarbonate solution (n = 8, $p < 0.05$). Frequency and amplitude of spontaneous phasic contractions were present in all preparations kept in Krebs-bicarbonate solution with calcium chloride, with an average frequency of 3.19 ± 0.43 cpm and amplitude of 0.34 ± 0.06 g (n = 8). In response to PGE_2 (10 μM), U&LP preparations that were kept in calcium-free Krebs-bicarbonate solution developed measurable spontaneous activity in 62.5% of all preparations (n = 5), with a frequency of 3.02 ± 0.33 cpm and amplitude of 0.25 ± 0.07 g (n = 8).

Detrusor preparations that were maintained in Krebs-bicarbonate solution with calcium chloride showed significantly greater increases in tonic contractions of 0.66 ± 0.09 g (n = 8) when compared to 0.39 ± 0.06 g developed by preparations kept in calcium-free Krebs-bicarbonate solution (n = 8, $p < 0.01$) ten minutes after treatment with PGE_2 (10 μM). The difference in tonic contractions remained significantly different twenty minutes after treatment with PGE_2 (10 μM). Preparations maintained in Krebs-bicarbonate solution with calcium chloride showed increases of 0.90 ± 0.13 g (n = 8), whereas preparations in calcium-free Krebs-bicarbonate solution showed increases of 0.61 ± 0.07 g (n = 8, $p < 0.05$).

5.4.5. Receptor-selective antagonists in U&LP preparations

The responses to prostaglandin E_2 were observed in the presence of the selective receptor antagonists: SC-19220 (EP1 antagonist, 10 μM), AH6809 (EP1/EP2 antagonist, 10 μM), L-798106 (EP3 antagonist, 100 nM) and AH23848 (EP4 antagonist, 10 μM). None of the prostaglandin E_2 receptor antagonists had any effect on tonic contractions (Table 5-3), frequency of phasic contractions (Table 5-4) or on the amplitude of phasic contractions (Table 5-5) in response to prostaglandin E_2 (1 μM).

The time required to reach maximal tonic contractions was investigated in U&LP tissues treated with PGE_2 in the presence and absence of EP receptor antagonists. In response to prostaglandin E_2 (1 μM), U&LP tissue strips took 4.48 ± 0.28 min to reach their maximal tonic contractions (n = 7). Tissues incubated with SC-19220 (1 μM, n = 8, $p < 0.05$) took 6.37 ± 0.39 min to reach maximal contractions. There were no significant differences in times taken to reach maximal tonic contractions in tissues treated with either AH6809, L-798106 or AH23848.

Table 5-3 – Tonic contractions: U&LP changes in tonic contractions after treatment with prostaglandin E_2 (1 μM) in the absence and presence of prostaglandin E_2 receptor antagonists (mean change ± SEM).

			ΔTension (g)		
Antagonist	Receptor antagonist	Conc.	Absence	Presence	n
SC-19220	EP1	10 μM	1.21 ± 0.21	1.52 ± 0.39	8
AH6809	EP1/EP2	10 μM	1.12 ± 0.19	1.14 ± 0.20	8
L-798106	EP3	100 nM	0.97 ± 0.09	0.82 ± 0.10	8
AH23848	EP4	10 μM	0.90 ± 0.10	0.99 ± 0.13	12

None of the antagonists had statistically significant effects using a paired Student's two-tailed t-test.

Table 5-4 – Frequency of phasic contractions: U&LP changes in the frequency of phasic contractions after treatment with prostaglandin E_2 (1 μM) in the absence and presence of prostaglandin E_2 receptor antagonists (mean change ± SEM).

			ΔFrequency (cpm)		
Antagonist	Receptor antagonist	Conc.	Absence	Presence	n
SC-19220	EP1	10 μM	1.19 ± 0.33	1.75 ± 0.57	8
AH6809	EP1/EP2	10 μM	1.42 ± 0.53	1.23 ± 0.41	8
L-798106	EP3	100 nM	0.68 ± 0.15	0.80 ± 0.18	8
AH23848	EP4	10 μM	1.05 ± 0.36	1.61 ± 0.44	12

None of the antagonists had statistically significant effects using a paired Student's two-tailed t-test.

Table 5-5 – Amplitude of phasic contractions: U&LP changes in amplitude of phasic contractions after treatment with prostaglandin E_2 (1 μM) in the absence and presence of prostaglandin E_2 receptor antagonists (mean change ± SEM).

Antagonist	Receptor antagonist	Conc.	ΔAmplitude (g)		n
			Absence	Presence	
SC-19220	EP1	10 μM	-0.16 ± 0.08	-0.07 ± 0.08	7
AH6809	EP1/EP2	10 μM	-0.13 ± 0.03	-0.13 ± 0.06	8
L-798106	EP3	100 nM	-0.26 ± 0.11	-0.13 ± 0.08	8
AH23848	EP4	10 μM	0.06 ± 0.05	-0.07 ± 0.05	12

None of the antagonists had statistically significant effects using a paired Student's two-tailed t-test.

The potential influence of receptor systems other than those activated by prostaglandin E_2 to contribute to contractile responses were investigated. Neither the tonic contractions (Table 5-6), frequency (Table 5-7), nor amplitude (Table 5-8) of spontaneous phasic contractions in response to prostaglandin E_2 (10 μM) were affected by the presence of the muscarinic receptor antagonist atropine (1 μM), cyclooxygenase (COX) inhibitor indomethacin (5 μM), nitric oxide synthase inhibitor N_ω-Nitro-L-arginine (L-NNA, 100 μM) or P2X receptor desensitizing agonist αβ-methylene ATP (αβm-ATP, 10 μM). The tissue did not exhibit changes in tension or in the frequency of phasic contractions in response to the addition of the FP receptor antagonist AL-8810 (5 μM).

However, the presence of AL-8810 (5 μM) significantly inhibited increases in tonic contractions in response to PGE_2 (10 μM, n = 8, $p < 0.02$, Figure 5-3). In the absence of any antagonist, contractions to PGE_2 increased tension by 103 ± 12%. In the presence of AL-8810 (5 μM), this increase was inhibited with the remaining contraction being 77 ± 8% (n = 8, $p < 0.05$, Table 5-6).

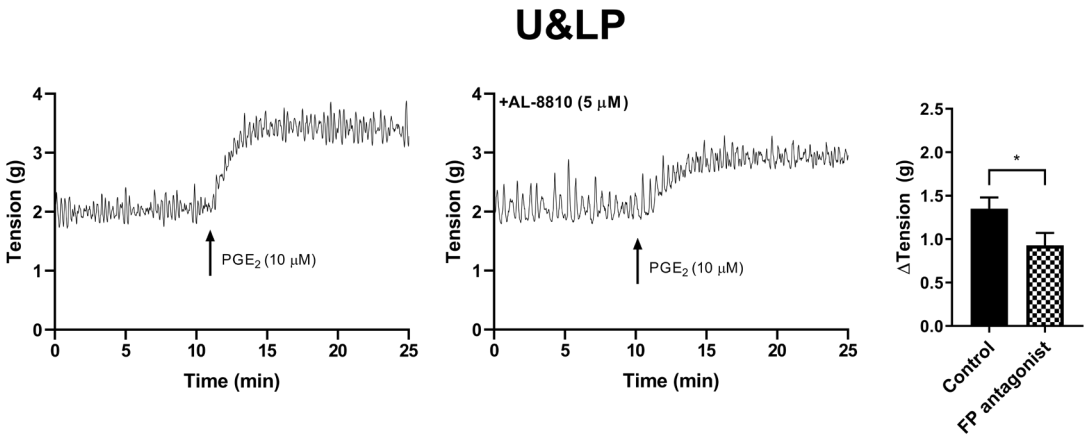

Figure 5-3: U&LP responses to PGE_2 (10 μM, *left*) and in the presence of FP receptor antagonist AL-8810 (5 μM, *middle*). Increases in tonic contractions in response to PGE_2 are represented as mean change ± SEM (*right*). Changes in tonic contractions between control and experimental conditions were evaluated using a paired Student's two tailed *t*-test, where *$p < 0.05$.

Table 5-6 – Tonic contractions: U&LP changes in tonic contractions in response to prostaglandin E_2 (10 μM) in the absence and presence of atropine (muscarinic receptor antagonist), indomethacin (cyclooxygenase inhibitor), Nω-Nitro-L-arginine (L-NNA, nitric oxide synthase inhibitor), αβ-methylene ATP (αβm-ATP, P2X purinergic receptor agonist) and AL-8810 (FP receptor antagonist) (mean change ± SEM).

			ΔTension (g)		
Chemical	Action	Conc.	Absence	Presence	n
Atropine	Muscarinic receptor antagonist	1 μM	1.45 ± 0.18	1.55 ± 0.12	6
Indomethacin	COX inhibitor	5 μM	1.90 ± 0.25	1.79 ± 0.21	4
L-NNA	NO synthase antagonist	100 μM	2.00 ± 0.32	2.31 ± 0.42	4
αβm-ATP	P2X purinergic receptor agonist	10 μM	1.58 ± 0.35	1.99 ± 0.48	4
AL-8810	FP receptor antagonist	5 μM	1.35 ± 0.13	0.93 ± 0.14*	8

*$p < 0.05$. Paired Student's two-tailed *t*-test.

Table 5-7 – Frequency of phasic contractions: U&LP changes in the frequency of phasic contractions in response to prostaglandin E_2 (10 μM) in the absence and presence of atropine (muscarinic receptor antagonist), indomethacin (cyclooxygenase inhibitor), Nω-Nitro-L-arginine (L-NNA, nitric oxide synthase inhibitor), αβ-methylene ATP (αβm-ATP, P2X purinergic receptor agonist) and AL-8810 (FP receptor antagonist) (mean change ± SEM).

			ΔFrequency (cpm)		
Chemical	Action	Conc.	Absence	Presence	n
Atropine	Muscarinic receptor antagonist	1 μM	0.53 ± 0.25	0.86 ± 0.30	6
Indomethacin	COX inhibitor	5 μM	0.73 ± 1.29	2.11 ± 1.45	4
L-NNA	NO synthase antagonist	100 μM	2.86 ± 1.32	3.51 ± 1.78	4
αβm-ATP	P2X purinergic receptor agonist	10 μM	0.46 ± 0.22	0.49 ± 0.23	4
AL-8810	FP receptor antagonist	5 μM	1.12 ± 0.38	0.68 ± 0.27	8

None of the chemicals had statistically significant effects using a paired Student's two-tailed t-test.

Table 5-8 – Amplitude of phasic contractions: U&LP changes in amplitude of phasic contractions in responses to prostaglandin E_2 (10 μM) in the absence and presence of atropine (muscarinic receptor antagonist), indomethacin (cyclooxygenase inhibitor), Nω-Nitro-L-arginine (L-NNA, nitric oxide synthase inhibitor), αβ-methylene ATP (αβm-ATP, P2X purinergic receptor agonist) and AL-8810 (FP receptor antagonist) (mean change ± SEM).

			ΔAmplitude (g)		
Chemical	Action	Conc.	Absence	Presence	n
Atropine	Muscarinic receptor antagonist	1 μM	-0.16 ± 0.12	-0.04 ± 0.09	6
Indomethacin	COX inhibitor	5 μM	-0.06 ± 0.04	-0.08 ± 0.07	4
L-NNA	NO synthase antagonist	100 μM	-0.30 ± 0.04	-0.21 ± 0.05	4
αβm-ATP	P2X purinergic receptor agonist	10 μM	-0.11 ± 0.01	0.002 ± 0.06	4
AL-8810	FP receptor antagonist	5 μM	-0.18 ± 0.09	-0.13 ± 0.07	8

None of the chemicals had statistically significant effects using a paired Student's two-tailed t-test.

5.4.6. Receptor-selective antagonists in detrusor preparations

Detrusor preparations did not reach a visible peak in response to PGE_2 (1 μM) within the experimental timeframe, so each tension recording was completed at set intervals of 10 and 20 minutes. As such, changes in detrusor tonic contractions were measured at 10 and 20 minutes after the addition of each agonist (Figure 5-4).

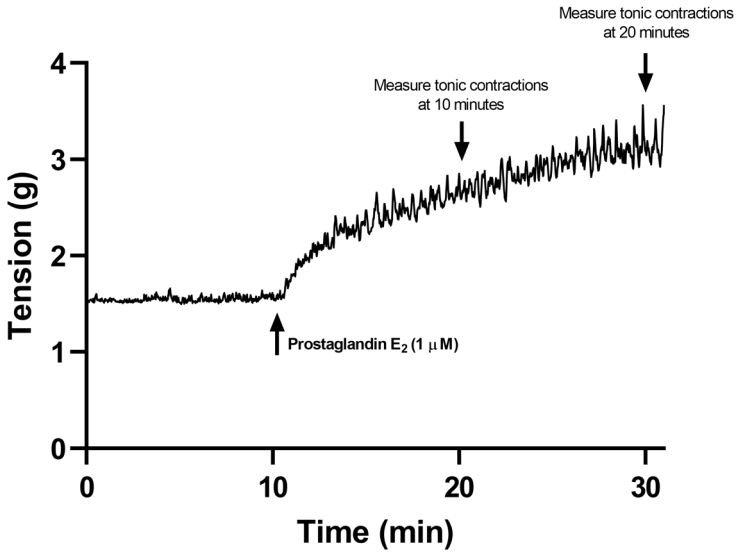

Figure 5-4: Detrusor example trace showing that after the addition of prostaglandin E2 (1 μM) the preparation did not reach a visible peak. Therefore, tonic contractions were measured 10 minutes and 20 minutes after the addition of the prostaglandin agonist.

The responses to prostaglandin E_2 were observed in the presence of the selective prostaglandin E_2 receptor antagonists: SC-19220 (EP1 antagonist, 10 μM), AH6809 (EP1/EP2 antagonist, 10 μM), L-798106 (EP3 antagonist, 100 nM) and AH23848 (EP4 antagonist, 10 μM). None of the prostaglandin E_2 receptor antagonists had any effect on the tonic contractions in detrusor tissue strips in response to prostaglandin E_2 (1 μM) after 10 minutes (Table 5-9) and 20 minutes (Table 5-10).

Table 5-9 – Tonic contractions at 10 min: Detrusor responses to prostaglandin E_2 (1 μM) measured 10 min after the addition of agonist in the absence and presence of PGE_2 receptor antagonists (mean change ± SEM). None of the antagonists had statistically significant effects (paired two-tailed Student's two-tailed *t*-test).

Antagonist	Receptor antagonist	Conc.	ΔTension (g) at 10 min		n
			Absence	Presence	
SC-19220	EP1	10 μM	0.60 ± 0.08	0.49 ± 0.11	8
AH6809	EP1/EP2	10 μM	0.64 ± 0.13	0.62 ± 0.10	8
L-798106	EP3	100 nM	1.12 ± 0.20	1.05 ± 1.16	10
AH23848	EP4	10 μM	0.46 ± 0.10	0.49 ± 0.14	8

None of the antagonists had statistically significant effects using a paired Student's two-tailed t-test.

Table 5-10 – Tonic contractions at 20 min: Detrusor responses to prostaglandin E_2 (1 μM) measured 20 min after the addition of agonist in the absence and presence of PGE_2 receptor antagonists (mean change ± SEM).

Antagonist	Receptor antagonist	Conc.	ΔTension (g) at 20 min		n
			Absence	Presence	
SC-19220	EP1	10 μM	1.12 ± 0.18	1.09 ± 0.15	8
AH6809	EP1/EP2	10 μM	0.96 ± 0.16	0.95 ± 0.16	8
L-798106	EP3	100 nM	1.25 ± 0.20	1.35 ± 0.26	10
AH23848	EP4	10 μM	0.81 ± 0.16	0.80 ± 0.16	8

None of the antagonists had statistically significant effects using a paired Student's two-tailed t-test.

The potential influence for receptor systems other than those activated by prostaglandin E_2, to contribute to contractile responses were investigated. It was found that increases in tonic contractions in detrusor preparations in response to prostaglandin E_2 (10 μM) were not affected by the presence of the muscarinic receptor antagonist atropine (1 μM), cyclooxygenase (COX) inhibitor indomethacin (5 μM), nitric oxide synthase inhibitor N_ω-Nitro-L-arginine (L-NNA, 100 μM) or P2X receptor desensitising agonist αβ-methylene ATP (αβm-ATP, 10 μM, Table 5). Treatment with the FP receptor antagonist AL-8810 (5 μM) did not affect the tonic contractions. However, in the presence of AL-8810, increases in tonic contractions mediated by prostaglandin E_2 (10 μM) were significantly inhibited both 10 minutes and 20 minutes after the addition of the agonist ($n = 8$, $p < 0.02$ for both, Figure 5-5). In the absence of any antagonists, contractions to PGE_2 increased the tension by 233 ±

60% at 10 min. In the presence of AL-8810 (5 μM) this increase was inhibited, with the remaining contraction of 127 ± 46% (n = 8, $p < 0.05$, Table 5-11, 5-12).

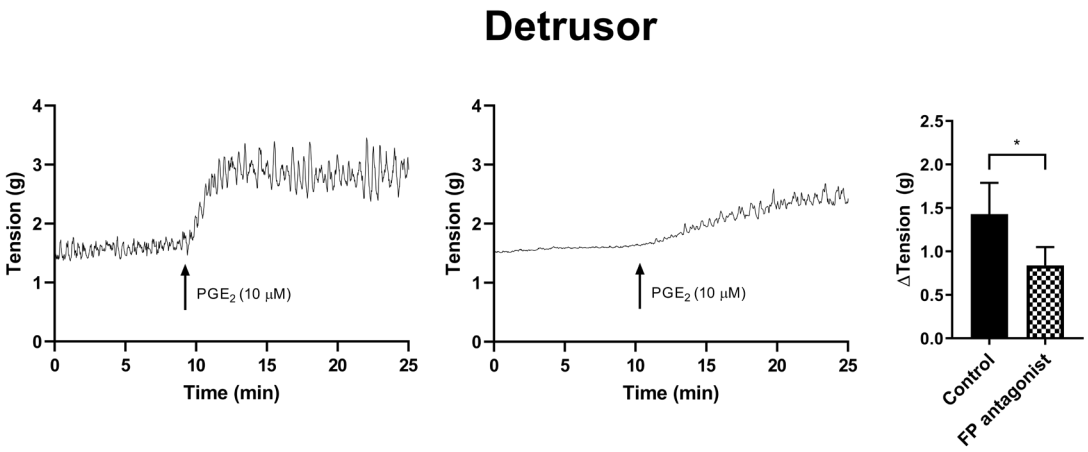

Figure 5-5: Detrusor responses to PGE_2 (10 μM, *left*) and in the presence of FP receptor antagonist AL-8810 (5 μM, *middle*) observed 10 minutes after the addition of agonist. Increases in tonic contractions in response to PGE_2 are represented as mean change ± SEM (*right*). Changes in tonic contractions between control and experimental conditions were evaluated using a paired Student's two tailed *t*-test, where *$p < 0.05$.

Table 5-11 – Tonic contractions at 10 min: Detrusor responses to prostaglandin E_2 (10 μM) measured 10 minutes after the addition of agonist in the absence and presence of atropine (muscarinic receptor antagonist), indomethacin (cyclooxygenase inhibitor), Nω-Nitro-L-arginine (L-NNA, nitric oxide synthase inhibitor), αβ-methylene ATP (αβm-ATP, P2X purinergic receptor agonist) and AL-8810 (FP receptor antagonist) (mean change ± SEM).

			ΔTension (g) at 10 min		
Chemical	Action	Conc.	Absence	Presence	n
Atropine	Muscarinic receptor antagonist	1 μM	1.38 ± 0.21	1.02 ± 0.09	6
Indomethacin	COX inhibitor	5 μM	1.55 ± 0.55	1.29 ± 0.42	4
L-NNA	NO synthase antagonist	100 μM	2.21 ± 0.28	1.90 ± 0.32	4
αβm-ATP	P2X purinergic receptor agonist	10 μM	1.40 ± 0.21	1.72 ± 0.49	4
AL-8810	FP receptor antagonist	5 μM	1.43 ± 0.36	0.84 ± 0.21*	8

*$p < 0.05$. Paired Student's two-tailed *t*-test.

Table 5-12 – Tonic contractions at 20 min: Detrusor responses to prostaglandin E_2 (10 μM) measured 20 minutes after the addition of agonist in the absence and presence of atropine (muscarinic receptor antagonist), indomethacin (cyclooxygenase inhibitor), Nω-Nitro-L-arginine (L-NNA, nitric oxide synthase inhibitor), αβ-methylene ATP (αβm-ATP, P2X purinergic receptor agonist) and AL-8810 (FP receptor antagonist) (mean change ± SEM).

			ΔTension (g) at 20 min		
Chemical	Action	Conc.	Absence	Presence	n
Atropine	Muscarinic receptor antagonist	1 μM	1.72 ± 0.11	1.47 ± 0.11	6
Indomethacin	COX inhibitor	5 μM	1.75 ± 0.55	1.35 ± 0.43	4
L-NNA	NO synthase antagonist	100 μM	2.05 ± 0.26	1.94 ± 0.27	4
αβm-ATP	P2X purinergic receptor agonist	10 μM	1.45 ± 0.29	1.94 ± 0.43	4
AL-8810	FP receptor antagonist	5 μM	1.39 ± 0.22	1.01 ± 0.18*	8

*$p < 0.05$. Paired Student's two-tailed *t*-test.

5.4.7. Prostaglandin $F_{2\alpha}$ on U&LP and detrusor preparations

In U&LP preparations, treatment with $PGF_{2\alpha}$ (10 μM) caused a significant increase in the tonic contractions of 65 ± 9 % ($n = 14$, $p < 0.001$). Additionally, the frequency of spontaneous phasic contractions increased by 13 ± 5 cpm ($n = 14$, $p < 0.05$), with no changes observed in the amplitude of these contractions. In the presence of AL-8810 (FP antagonist, 5 μM), increases in the tonic contractions in response to $PGF_{2\alpha}$ (10 μM) were significantly inhibited ($n = 8$, $p < 0.001$, Table 5-13, Figure 5-6). However, the antagonism of the FP receptor did not affect the frequency or amplitude of spontaneous phasic contractions.

In detrusor preparations, an increase in tonic contractions of 88 ± 10 % ($n = 14$, $p < 0.001$) was observed in response to $PGF_{2\alpha}$ (10 μM). The presence of FP antagonist AL-8810 (5 μM) significantly inhibited increases in the tonic contractions in the first 10 minutes after the addition of agonist ($n = 8$, $p < 0.05$, Figure 5-7). This inhibition was maintained 20 minutes after the addition of $PGF_{2\alpha}$ (10 μM, Table 5-13). However, the differences between experimental and control preparations were no longer significant ($p = 0.11$).

Table 5-13: U&LP responses to $PGF_{2\alpha}$ (10 μM) in the absence and presence of AL-8810 (5 μM) on tonic contractions and the frequency and amplitude of phasic contractions (top). Detrusor tonic contractions responses at 10 minutes and 20 minutes to $PGF_{2\alpha}$ (10 μM) in the absence and presence of AL-8810 (bottom). Results represented as mean change ± SEM.

		$PGF_{2\alpha}$	$PGF_{2\alpha}$ with AL-8810	n
Urothelium with lamina propria (U&LP)	ΔTension (g)	0.85 ± 0.09	0.49 ± 0.08***	8
	ΔAmplitude (g)	-0.11 ± 0.06	-0.13 ± 0.07	8
	ΔFrequency (cpm)	0.67 ± 0.23	0.73 ± 0.14	8
		$PGF_{2\alpha}$	$PGF_{2\alpha}$ with AL-8810	
Detrusor	ΔTension (g) at 10 min	0.78 ± 0.18	0.36 ± 0.04*	8
	ΔTension (g) at 20 min	0.91 ± 0.19	0.57 ± 0.08	8

*p < 0.05, ***p < 0.001. Paired Student's two-tailed *t*-test.

U&LP

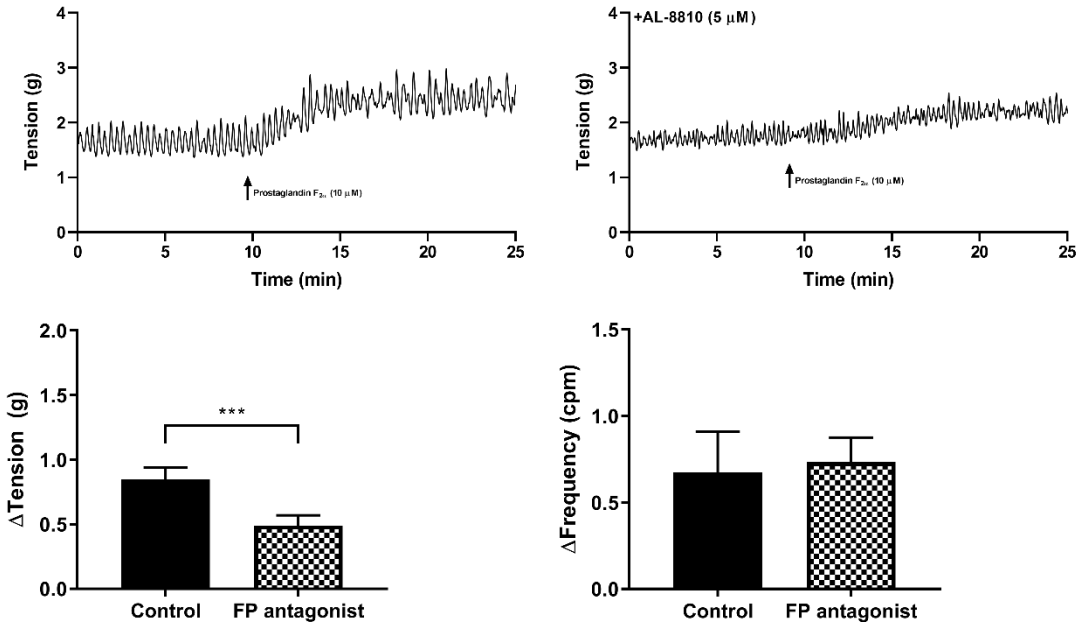

Figure 5-6: U&LP responses to $PGF_{2\alpha}$ (10 μM, *top left*) and in the presence of FP receptor antagonist AL-8810 (5 μM, *top right*). Increases in tonic contractions (*bottom left*) and in the frequency of phasic activity (*bottom right*) in response to $PGF_{2\alpha}$ are represented as mean change ± SEM. Changes in the tonic contractions between control and experimental conditions were evaluated using a paired Student's two-tailed t-test, where ***$p < 0.001$

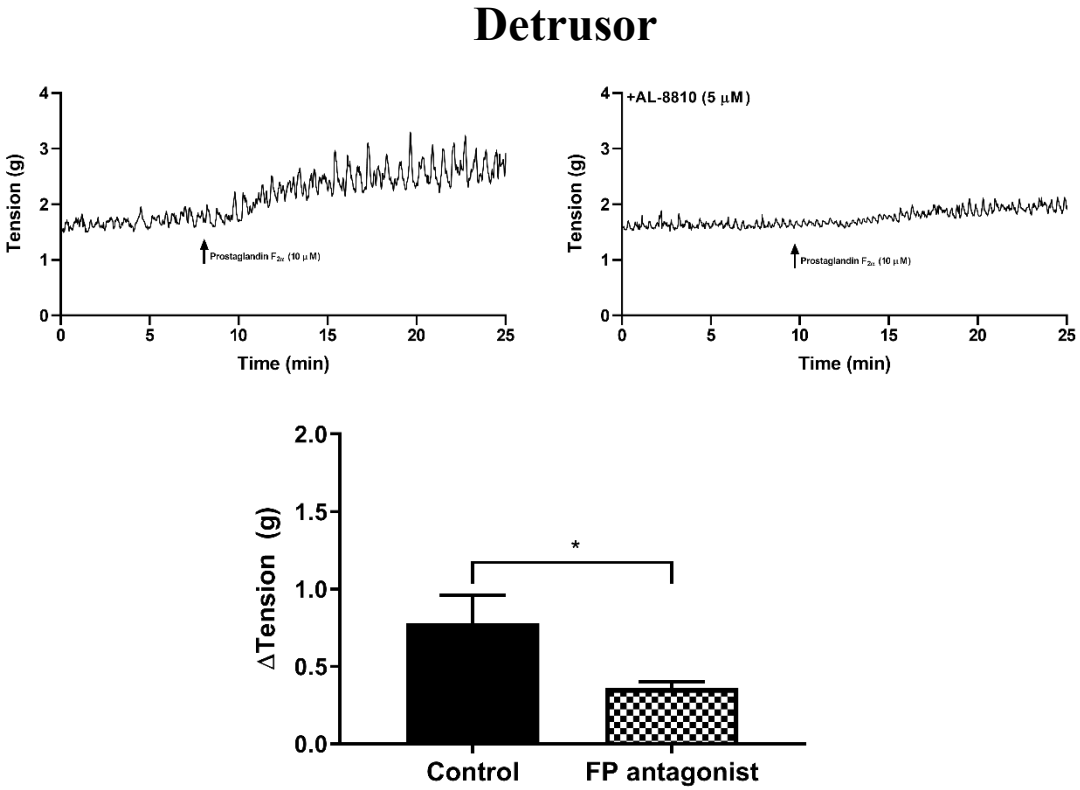

Figure 5-7: Detrusor responses to $PGF_{2\alpha}$ (10 μM, *top left*) and in the presence of FP receptor antagonist AL-8810 (5 μM, *top right*). Increases in tonic contractions after treatment with $PGF_{2\alpha}$ are represented as mean change ± SEM (*bottom*). Changes in the tonic contractions between control and experimental conditions were evaluated using a paired Student's two-tailed *t*-test, where *$p < 0.05$.

5.5. Discussion

This study presents prostaglandin E_2 as a potential contributor to the overall urinary bladder contractility and showcases the ability of isolated urothelium with lamina propria to respond to this prostanoid. Prostaglandin E_2 causes increases in tonic contractions in both U&LP and detrusor tissue, as well as influences the frequency and amplitude of U&LP spontaneous phasic contractions. In addition, this study is the first to identify responses to PGE_2 in the urothelium with lamina propria when separated from its underlying detrusor layer. The two layers are distinct in both structure and function, with the U&LP comprised of several layers of epithelial cells and an underlying connective tissue layer with muscularis mucosae, while the detrusor is composed of primarily smooth muscle cells.

The U&LP not only provides a physical barrier between urine and the bladder wall but is also capable of releasing chemicals like acetylcholine, which can influence the contractility of the detrusor (Moro et al., 2011). Furthermore, it is also capable of responding to external signals like noradrenaline (Moro et al., 2013), nitric oxide (Moro et al., 2012), the five primary prostaglandins (Stromberga et al., 2020) and histamine (Stromberga et al., 2019), as well as evoking ATP release (Andersson et al., 2018). It is known that cells found in U&LP can express both EP1 and EP2 receptors (Rahnama'i et al., 2010), indicating that they are capable of responding to PGE_2 and eliciting a contractile response. However, the exact mechanisms and the involvement of the different PGE_2 receptor subtypes in the contractile responses observed are still unclear.

In this study, increases in the tonic contractions in both U&LP and detrusor tissues that were treated with varying doses of PGE_2 (100 nM - 10 μM) were observed. The presence of PGE_2 increased the frequency of spontaneous contractile activity, which was similar to responses observed after muscarinic receptor activation (Moro et al., 2011), the target of many first-line pharmaceuticals to treat overactive bladder. In detrusor preparations, which generally do not exhibit spontaneous phasic contractions, these contractions were initiated in most preparations in response to the addition of PGE_2. Previous studies have shown that contractions in detrusor smooth muscle in response to acetylcholine and ATP are enhanced by the presence of prostaglandins in both rabbit (Anderson, 1982) and guinea-pig animal models (Nile and Gillespie, 2012). There are complex interactions between these three molecules, as ATP enhances PGE_2 release via a P2-purinoreceptor-mediated mechanism (Kasakov and Vlaskovska, 1985) and acetylcholine release has been detected when unstretched preparations were exposed to PGE_2 (Nile and Gillespie, 2012).

Therefore, interactions between these three mediators can potentially result in the amplification of molecule signalling and an increase in contractile activity. However, the involvement of acetylcholine, ATP and nitric oxide in mediating the increases in both the tonic contractions and the frequency of spontaneous phasic contractions were ruled out, as the contractile responses were the same when respective receptors were selectively antagonised or desensitised. Therefore, even though previous research has shown that acetylcholine is released when tissue is exposed to PGE_2 (Nile and Gillespie, 2012), the inhibition of muscarinic receptors revealed no changes in the magnitude of contractions in response to PGE_2. Furthermore, treatment with indomethacin, which inhibits prostaglandin production via cyclooxygenase (COX), did not affect contractile responses indicating that addition of PGE_2 did not initiate the production of additional PGE_2 in the tissue. It is not

clear how calcium channels are involved, as depending on the preparations studied, Nicol et al. (1992) showed that 1 μM PGE_2 either enhanced or inhibited the high-voltage-activated calcium current I_{ca}.

In order to isolate the role of a specific PGE_2 receptor subtype in mediating these changes to tonic contractions and the frequency of spontaneous phasic contractions, selective receptor antagonists for EP1-EP4 receptors were used. The antagonists and concentrations selected for this study were based on prior research investigating smooth muscle contractility in rat prostate gland (Tokanovic et al., 2010). When U&LP and detrusor smooth muscle tissues were treated with each individual PGE_2 receptor subtype antagonists (EP1 – EP4), there were no differences observed in the contractile responses to PGE_2 when compared to tissues with no antagonist treatment. Activation of EP1 and EP3 receptor subtypes mediate contractions in smooth muscle, whereas EP2 and EP4 are involved in the relaxatory responses (Woodward et al., 2011). In this study, when both U&LP and detrusor tissues were treated with the highly selective EP1 receptor antagonist SC19220, with a pA_2 value of approximately 5.9 (Jones et al., 2009) and EP3 receptor antagonist L798106 with a K*i* of 0.3 nM (Juteau et al., 2001), there was no change in the magnitude of contractions to PGE_2.

However, when U&LP was incubated with the EP1-selective receptor antagonist SC-19220, it took significantly more time to reach peak contraction in response to PGE_2 compared to control tissues. Even though the stimulation of the EP2 receptors have previously been shown to relax non-vascular urogenital smooth muscle tissue (Coleman et al., 1994, Tokanovic et al., 2010), no changes in contractile response were observed in tissue preparations that were treated with the dual EP1 and EP2 antagonist AH6809 [pA for EP1 approx. 7.3 and pA_2 for EP2 approx. 5.7 (Jones et al., 2009)]. The involvement of EP4

receptors in the inhibition of muscle contractions was also ruled out using AH23848, an antagonist for EP4 receptors (pA_2 value 5.4), with no activity towards other EP receptor subtypes (Coleman et al., 1994). The lack of responses also aligns with previous findings suggesting that the EP4 receptor is involved in vascular smooth muscle relaxation instead of the smooth muscle of the urinary bladder (Coleman et al., 1994, Tokanovic et al., 2010). Findings of this study suggest that the contractile responses observed to prostaglandin E_2 in both U&LP and detrusor may be mediated by receptors systems other than EP, purinergic and cholinergic.

The ability of AL-8810 to significantly inhibit the contractile response to PGE_2 suggests a mechanism of action at the FP receptor. This response is most likely a result of the PGE_2 chemical undergoing a conversion from PGE_2 into $PGF_{2\alpha}$ through 9-ketoreductase activity upon contact with the tissue. The potential for these conversions has been known for some time (Canete Soler et al., 1987, Cheung and Challis, 1989, Abadir and Siragy, 2015). There is also evidence to support a partial agonist effect of AL-8810 (Griffin et al., 1999), although the addition of this antagonist showed no response or any effect on the baseline activity. The potential residual contraction to $PGF_{2\alpha}$ may also occur though $PGF_{2\alpha}$-1-ethanolamide receptors. This has been explained in terms of heterodimerization of the wild-type FP receptor and an alternative mRNA splicing variant to give a 'prostamide F receptor' (Liang et al., 2008). The contraction remaining after FP receptor inhibition is unknown, although it does not appear to be related to stimulation of the muscarinic, purinergic or nitric oxide receptor systems, nor from the creation of new prostaglandins. One potential mechanism of action could be via a receptor unspecified pathway, such as affecting calcium channels, and this could be considered for future experiments.

A limitation of this study was the use of single-concentration applications of PGE_2 and $PGF_{2\alpha}$ to examine changes in frequencies and amplitudes of phasic contractions over a 20-minute timeframe. It would be of interest to examine a larger range of concentrations, as well as incorporate alternative agonists to obtain a wider understanding of the prostaglandin responses. Finally, there remains no literature demonstrating FP receptor expression or localisation in porcine or any other animal model bladders, and its identification throughout this region would assist in a better understanding of this receptor system. It should also be considered that PGE_2 exhibits a similar affinity to $PGF_{2\alpha}$ on the FP receptor (Abramovitz et al., 2000), and as such, there is the potential for an influence of FP receptors in the responses observed. This cross-sensitivity of FP receptors to PGE_2 may also present a mechanism underlying the inhibitory effect observed from treatment with AL-8810 and should be further explored in follow-up studies. Future studies could also investigate whether the suppressed responses observed after AL-8810 treatment is due to the actions of PGE_2 after a possible conversion to $PGF_{2\alpha}$.

This study identifies a functional response in both U&LP and detrusor preparations to PGE_2, and its ability to be inhibited by an FP receptor antagonist suggests some conversion to $PGF_{2\alpha}$ upon contact with the tissue. The response to direct application of $PGF_{2\alpha}$, which appears to be similar to the PGE_2 response also supports this hypothesis. Therefore, the finding of this study supports and further advances prior research, where PGE_2 and $PGF_{2\alpha}$ had previously been found to contract isolated detrusor smooth muscle in human preparations (Abrams and Feneley, 1975, Andersson et al., 1977, Palea, 1998).

5.6. Conclusions

Prostaglandin E_2 and prostaglandin $F_{2\alpha}$ can elicit clear contractile responses in both urinary bladder urothelium with lamina propria and detrusor smooth muscle preparations. Furthermore, the addition of these prostaglandins in U&LP preparations increases the frequency of spontaneous phasic contractions. In the detrusor, which typically does not exhibit spontaneous contractions, these contractions were initiated in most preparations. The antagonism of all four PGE_2 receptor subtypes, cholinergic receptors and purinergic receptors failed to inhibit contractile responses observed to the prostaglandins in either U&LP or detrusor tissues. However, the inhibition of the EP1 receptor in U&LP resulted in a significant decrease in the time taken to reach peak contractile responses to PGE_2, suggesting partial mediation via the EP1 receptor system. Antagonism of the FP receptor inhibited responses to $PGF_{2\alpha}$, but also to PGE_2, suggesting some conversion of this into $PGF_{2\alpha}$ after contact with the tissue. Overall, contractile responses to both prostaglandin E_2 and prostaglandin $F_{2\alpha}$ appear mediated, in part, by the FP receptor in both U&LP and detrusor smooth muscle.

Chapter 6

Alterations in histamine responses between juvenile and adult urinary bladder urothelium, lamina propria and detrusor tissues

The content in the following chapter is presented as the author's copy of the published document, prior to being accepted for publishing. The final version of this manuscript can be accessed from the relevant peer-reviewed journal. Formatting and terminology changes have been made to maintain consistency throughout this thesis.

All data presented in this manuscript are the sole work of Zane Stromberga.

Prelude

This thesis has so far examined the influence of the inflammatory mediators histamine (Chapter 4) and the five primary prostanoids (Chapters 5 & 6) on U&LP and detrusor contractile function (tonic contractions and the frequency and amplitude of phasic contractions). It is known that bladder contractile properties are altered with ageing, and therefore an important avenue to explore further. As will be outlined in the Discussion, between each of the inflammatory mediators considered in this thesis, histamine was the most suitable candidate for examining the influences of ageing, as it showed differentiation in the control responses after stimulation with an agonist between the two age groups. Results from preliminary studies involving PGE_2 showed no differences in the contractile responses between the age groups in both U&LP and detrusor (Figure 6-1). As such, this Chapter will focus specifically on the effect of ageing on the responses to histamine.

The porcine preparations that were used in the previous three chapters were obtained from pre-pubescent animals and will further be considered as a "juvenile" sample. The following Chapter examines the influence of histamine and its receptor antagonists on aged tissues to determine whether or not responses in aged preparations were altered. As preparations obtained from juvenile and adult animals differ in size and weight, the changes in tonic contractions and amplitudes of phasic contractions were converted into mN/g to enable comparison between the two age groups.

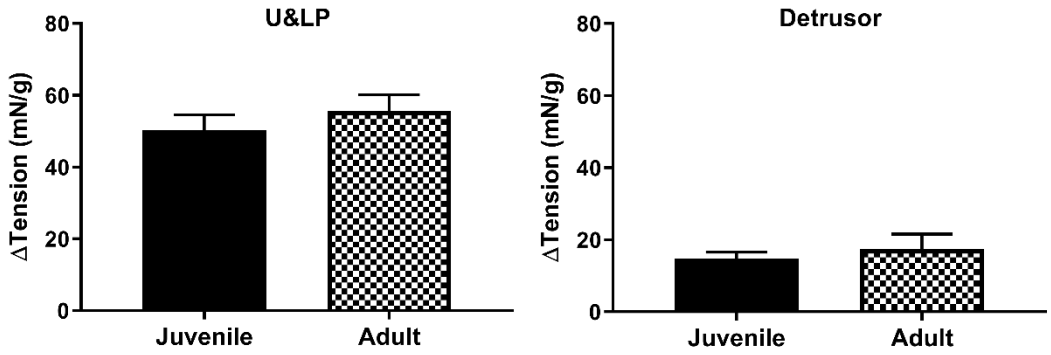

Figure 6-1: U&LP (left) and detrusor (right) responses to PGE_2 (1 μM) in juvenile (n = 38 both U&LP and detrusor) and adult (n = 6 both U&LP and detrusor) tissue samples. Increases in tonic contractions after treatment with histamine are represented as mean change ± SEM. Changes in tonic contractions between juvenile and adult age groups were evaluated using an unpaired Student's two-tailed *t*-test, with no statistically significant differences between the two groups.

PUBLISHED MANUSCRIPT

Stromberga, Z., Chess-Williams, R., & Moro, C. (2020). Alterations in histamine responses between juvenile and adult urinary bladder urothelium, lamina propria and detrusor tissues. Scientific reports. *Scientific Reports*, *10*(1), 4116.

Published abstracts and conference presentations arising from this chapter:

Stromberga, Z., Chess-Williams, R., & Moro, C. (2020, July). Inflammatory mediators as contributors to age-related urinary bladder dysfunction. *Future Physiology 2020, virtual conference.*

Stromberga, Z., Chess-Williams, R., Moro, C. (2019, November). Age-related changes in histamine receptor mediated contractions of the urinary bladder. *Proceedings of the ASCEPT-PAGANZ joint scientific meeting, Queenstown, New Zealand*. Can be accessed on https://www.asceptasm.com/wp-content/uploads/2019/11/ASCEPT-PAGANZ-2019-oral-abstracts-updated.pdf

6.1. Abstract

Introduction: Inflammatory mediators may have a role in various lower urinary tract disorders. Histamine is known to induce significant increases to both tension and frequency of spontaneous phasic contractions in both urothelium with lamina propria (U&LP) and detrusor muscle via the activation of H1 receptors in juvenile animal models. However, it is unclear whether age affects these contractile responses to histamine. This study assessed the contractile histamine receptor subtypes in juvenile and adult porcine bladders and compared the responses to histamine in urothelium with lamina propria and detrusor.

Methods: Isolated tissue bath studies were conducted using strips of porcine U&LP and detrusor obtained from juvenile (6 months) and adult (3 years) animals exposed to histamine receptor agonists and antagonists.

Results: Treatment with histamine (100 μM) in U&LP of juvenile animals caused increases in tonic contractions by 47.84 ± 6.52 mN/g ($p < 0.001$, $n = 51$) and by 50.76 ± 4.10 mN/g ($p < 0.001$, $n = 55$) in adult animals. Furthermore, the frequency of spontaneous phasic contractions was significantly enhanced in response to histamine in U&LP of both juvenile and adult tissues ($p < 0.001$ for both age groups). Treatment with an H2 agonist in U&LP of juvenile animals decreased tonic contractions by 13.97 ± 3.45 mN/g ($n = 12$, $p < 0.05$), but had no effect om adult animals. Inhibition of H1 receptors resulted in significantly reduced contractile responses of U&LP and detrusor to histamine in both juvenile and adult animals ($p < 0.05$). Treatment with H2 receptor antagonist significantly enhanced contractions in juvenile preparations ($n = 10$, $p < 0.05$) but had no effect in adult preparations ($n = 8$). In

detrusor, treatment with histamine (100 μM) in juvenile tissues showed a significantly higher increase in tonic contractions of 19.10 ± 4.92 mN/g (n = 51) when compared to adult tissues exhibiting increases of 8.21 ± 0.89 mN/g (n = 56, $p < 0.05$).

Conclusions: The increases in tonic contractions were significantly inhibited by the presence of H1 receptor antagonists in both juvenile and adult detrusor preparations. Treatment with both H2 receptor antagonist or agonist in detrusor did not affect both juvenile and adult tissues. Therefore, the histamine receptor system may play an essential role in the maintenance of bladder function or bladder dysfunction observed in some lower urinary tract disorders.

6.2. Introduction

Normal bladder function deteriorates throughout adult life with age appearing to have a direct effect on bladder sensation, contractility and the ability to postpone voiding (Pfisterer et al., 2006). Ageing is associated with several changes that occur in the urinary bladder, such as a reduction in bladder capacity (Hald and Horn, 1998) and an increase in bladder sensitisation (Homma et al., 1994). It is also linked with marked increases in the prevalence of lower urinary tract symptoms, such as urinary frequency, voiding difficulties, decreased bladder contractions (as seen in underactive bladder) or uninhibited bladder contractions (as seen in overactive bladder) (Pfisterer et al., 2006). Urinary frequency and incontinence are common in the general population, with a significant increase over the age of 65 years (Irwin et al., 2006). With a worldwide ageing population increase (Harper, 2014), the incidence of lower urinary tract symptoms is likely to rise.

The mechanisms underlying age-related bladder dysfunction are largely unclear. However, it is known that the prevalence of these symptoms generally increases with age (Takanashi et al., 2019). At this current moment, there are a limited number of studies exploring the physiology of the lower urinary tract throughout ageing.

Immunohistochemical analysis of cultured human detrusor cells identified the presence of all four histamine receptor subtypes: H1, H2, H3 and H4 (Neuhaus et al., 2006). However, no immunohistochemical studies have been performed to determine the presence of these receptor subtypes in urothelium or lamina propria of the urinary bladder. Administration of histamine has previously shown to elicit a contractile response via the activation of the H1 receptors in the isolated guinea pig (Khanna et al., 1977, Kondo et al., 1985, Poli et al., 1988) and rabbit (Fredericks, 1975) bladders. Furthermore, previous findings presented in Chapter 3 established functional responses to histamine not only in detrusor but also in urothelium with lamina propria layer of the urinary bladder, via stimulation of H1 and H2 receptors in juvenile animals. However, it remains unclear how ageing impacts this receptor system.

There are currently no studies that have investigated the impacts of ageing on the contractile responses to histamine in the urinary bladder. In the mouse brain, age is associated with significant changes in the histamine receptor mRNA levels and subsequent reduction in the expression of the H1, H2 and H3 receptors (Terao et al., 2004). In the human brain, similar observations were noted, where H1 receptors showing significant decreases with age (Yanai et al., 1992). The ability of histamine to induce vasodilation via the activation of H1 and H2 receptors is altered with age (Bedarida et al., 1995). Ageing has a direct effect on the signal transduction pathway activated via the H2 receptor, while maintaining the function of the H1 receptor. Furthermore, functional studies show that decreases in the histamine-stimulated

AC activity occur in aged rabbits, appearing to be represented as reductions to maximal responses rather than an alteration in receptor affinity (Makman et al., 1978), while K^+-induced histamine release is lowered in *in vitro* experiments involving aged rat hypothalamus (Ferretti et al., 1998).

As a potent inflammatory mediator released from mast cells, histamine may play a key role in the pathogenesis of various lower urinary tract disorders. Several research studies have reported signs of inflammation in urinary bladder biopsies obtained from patients suffering from bladder overactivity (Compérat et al., 2006, Loran et al., 2007) and established a central role of inflammation on the pathogenesis of IC/BPS (Grover et al., 2011). An increased expression of monocyte chemoattractant protein-1 (MCP-1), which stimulates the release of inflammatory mediators from mast cells (Tyagi et al., 2010, Ghoniem et al., 2011), has been suggested as a contributor to the inflammation observed in the tissue (Chao et al., 2011). Specifically, in the urinary bladder, mast cells can be found in all layers of the bladder wall, including urothelium, lamina propria and detrusor smooth muscle (Christmas and Rode, 1991, Yamada et al., 2000). When urothelial cells of the bladder are damaged or stressed, they are capable of releasing ATP (Säve and Persson, 2010), IL-33 (Jang and Kim, 2015) and β-defensins that directly trigger the degranulation of mast cells and subsequent release of pro-inflammatory mediators, such as histamine, prostaglandins, proteases and cytokines (Amin, 2012).

Increased cellular responsiveness to histamine is a key feature of many inflammatory conditions (Bouchelouche and Bouchelouche, 2006). However, it is unknown whether the histamine receptor system directly mediates contractions of the bladder dome and is associated with bladder pathologies. Histamine is known to induce significant increases in

both the tension and frequency of spontaneous phasic contractions in both the urothelium with lamina propria and in the underlying detrusor muscle through the activation of the H1 receptors (Chapter 3). Histamine also enhances the mechanosensitivity of nearby afferent nerves to bladder distension, resulting in increased neuronal activation in the spinal cord (Grundy et al., 2019). In addition, this effect may work in conjunction with other inflammatory-cell released chemicals such as 5-HT (Moro et al., 2016). These pro-inflammatory mediators may hence work together to induce or maintain various bladder lower urinary tract disorders.

Based on prior research investigating the expression of histamine receptors in ageing, it is hypothesised that responses from stimulation of the H1 receptor will be reduced with ageing in both U&LP and detrusor smooth muscle. It is also hypothesised that stimulation of the H2 receptor, shown to be involved in inhibitory responses in urothelium with lamina propria (Chapter 3), will show greater inhibition of contractions in juvenile tissues when compared to adults. This study aimed to compare responses to histamine in both U&LP and detrusor tissues from porcine juvenile and adult bladders.

6.3. Materials and methods

6.3.1. Tissue source and acquisition

Urinary bladders of crossbred Large-White-Landrace-Duroc (*Suf scrofa domestica*) pigs were used as the tissue in this study. Juvenile samples were obtained from prepubescent pigs aged 6 months old at 80 kg live weight. Adult tissues were taken from sow animals, aged 2-

3 years old at ~200 kg live weight. 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, 2016).

Immediately after the slaughter of pigs at the abattoir, the bladders were removed and directly placed in a cold Krebs-bicarbonate solution at 4°C (NaCl 118.4 mM, $NaHCO_3$ 24.9 mM, $CaCl_2$ 1.9 mM, $MgSO_4$ 2.41 mM, KCl 4.6 mM, KH2PO4 1.18 mM and D-glucose 11.7 mM). Upon collection, a container with porcine bladders was stored in a portable cooler and transported back to Bond University's research laboratory to be set up within 3 hours of the animal's slaughter. Upon return, tissues were prepared by removing the ureters, urethra, serosa and arteries on the outside of the bladder.

6.3.2. Tissue preparation

Tissue strips of U&LP and detrusor were prepared and set up, as described previously in Chapter 2. After the equilibration period, a single dose of histamine was added to both control and experimental tissues. The increases in tonic contractions and the frequency and amplitude of spontaneous phasic contractions were recorded simultaneously through an isometric force transducer on a Powerlab system using LabChart v7 software (MCT050/D, ADInstruments, Castle Hill, Australia). The viability of the tissue was ensured by adding a single dose of carbamoylcholine chloride (10 μM, Sigma Aldrich, Missouri, USA) to all tissues at the very end of each experiment. U&LP or detrusor strips that did not respond to carbamoylcholine chloride were deemed as non-viable and were not included in the analysis.

After each experiment, tissues were removed from each organ bath and measured on a weighing scale to an accuracy of 1 mg.

6.3.3. Measurements and data collection

Measurements of the tonic contractions and the frequency and amplitude of spontaneous phasic contractions were taken before the agonist was added and during peak contractile response after the addition of the agonist. The frequency of spontaneous contractions was measured from the total number of phasic waves occurring over 2-3 minutes, calculated as an average and expressed as contractions per minute (cpm). The amplitude of each contraction was measured as the tension differences between the averaged lowest and highest point of each phasic wave. Tonic contractions were measured from the lowest point of each spontaneous phasic contraction before treatment with an agonist and during peak contractile response. Changes in both tonic contractions and the amplitudes of spontaneous phasic contractions were expressed as Newton force per gram tissue weight (mN/g).

6.4.4. Pharmacological agents

Histamine dihydrochloride, amthamine dihydrobromide, pyrilamine maleate salt, cimetidine, thioperamide maleate salt, indomethacin, atropine and Nω-Nitro-L-arginine were obtained from Sigma-Aldrich (Missouri, USA), fexofenadine hydrochloride, αβ-methylene ATP (sodium salt) and cyproheptadine hydrochloride hydrate from Cayman Chemicals (Michigan, USA). Concentrations chosen for the agonists and antagonists were selected based on their selectivity at each receptor and consistent with concentrations used in Chapter 3 to enable comparison between the two age groups.

6.4.5. Statistical analysis

Data were graphed and analysed using GraphPad Prism version 8.3 for Windows (GraphPad Software, La Jolla California USA) and results expressed as the mean change ± SEM. Within experiments using only juvenile, or only adult tissues, data were calculated as the total change between the control and its paired experimental sample. For comparisons between age groups, data comparing juvenile with adult samples were calculated from the averaged changes between each tissue's responses when placed under identical experimental parameters. All responses were compared using a Student's two-tailed *t*-test, with $p < 0.05$ considered as statistically significant. A paired Student's two-tailed *t*-test was applied to tissues with direct controls (within each juvenile or adult experiment), and an unpaired Student's two-tailed *t*-test was applied to make comparisons between juvenile and aged groups.

6.5. Results

6.5.1. Histamine agonists stimulating U&LP phasic activity in juvenile and adult tissues

Strips of U&LP developed spontaneous phasic contractions in the absence of any stimulation during the baseline (also noted in Chapters 3, 4 and 5). In juvenile animals, these contractions occurred at 3.50 ± 0.13 cycles per minute (cpm, n = 51) with an amplitude of 23.11 ± 1.76 mN/g (n = 51). The frequency of spontaneous contractions in adult animals occurred at a frequency of 3.37 ± 0.11 cpm (n = 59) with an amplitude of 34.88 ± 3.79 mN/g (n = 59). While there were no significant differences in the frequency of spontaneous phasic

contractions between juvenile (n = 52) and adult (n = 59) tissues, the amplitude of phasic contractions was significantly smaller in juvenile tissues ($p < 0.001$).

When histamine (100 μM) was added to U&LP strips of juvenile animals, the frequency of spontaneous phasic contractions increased by 1.29 ± 0.26 cpm ($p < 0.001$, n = 51) and the amplitude of each contraction decreased by 5.54 ± 1.60 mN/g ($p < 0.001$, n = 51). In U&LP strips of adult animals, the frequency of spontaneous phasic contractions increased by 1.18 ± 0.16 cpm ($p < 0.001$, n = 55) and amplitude decreased by 10.05 ± 2.06 mN/g ($p < 0.001$, n = 55). The increases in the tonic contractions and the frequency and amplitude of spontaneous phasic contractions in response to histamine (100 μM) were not significantly different between juvenile (n = 51) and adult (n = 55) tissues.

Treatment with the H2 receptor agonist amthamine did not affect the frequency or the amplitude of the spontaneous phasic contractions in either juvenile or adult U&LP preparations.

6.5.2. Histamine agonists stimulating detrusor spontaneous activity in juvenile and adult tissues

In detrusor preparations of juvenile animals, spontaneous activity in the absence of any stimulation was present in 13% of preparations that exhibited phasic contractions at a frequency of 2.06 ± 0.30 cpm and amplitude of 18.29 ± 3.07 mN/g (n = 7). After the addition of histamine (100 μM), the frequency was significantly enhanced, increasing to 4.47 ± 0.69 cpm (n = 7, $p < 0.01$) with an amplitude of 29.07 ± 6.40 mN/g (n = 7, NSD). In adult preparations, this activity was present in 30% of all preparations (n = 17) with an average

frequency of 1.95 ± 0.15 cpm and amplitude of 14.26 ± 2.15 mN/g. In response to histamine (100 μM), frequency of spontaneous contractions increased significantly to 2.39 ± 0.16 cpm ($n = 17$, $p < 0.01$) with an amplitude of 24.22 ± 4.36 mN/g ($n = 17$, $p < 0.05$). There were no significant differences in the frequency or amplitude of spontaneous phasic contractions exhibited by detrusor preparations of both juvenile and adult animals before the treatment with histamine.

During peak response to histamine (100 μM), strips of detrusor from juvenile animals ($n = 7$) exhibited a significantly higher frequency of spontaneous contractions when compared to adult tissues ($n = 17$, $p < 0.001$). The amplitude of spontaneous contractions exhibited during peak response to histamine (100 μM) was not significantly different between the two age groups. The spontaneous phasic activity was not present at any point during the experiment in 35% of juvenile preparations and 34% ($n = 18$) of adult preparations ($n = 19$).

In those detrusor strips that did not develop spontaneous activity during baseline, treatment with histamine (100 μM) initiated contractions in 59% ($n = 26$) of preparations of juvenile animals with a frequency of 2.90 ± 0.20 cpm and amplitude of 24.46 ± 8.40 mN/g. In adult animals, contractions were evoked in 51% of preparations ($n = 20$) with an average frequency of 2.83 ± 0.32 cpm and amplitude of 14.79 ± 1.61 mN/g. An example of spontaneous phasic contractions being initiated after treatment with histamine (100 μM) in both juvenile and adult preparations can be seen in Figure 6-2. There were no significant differences in the frequency and amplitude of spontaneous contraction developed after treatment with histamine (100 μM) between the two age groups.

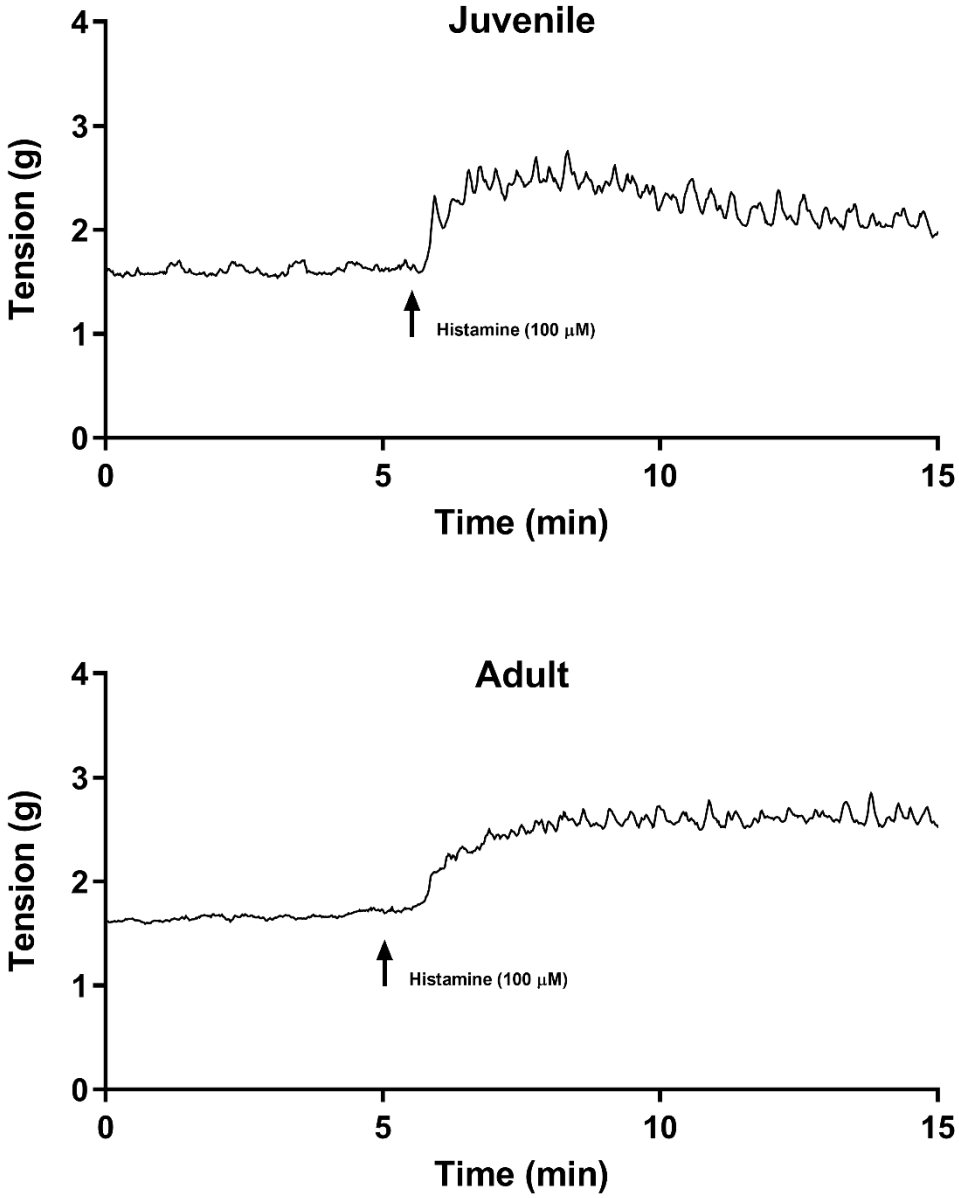

Figure 6-2: Example of how histamine agonist was able to initiate spontaneous contractions in detrusor preparations. These contractions occurred in both juvenile (*top*) and adult (*bottom*) tissue preparations.

6.5.3. Histamine agonists stimulating contractions in U&LP and detrusor in juvenile and adult tissues

When histamine (100 μM) was added to U&LP strips of juvenile animals, tonic contractions increased by 47.84 ± 6.52 mN/g ($p < 0.001$, $n = 51$, Figure 6-3). Similarly, the addition of histamine (100 μM) to U&LP strips of adult animals increased tonic contractions by 50.76 ± 4.10 mN/g ($p < 0.001$, $n = 55$, Figure 6-3). Both age groups exhibited similar increases in the tonic contractions with no statistically significant differences between the two groups.

In detrusor preparations, young tissues showed significantly greater ($p < 0.05$) increases in tonic contractions of 19.10 ± 4.92 mN/g ($n = 51$) when compared to aged tissues, which exhibited increases of 8.21 ± 0.89 mN/g ($n = 56$, Figure 6-4) in response to histamine (100 μM).

In bladders of juvenile animals, U&LP responses to the H2 agonist amthamine (1 μM) resulted in a decrease in tonic contractions of 13.97 ± 3.45 mN/g ($n = 12$, $p < 0.05$). The preparation of juvenile U&LP tissues showed a significantly greater reduction in tonic contractions in response to amthamine (1μM) when compared to adult U&LP tissues ($p < 0.05$, Figure 6-5). In detrusor preparations, the addition of amthamine (100 μM) in both juvenile ($n = 4$) and adult ($n = 8$) tissues did not affect the tonic contractions (Figure 6-5).

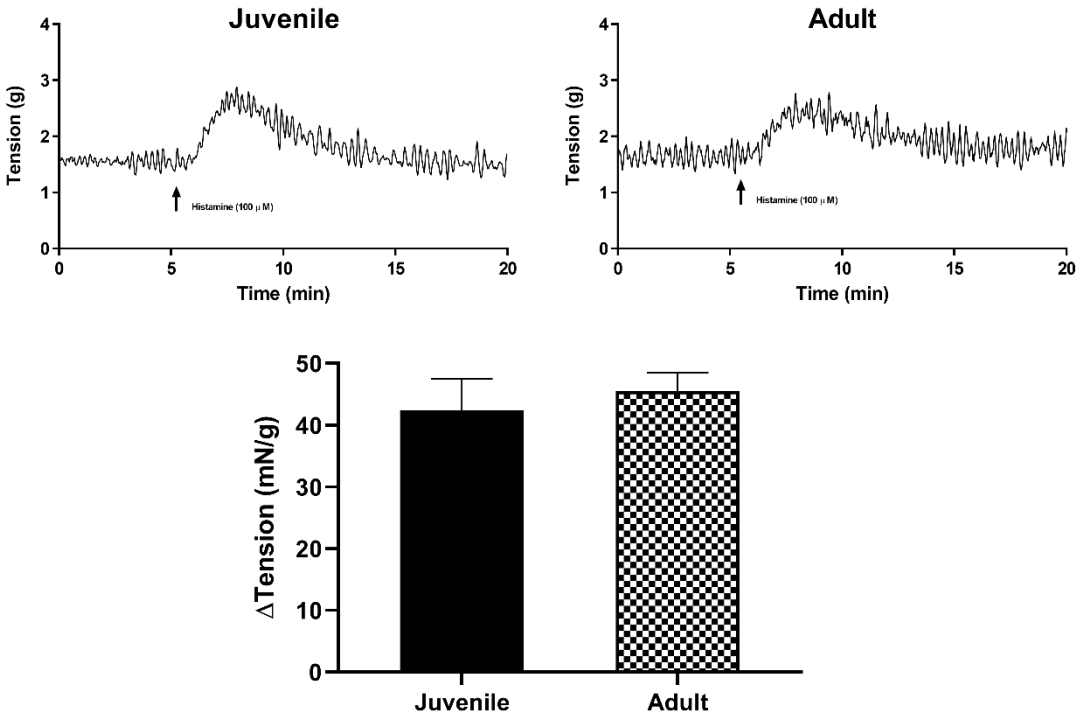

Figure 6-3: U&LP responses to histamine (100 μM, *top row*) in juvenile (*left*) and adult (*right*) tissue samples. Increases in tonic contractions after treatment with histamine are represented as mean change ± SEM (*bottom*). Changes in tonic contractions between juvenile and adult age groups were evaluated using an unpaired Student's two-tailed *t*-test, NSD.

Detrusor

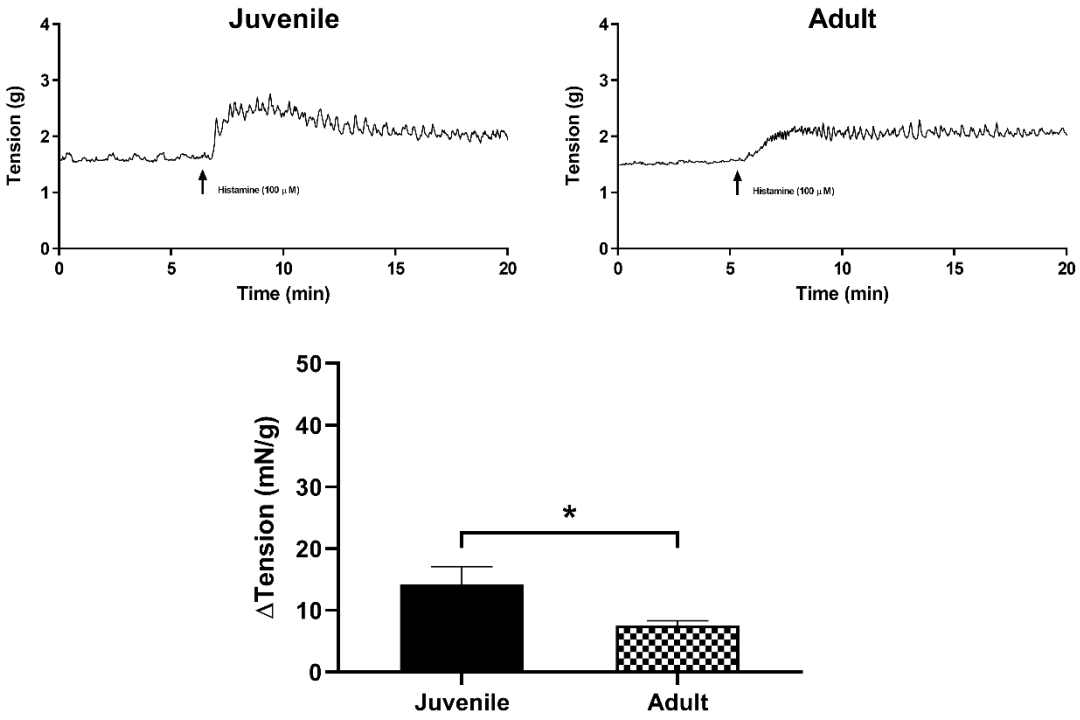

Figure 6-4: Detrusor responses to histamine (100 μM) in juvenile (*top*) and adult (*middle*) tissue samples. Increases in tonic contractions after treatment with histamine are represented as mean change ± SEM (*bottom*). Changes in tonic contractions between juvenile and adult age groups were evaluated using an unpaired Student's two-tailed *t*-test, where *$p < 0.05$.

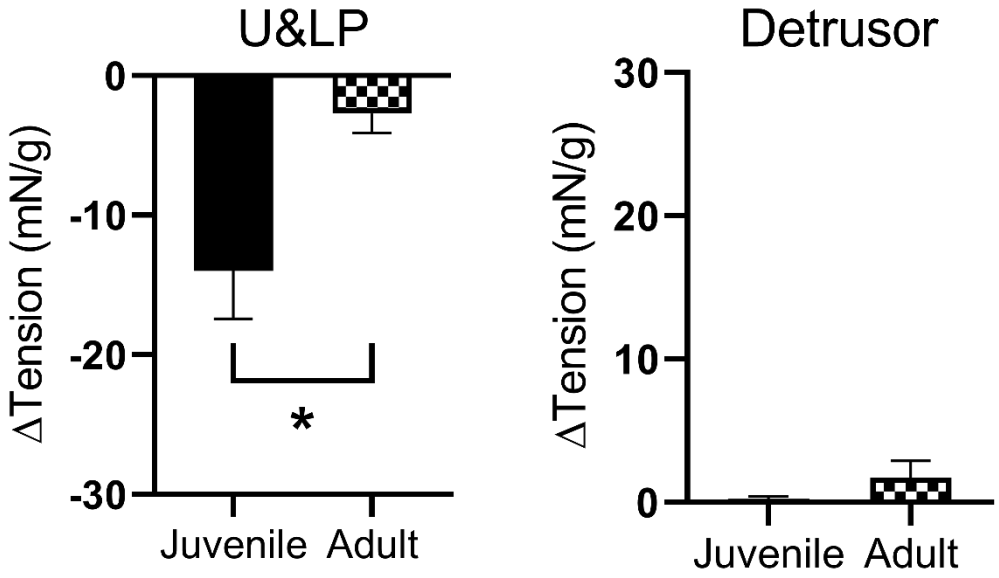

Figure 6-5: Influence of age on the tonic contractions in the U&LP (*left*) and detrusor (*right*) in responses to the H2 agonist amthamine (1 μM). *$p < 0.05$

6.5.4. Selective histamine receptor antagonists in U&LP of adult animals

The effects of histamine in adult U&LP preparations was investigated in the presence of five selective histamine receptor antagonists: pyrilamine, cyproheptadine, fexofenadine, cimetidine and thioperamide. When tissues were treated with pyrilamine (H1 antagonist, 30 nM), increases in both the tonic contractions and frequency of spontaneous contractions to histamine (100 μM) were significantly inhibited when compared to the control tissues ($n = 14$, $p < 0.01$ for both, Figure 6-6). In the presence of pyrilamine, tonic contractions increased by 15.25 ± 2.30 mN/g ($n = 14$) and the frequency of phasic contractions by 0.34 ± 0.10 cpm ($n = 14$), with no significant differences observed in the amplitude of phasic contractions.

Alternative H1 antagonists, cyproheptadine and fexofenadine, were also used to ascertain the involvement of the H1 receptor, consistent with the approach used in Chapter 3. In the presence of cyproheptadine (30 nM), significant inhibition to increases in tonic contractions in response to histamine (100 μM) were observed, exhibiting contractions of 28.46 ± 6.04 mN/g ($n = 8$, $p < 0.01$, Figure 6-7). The presence of cyproheptadine had no impact on the frequency or amplitude of phasic contractions. The presence of fexofenadine (1 μM) completely inhibited increases in tonic contractions ($n = 8$, $p < 0.001$, Figure 6-8), with most tissues showing decreases in tonic tension below the baseline in response to histamine (100 μM). Furthermore, the frequency of phasic contractions decreased by 0.13 ± 0.09 cpm ($n = 8$, $p < 0.01$, Figure 6-8) in response to histamine (100 μM), with no significant differences in the amplitude of phasic contractions.

Preparations treated with cimetidine (1 μM, H2 antagonist) demonstrated no impact on increases in tonic contractions and on the frequency and amplitude of spontaneous phasic

contractions in response to histamine (100 μM, Figure 6-9). In the presence of thioperamide (1 μM, H3 and H4 antagonist), increases to phasic contractions in response to histamine (100 μM) were significantly inhibited, exhibiting contractions of 33.82 ± 5.19 mN/g ($n = 8$, $p < 0.05$, Figure 6-10). The frequency or amplitude of spontaneous phasic contractions was not influenced in the presence of thioperamide.

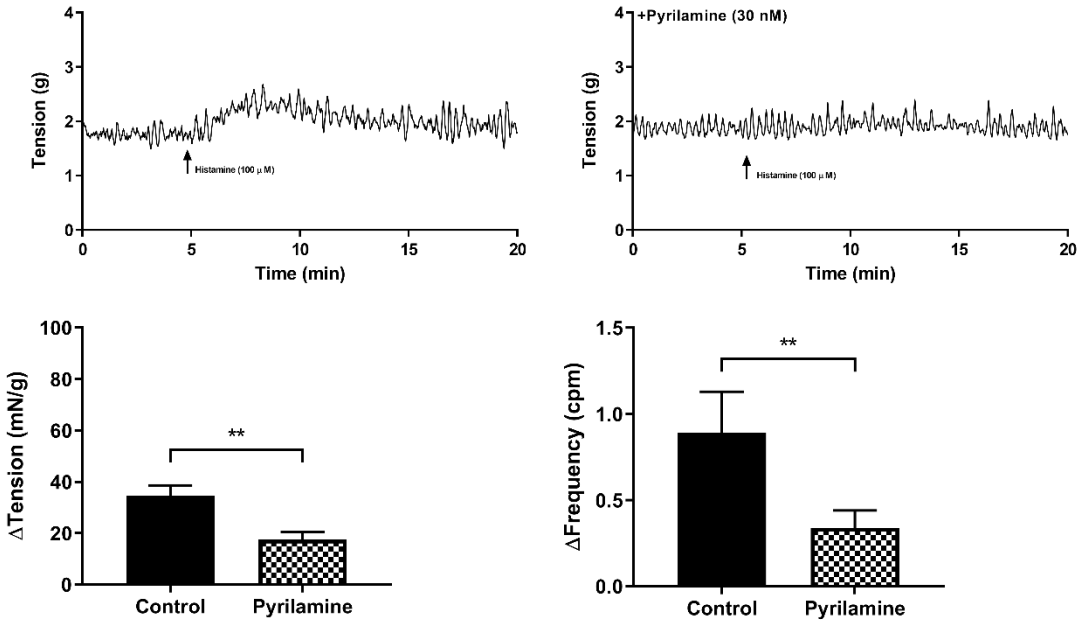

Figure 6-6: U&LP tonic contractions in response to histamine (100 µM, *top row*) as control (*left*) and in the presence of the histamine 1 (H1) receptor antagonist pyrilamine (30 nM, *right*) in adult preparations. Changes in tonic contractions (*bottom left*) and in the frequency of phasic activity (*bottom right*) in response to histamine are represented as mean change ± SEM. Changes in tonic contractions between control and experimental conditions were evaluated using a paired Student's two-tailed *t*-test, where **p < 0.01

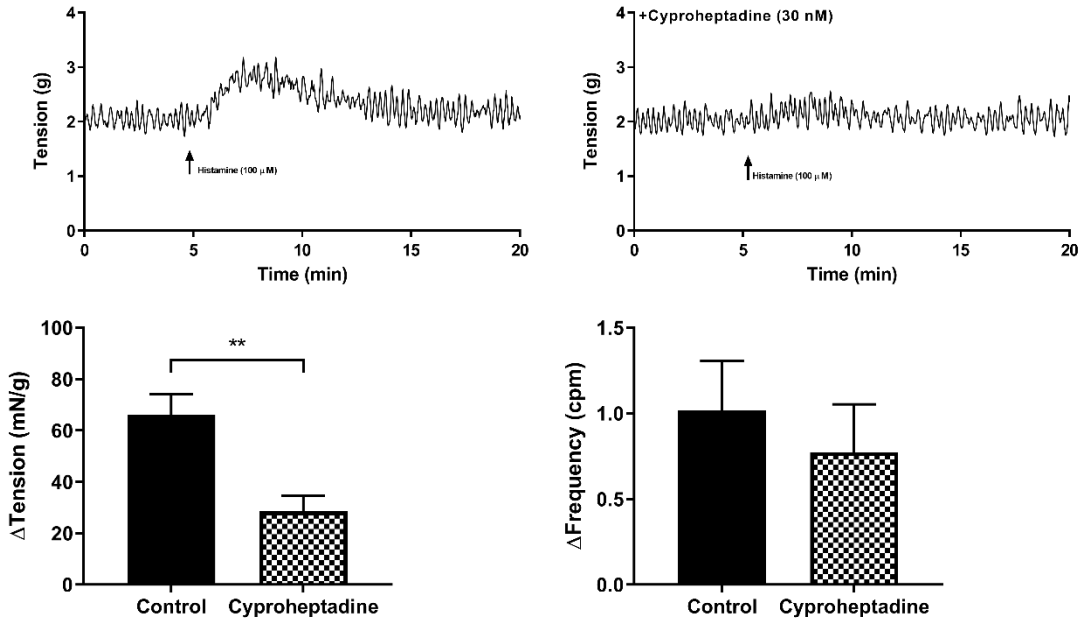

Figure 6-7: U&LP tonic contractions in response to histamine (100 μM, *top row*) as control (*left*) and in the presence of the histamine 1 (H1) receptor antagonist cyproheptadine (30 nM, *right*) in adult preparations. Changes in tonic contractions (*bottom left*) and in the frequency of phasic activity (*bottom right*) in response to histamine are represented as mean change ± SEM. Changes in tonic contractions between control and experimental conditions were evaluated using a paired Student's two-tailed *t*-test, where **$p < 0.01$

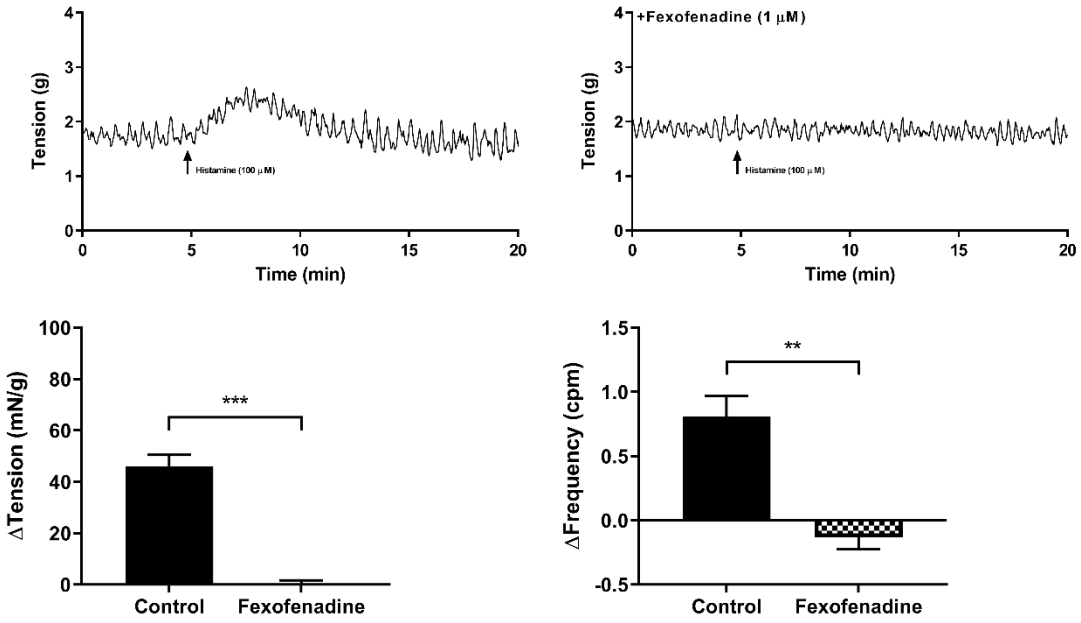

Figure 6-8: U&LP tonic contractions in response to histamine (100 μM) as control (*top*) and in the presence of the histamine 1 (H1) receptor antagonist fexofenadine (1 μM, *middle*) in adult preparations. Changes in tonic contractions (*bottom left*) and in the frequency of phasic activity (*bottom right*) in response to histamine are represented as mean change ± SEM. Changes in tonic contractions between control and experimental conditions were evaluated using a paired Student's two-tailed *t*-test, where **p < 0.01, ***p < 0.001

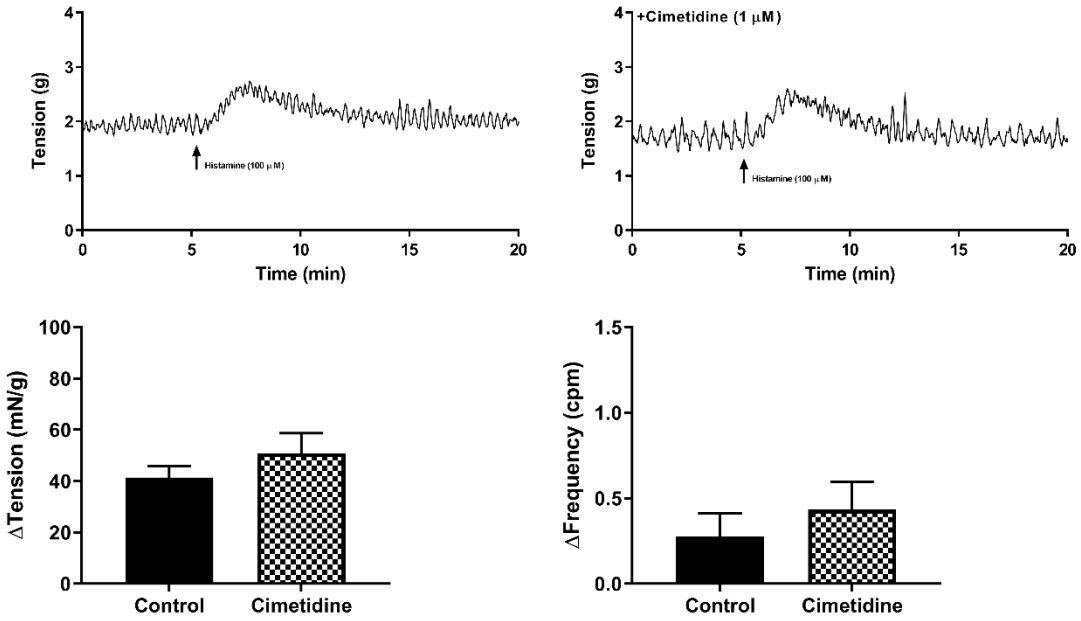

Figure 6-9: U&LP tonic contractions in response to histamine (100 μM) as control (*top*) and in the presence of the histamine 2 (H2) receptor antagonist cimetidine (1 μM, *middle*) in adult preparations. Changes in tonic contractions (*bottom left*) and in the frequency of phasic activity (*bottom right*) in response to histamine are represented as mean change ± SEM. Changes in the tonic contractions between control and experimental conditions were evaluated using a paired Student's two-tailed *t*-test, NSD.

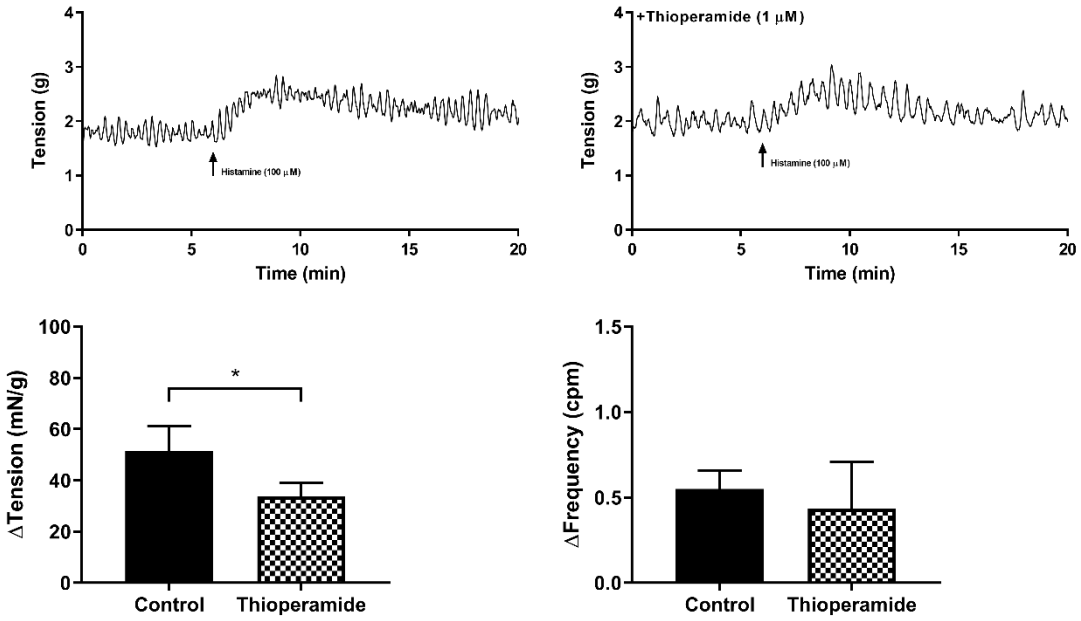

Figure 6-10: U&LP tonic contractions in response to histamine (100 µM) as control (*top*) and in the presence of the histamine 3 and 4 (H3 and H4) receptor antagonist thioperamide (1 µM, *middle*) in adult preparations. Changes in tonic contractions (*bottom left*) and in the frequency of phasic activity (*bottom right*) in response to histamine are represented as mean change ± SEM. Changes in tonic contractions between control and experimental conditions were evaluated using a paired Student's two-tailed *t*-test, where *$p < 0.05$

6.5.5. Combination of selective histamine receptor antagonist in U&LP of adult animals

Experiments involving a combination of histamine receptor antagonists were used to elucidate the involvement of a specific receptor subtype in observed increases in tonic contractions in response to histamine (100 μM). The effectiveness of pyrilamine in significantly inhibiting increases to histamine (100 μM) was demonstrated in experiments comparing effects of tissues solely treated with cimetidine (1 μM, n = 8) and tissues treated with a combination of cimetidine (1 μM) and pyrilamine (30 nM, n = 8, $p < 0.01$, Figure 6-11).

Furthermore, the efficacy of pyrilamine (30 nM) in inhibiting increases in tonic contractions to histamine (100 μM) were equally effective when tissues were treated solely with pyrilamine (30 nM, n = 8) and when it was used in combination with cimetidine (1 μM, n = 8, Figure 6-12).

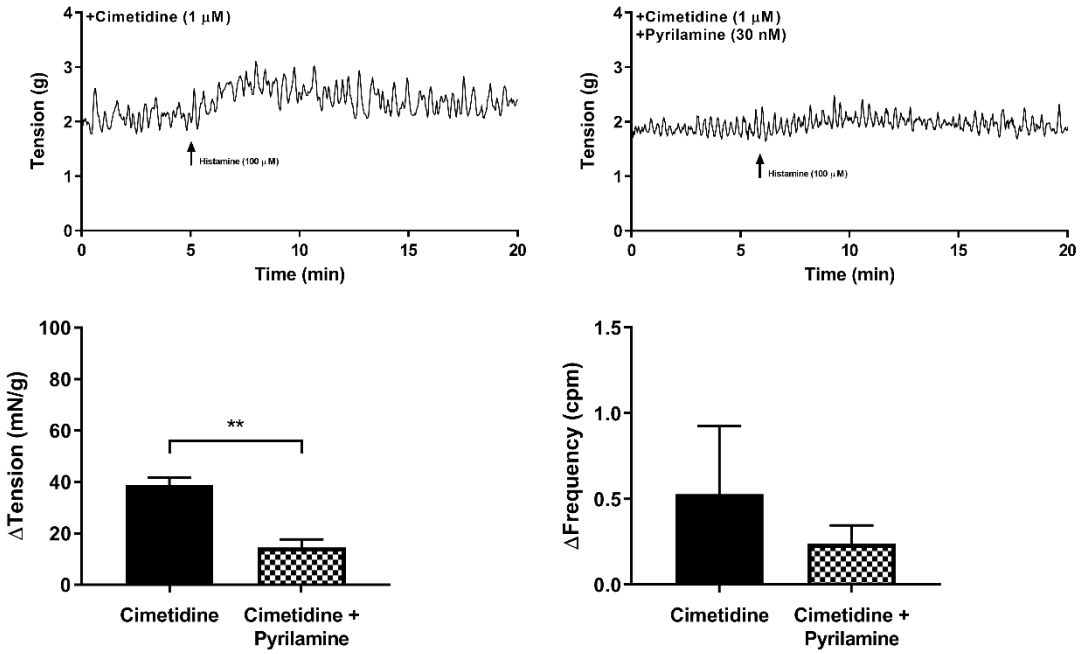

Figure 6-11: Comparison of U&LP tonic contractions in response to histamine (100 μM) in the presence of the H2 receptor antagonist cimetidine (1 μM, *top left*) and the presence of the combination of H1 and H2 receptor antagonists cimetidine (1 μM) and pyrilamine (30 nM, *top left*) in adult preparations. Changes in tonic contractions (*bottom left*) and in the frequency of phasic activity (*bottom right*) in response to histamine are represented as mean change ± SEM. Changes in tonic contractions between control and experimental conditions were evaluated using a paired Student's two-tailed *t*-test, where **$p < 0.01$

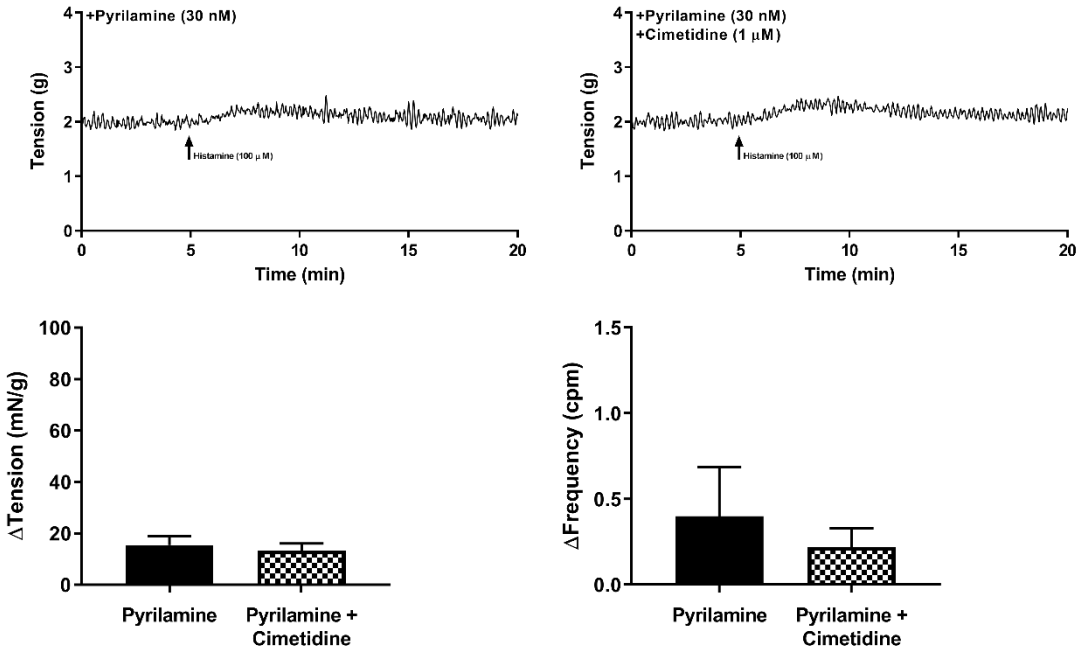

Figure 6-12: Comparison of U&LP tonic contractions in response to histamine (100 μM) in the presence of the H1 receptor antagonist pyrilamine (30 nM, *top left*) and the presence of the combination of H1 and H2 receptor antagonists pyrilamine (30 nM) and cimetidine (1 μM, *top right*). Changes in tonic contractions (*bottom left*) and in the frequency of phasic activity (*bottom right*) in response to histamine are represented as mean change ± SEM. Changes in tonic contractions between control and experimental conditions were evaluated using a paired Student's two-tailed *t*-test, NSD.

6.5.6. Selective histamine receptor antagonists in detrusor of adult animals

In the presence of 30 nM pyrilamine (H1 receptor antagonist), responses to histamine (100 μM) were significantly inhibited in comparison to the control tissues exhibiting increases of 5.44 ± 1.24 mN/g ($n = 8$, $p < 0.05$, Figure 6-13). Responses to histamine (100 μM) in the presence of an alternative H1 antagonist cyproheptadine (30 nM) also resulted in significantly inhibited increases to tonic contractions of 1.37 ± 0.33 mN/g ($n = 8$, $p < 0.05$, Figure 6-14). Finally, the H1 receptor antagonist fexofenadine (1 μM) completely inhibited all increases in tonic contractions to histamine (100 μM, $p < 0.05$, Figure 6-15).

In the presence of cimetidine (H2 receptor antagonist, 1 μM, $n = 8$, Figure 6-16) and thioperamide (H3 and H4 receptor antagonist, 1 μM, $n = 8$, Figure 6-17), no significant differences in tonic contractions between control and experimental tissues were observed.

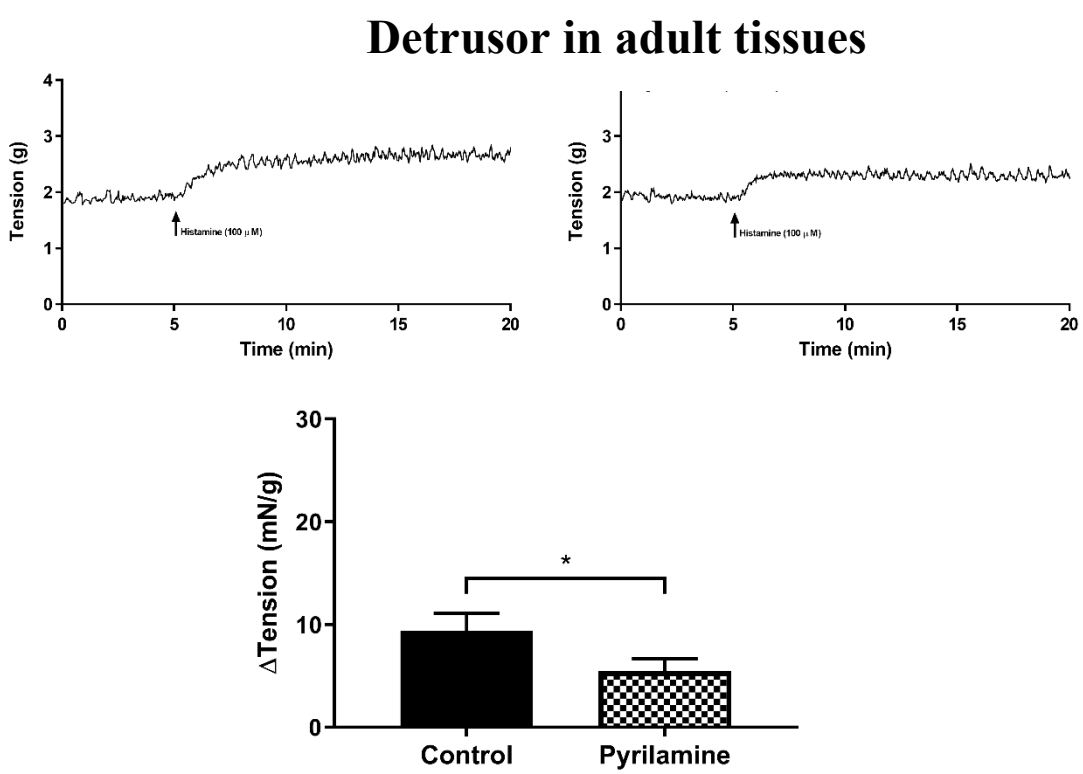

Figure 6-13: Detrusor tonic contractions in response to histamine (100 μM) as control (*top left*) and in the presence of the histamine 1 (H1) receptor antagonist pyrilamine (30 nM, *top right*) in adult preparations. Changes in the tonic contractions after treatment with histamine are represented as mean change ± SEM (*bottom*). Changes in tonic contractions between control and experimental conditions were evaluated using a paired Student's two-tailed *t*-test, where *$p < 0.05$.

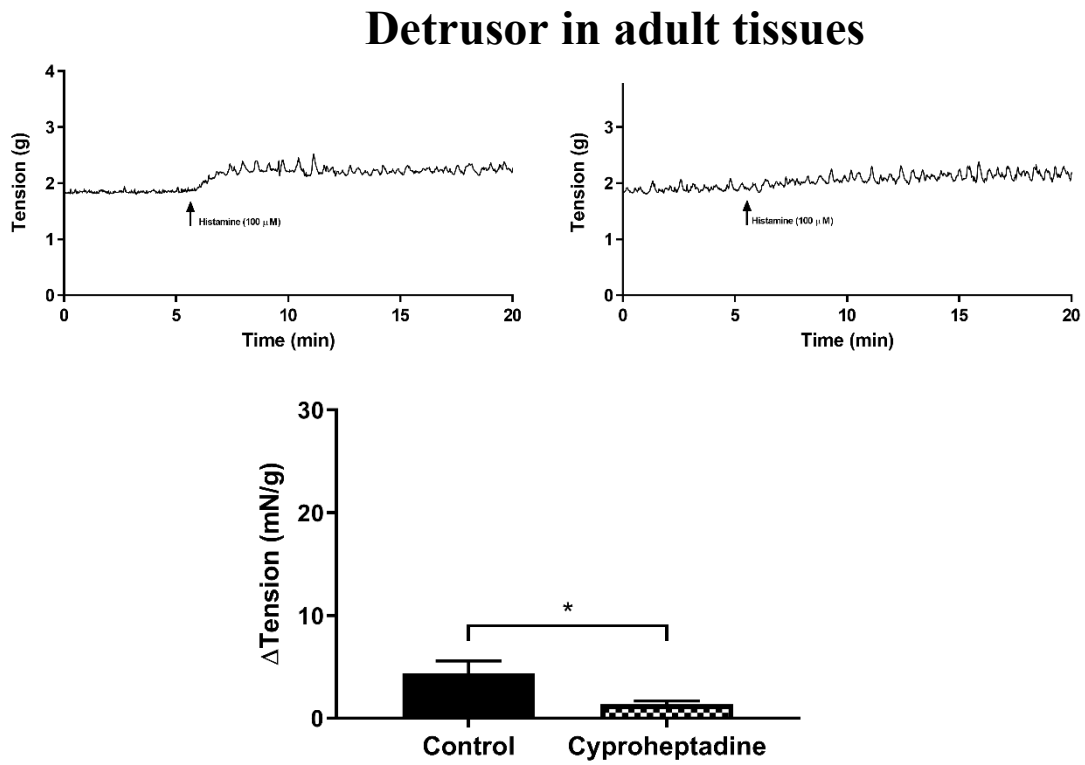

Figure 6-14: Detrusor tonic contractions in response to histamine (100 μM) as control (*top left*) and in the presence of the histamine 1 (H1) receptor antagonist cyproheptadine (30 nM, *top right*) in adult preparations. Changes in the tonic contractions after treatment with histamine are represented as mean change ± SEM (*bottom*). Changes in tonic contractions between control and experimental conditions were evaluated using a paired Student's two-tailed *t*-test, where *$p < 0.05$.

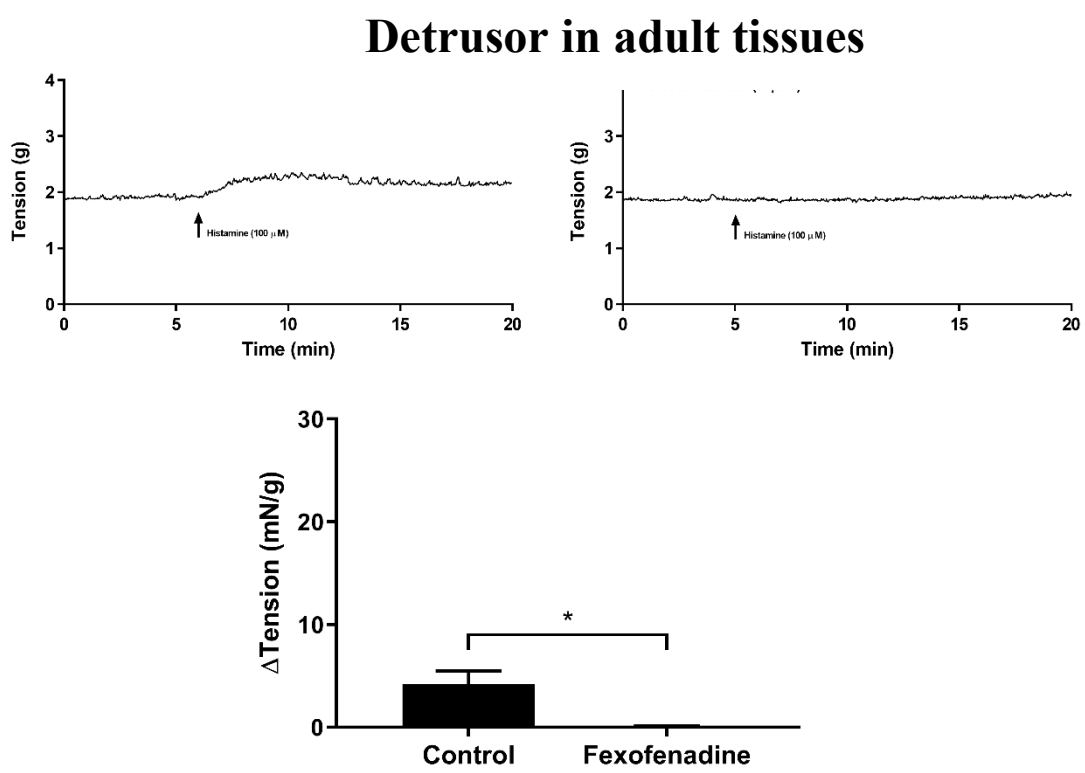

Figure 6-15: Detrusor tonic contractions in response to histamine (100 μM) as control (*top left*) and in the presence of the histamine 1 (H1) receptor antagonist fexofenadine (1 μM, *top right*) in adult preparations. Changes in the tonic contractions after treatment with histamine are represented as mean change ± SEM (*bottom*). Changes in tonic contractions between control and experimental conditions were evaluated using a paired Student's two-tailed *t*-test, where *$p < 0.05$.

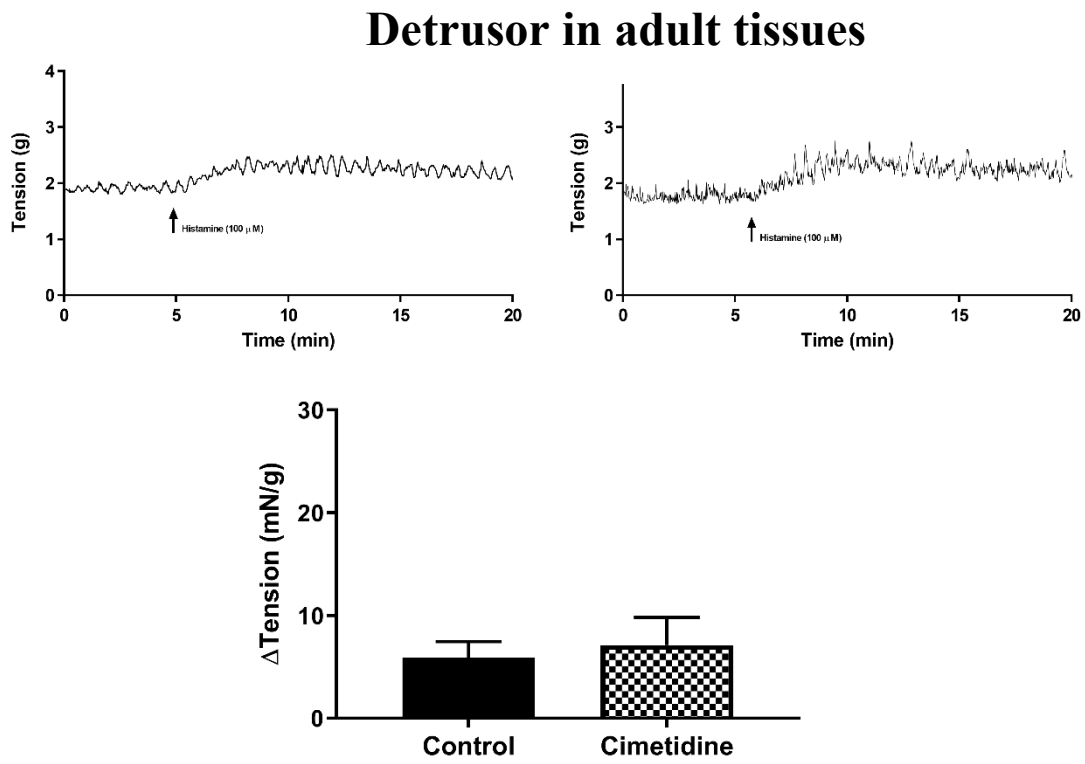

Figure 6-16: Detrusor tonic contractions in response to histamine (100 μM) as control (*top left*) and in the presence of the histamine 2 (H2) receptor antagonist cimetidine (1 μM, *top right*) in adult preparations. Changes in tonic contractions after treatment with histamine are represented as mean change ± SEM (*bottom*). Changes in tonic contractions between control and experimental conditions were evaluated using a paired Student's two-tailed *t*-test, NSD.

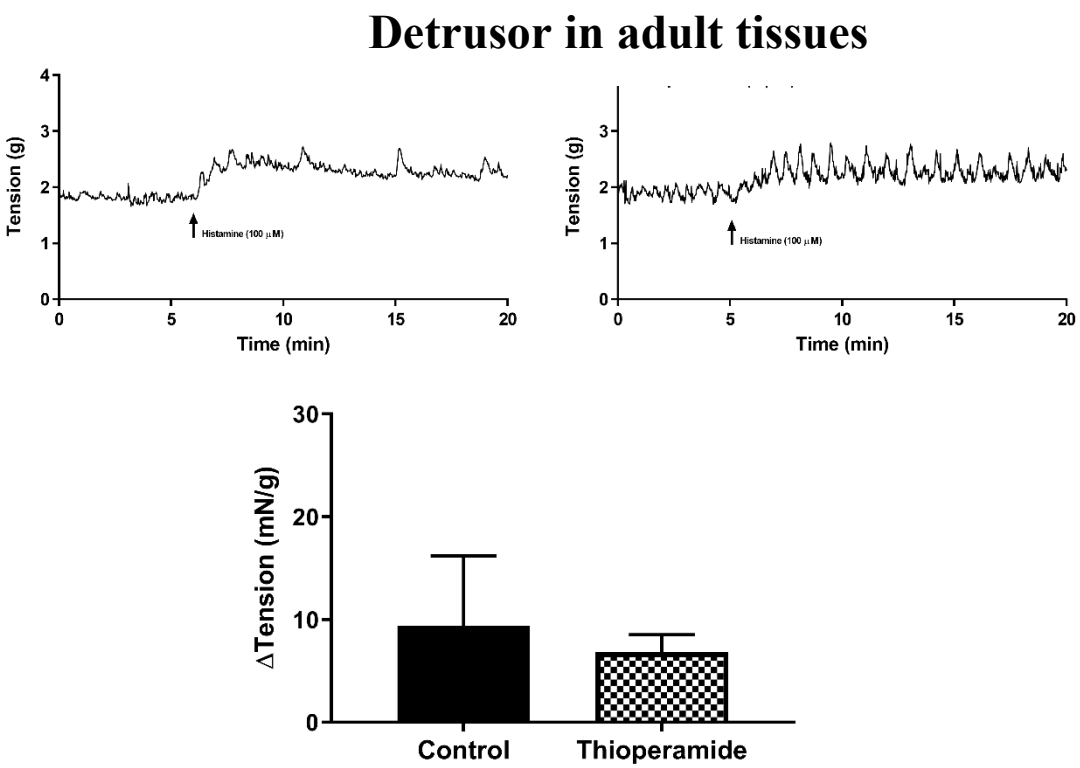

Figure 6-17: Detrusor tonic contractions in response to histamine (100 µM) as control (*top left*) and in the presence of the histamine 3 and 4 (H3/H4) receptor antagonist thioperamide (1 µM, *top right*) in adult preparations. Changes in tonic contractions after treatment with histamine are represented as mean change ± SEM (*bottom*). Changes in tonic contractions between control and experimental conditions were evaluated using a paired Student's two-tailed *t*-test, NSD.

6.5.7. Comparison of receptor-selective antagonists in U&LP preparations of juvenile and adult animals

The influence of three different H1 receptor antagonists on tonic contraction responses to histamine was compared between juvenile and adult tissues. Strips of U&LP treated with the H1 receptor antagonists pyrilamine (30 nM), fexofenadine (1 μM) and cyproheptadine (30 nM) showed significant inhibition of the increases in tonic contractions in response to histamine in both juvenile and adult preparations (100 μM, $p < 0.01$ for all, Table 6-1). None of the increases in tonic contraction to histamine (100 μM) in the presence of any of the three H1 antagonists were significantly different between juvenile or adult tissues (unpaired Student's *t*-test, p = NSD for all).

Treatment with cimetidine (1 μM, H2 receptor antagonist) in juvenile U&LP tissues significantly enhanced increases in tonic contractions by 19.05 ± 8.32 mN/g ($n = 10$, $p < 0.05$) in response to histamine (100 μM), but had no effect in adult tissues ($n = 8$, Table 6-1). Treatment with thioperamide (1 μM, H3/H4 receptor antagonist) in juvenile tissues caused significantly enhanced increases in tonic contractions of 27.33 ± 8.67 mN/g ($n = 6$, $p < 0.05$) in response to histamine (100 μM). The opposite effect was observed in adult tissues in response to histamine (100 μM), where increases in tonic contractions were significantly inhibited by 17.64 ± 7.22 mN/g ($n = 8$, $p < 0.05$, Table 1). However, upon further investigation involving a combination of histamine receptor antagonists, it was revealed that thioperamide did not affect tonic contractions in response to histamine (100 μM) in both juvenile and adult tissues.

Table 6-1 – Tonic contractions: Comparison of U&LP changes to tonic contractions (mN/g) in response to histamine (100 μM) in the absence and presence of histamine receptor antagonists (mean change ± SEM) in juvenile and adult urinary bladders.

	Juvenile (mN/g)			Adult (mN/g)		
Antagonist	Absence	Presence	n	Absence	Presence	n
Pyrilamine	34.82 ± 11.09	6.39 ± 5.74***	14	34.52 ± 4.14	17.56 ± 2.81**	14
Fexofenadine	44.63 ± 11.78	-1.63 ± 1.01**	8	46.03 ± 4.44	-0.59 ± 2.21**	8
Cyproheptadine	59.56 ± 18.97	21.00 ± 8.11*	8	66.18 ± 7.97	28.46 ± 6.04**	8
Cimetidine	61.70 ± 22.26	80.75 ± 28.12*	10	41.40 ± 4.45	50.81 ± 7.85	8
Thioperamide	59.92 ± 14.35	87.25 ± 14.71*	6	51.47 ± 9.78	33.82 ± 5.19*	8

*p < 0.05, **p < 0.01, ***p < 0.001. Paired Student's two-tailed *t*-test.

In both juvenile and adult preparations, increases in the frequency of spontaneous phasic contractions were significantly inhibited in response to histamine (100 μM) when the tissues were treated with pyrilamine (30 nM, $p < 0.05$ for both). Treatment with fexofenadine (1 μM) caused complete inhibition to the increases in tonic contractions in response to histamine (100 μM) in both juvenile ($n = 8$, $p < 0.01$) and adult tissues ($n = 8$, $p < 0.01$). In tissues from juvenile animals, cyproheptadine (30 nM) significantly inhibited increases in spontaneous activity ($n = 8$, $p < 0.05$) in response to histamine (100 μM), although this was not replicated in adult tissues ($n = 8$, Table 6-2).

Treatment with cimetidine (H2 receptor antagonist) did not affect the frequency and amplitude of spontaneous phasic contractions in both juvenile and adult tissues in response to histamine (100 μM). The addition of thioperamide (H3/H4 receptor antagonist) caused a significant inhibition in the frequency of spontaneous activity in tissues from adult animals ($n = 8$, $p < 0.01$), but did not affect tissues obtained from juvenile animals ($n = 6$). None of the five histamine receptor antagonists had any influence on the amplitude of spontaneous phasic contractions in response to histamine (100 μM), in either juvenile or adult preparations (Table 6-3).

Table 6-2 – Frequency of phasic contractions: Comparison of U&LP changes in the frequency of spontaneous phasic contractions (cpm) in response to histamine (100 μM) in the absence and presence of histamine receptor antagonists (mean change ± SEM) in juvenile and adult urinary bladders.

	Juvenile (cycles per minute)			Adult (cycles per minute)		
Antagonist	Absence	Presence	n	Absence	Presence	n
Pyrilamine	1.27 ± 0.29	0.23 ± 0.22**	12	0.89 ± 0.24	0.34 ± 0.10*	14
Fexofenadine	1.11 ± 0.28	-0.09 ± 0.14**	8	0.81 ± 0.15	-0.13 ± 0.09**	8
Cyproheptadine	1.87 ± 0.78	0.56 ± 0.28*	8	1.01 ± 0.29	0.77 ± 0.28	8
Cimetidine	1.77 ± 0.57	1.32 ± 0.75	10	1.40 ± 0.77	1.18 ± 0.26	8
Thioperamide	1.49 ± 0.63	1.76 ± 0.55	6	1.84 ± 0.41	0.63 ± 0.27**	8

*$p < 0.05$, **$p < 0.01$. Paired Student's *t*-test.

Table 6-3 – Amplitude of phasic contractions: Comparison of U&LP changes in the amplitude of spontaneous phasic contractions (mN/g) in response to histamine (100 μM) in the absence and presence of histamine receptor antagonists (mean change ± SEM) in juvenile and adult urinary bladders. None of the antagonists had statistically significant effects on the amplitude of phasic contractions using a paired two-tailed Student's *t*-test.

	Juvenile (mN/g)			Adult (mN/g)		
Antagonist	Absence	Presence	n	Absence	Presence	n
Pyrilamine	-9.14 ± 3.71	-10.36 ± 5.10	12	-7.38 ± 3.13	-2.71 ± 2.36	14
Fexofenadine	-2.88 ± 5.57	-3.69 ± 3.05	8	-6.18 ± 1.94	-5.00 ± 3.63	8
Cyproheptadine	-5.69 ± 1.95	-4.50 ± 3.27	8	-21.69 ± 7.88	-9.04 ± 4.33	8
Cimetidine	-4.55 ± 1.30	-7.40 ± 2.50	10	-8.53 ± 3.22	-13.97 ± 7.10	8
Thioperamide	-8.67 ± 3.80	-9.67 ± 6.00	6	-8.09 ± 3.57	-7.35 ± 2.93	8

The potential contributions of other receptor systems present in the U&LP to histamine-induced (100 μM) contractile responses were investigated. Neither the tonic contractions nor the frequency or amplitude of spontaneous phasic contractions in response to histamine in both juvenile and adult strips of U&LP were affected by the presence of the muscarinic receptor antagonist atropine (1 μM), cyclooxygenase (COX) inhibitor indomethacin (5 μM), nitric oxide synthase inhibitor N_{ω}-Nitro-L-arginine (L-NNA, 100 μM) or the P2X receptor desensitising agonist αβ-methylene ATP (αβm-ATP, 10 μM).

6.5.8. Influence of histamine receptor antagonists in the detrusor of juvenile and adult urinary bladders

The presence of pyrilamine in juvenile detrusor preparations showed significantly greater inhibition of the contractile response to histamine (100 μM) when compared to adult tissues ($p < 0.05$). In juvenile preparations, increases in tonic contractions were inhibited by 12.46 ± 3.88 mN/g ($n = 14$, $p < 0.05$), whereas in adult tissues these increases were inhibited by 3.91 ± 1.73 mN/g ($n = 14$, $p < 0.05$, Table 6-4). Treatment with an alternative H1 receptor antagonist fexofenadine inhibited increases in tonic contractions in response to histamine (100 μM) by 9.61 ± 3.95 mN/g ($n = 8$, $p < 0.05$) in juvenile preparations and by 4.17 ± 1.33 mN/g ($n = 8$, $p < 0.05$) in adult tissues. Similarly, the addition of the H1 receptor antagonist cyproheptadine caused inhibition of the contractile responses to histamine (100 μM) by 5.57 ± 1.22 mN/g ($n = 8$, $p < 0.01$) in juvenile tissues and by 3.00 ± 1.22 mN/g ($n = 8$, $p < 0.05$, Table 6-4) in adult tissue preparations. None of the changes to tonic contractions in response to histamine (100 μM) in the presence of any of the three H1 antagonists were significantly

different between juvenile or adult detrusor tissues (unpaired Student's *t*-test, p = NSD for all).

Treatment with the H2 receptor antagonist cimetidine in detrusor from juvenile animals (n = 8) demonstrated some inhibition of the contractile responses to histamine (100 μM), but this response was not significant. In adult tissues (n = 8), cimetidine did not exert any effect on the increases to tonic contractions in response to histamine (100 μM). Similarly, the H3 and H4 receptor dual antagonist thioperamide did not affect the contractile responses to histamine (100 μM) in both juvenile (n = 6) and adult (n = 8) detrusor preparations (Table 6-4).

Tonic contractions in response to histamine (100 μM) in both young and aged strips of detrusor were not affected by the presence of the muscarinic receptor antagonist atropine (1 μM), cyclooxygenase (COX) inhibitor indomethacin (5 μM), nitric oxide synthase inhibitor N_{ω}-Nitro-L-arginine (L-NNA, 100 μM) or P2X receptor desensitising agonist αβ-methylene ATP (αβm-ATP, 10 μM).

Table 6-4: Comparison of detrusor changes to tonic contractions (mN/g) in response to histamine (100 μM) in the absence and presence of histamine receptor antagonists (mean change ± SEM) in juvenile and adult urinary bladders.

	Juvenile (mN/g)			Adult (mN/g)		
Antagonist	Absence	Presence	n	Absence	Presence	n
Pyrilamine	17.11 ± 4.93	4.65 ± 1.90*	8	11.20 ± 2.00	5.72 ± 1.15**	13
Fexofenadine	10.88 ± 3.65	1.27 ± 0.50*	8	4.53 ± 1.39	0.06 ± 0.13*	8
Cyproheptadine	7.06 ± 0.86	1.49 ± 0.66**	8	4.81 ± 1.38	1.52 ± 0.42*	8
Cimetidine	12.12 ± 3.28	5.48 ± 1.55	10	6.62 ± 2.02	8.30 ± 2.73	8
Thioperamide	18.88 ± 6.09	13.25 ± 3.33*	4	8.04 ± 2.02	5.64 ± 1.24	8

*$p < 0.05$, **$p < 0.01$. Paired Student's *t*-test.

6.6. Discussion

This study shows that ageing has varying effects on the contractile responses of U&LP or detrusor smooth muscle of the porcine bladder. This research has provided several novel findings that further the understanding of histamine's influence on bladder contractility throughout ageing. There are five main findings of this study: (1) Age does not affect increases in the tonic contractions or the frequency of spontaneous phasic contractions in response to histamine in U&LP. (2) Maximal contractile responses in detrusor smooth muscle of adult tissues were significantly smaller when compared to contractions observed in juvenile animals. (3) Inhibition of H2 receptors significantly enhances increases to tonic contractions in response to histamine in juvenile animals but does not affect adult animals. (4) Stimulation of the H2 receptors causes relaxation of U&LP in juvenile but not in adult tissues. (5) Neither the muscarinic nor purinergic receptor systems are involved in the contractile response to histamine.

The current focus of pharmacological interventions for lower urinary tract symptoms has been primarily focused on muscarinic receptor antagonists, due to the involvement of this receptor system in bladder voiding (Yamada et al., 2018). However, the use of antimuscarinics is associated with several side effects, due to muscarinic receptor localisation throughout the body that limits their overall tolerability (Abrams et al., 2006). Due to the troublesome side effects, the long-term adherence and persistence to this treatment are generally low (Basra et al., 2008, Wagg et al., 2012, Veenboer and Bosch, 2014) and declines rapidly after the initiation of the treatment (Sexton et al., 2011). Furthermore, the symptoms associated with uninhibited contractions that occur in patients

with an overactive bladder are considered to occur, in part, through stimulation of receptor pathways other than the muscarinic system (Takanashi et al., 2019). The influence of different receptor systems within the U&LP has been of particular interest, specifically their impact on the spontaneous contractile activity that is known to occur during the filling phase (Pel et al., 2006, Moro et al., 2011). These contractions may also play a role in the regulation of tone and overall continence mechanisms, as previously hypothesised in the literature (Pel et al., 2006).

Some of the chemicals that are capable of mediating contractions in the bladder include ATP (Silva et al., 2015), 5-HT or serotonin (Moro et al., 2016), prostaglandins (Schroder et al., 2004) and histamine (Stromberga et al., 2019). Specifically, histamine exerts its function in both U&LP and detrusor smooth muscle via the activation of the H1 receptor as confirmed in the guinea pig (Khanna et al., 1977, Kondo et al., 1985, Poli et al., 1988), rabbit (Fredericks, 1975) and pig (Stromberga et al., 2019) animal models. The stimulation of the H2 receptor in porcine U&LP has recently been associated with inhibited contractions to histamine (Stromberga et al., 2019). However, this finding was not present in the detrusor tissue. Having established a clear involvement of the histamine receptor system in mediating bladder contractions and spontaneous activity in tissues obtained from juvenile animals (Stromberga et al., 2019), this study was undertaken to determine how ageing impacts the responses to histamine and the involvement of the different histamine receptor subtypes. This study also confirmed the lack of involvement of purinergic or cholinergic receptor systems in the mediation of histamine-induced contractile responses that was first noted in the Stromberga et al. (2019) study.

This study found that maximal responses to histamine in detrusor were significantly lower in adult preparations when compared to juvenile preparations. Interestingly, a general decline in the contractile responses evoked by high potassium and muscarinic receptor agonist in adult rats has previously been reported in detrusor smooth muscle (Munro and Wendt, 1993, Pagala et al., 2001). The mechanisms underlying this are unclear, but the urinary bladder contains elastic and collagen fibres that are responsible for the distensibility of the bladder wall and thus play a role in the generation of contractions and intravesical pressure (Macarak and Howard, 1999). As there is a greater deposition of collagen observed in the ageing bladder (Lepor et al., 1992, Nordling, 2002), decreases in the maximal contractions likely occurred due to reduced compliance in adult detrusor.

Maximal contractile responses in U&LP and the frequency of spontaneous phasic contractions remained the same in both juvenile and adult tissues, indicating that the contractile properties of this layer were not affected by age. However, it appears that only the H1 receptor is functional in adult preparations of U&LP. In juvenile tissues, the H2 receptor has been shown to inhibit contractile responses to histamine (Stromberga et al., 2019), yet neither stimulation or inhibition of this receptor had any effect in adult tissues. This lack of response might have occurred due to a general reduction in the responsiveness of H2 receptors within the U&LP layer or due to changes in sensory or motor function of the urinary bladder that occurs with age (Griffiths et al., 2009, Daly et al., 2014). The loss of a seemingly modulatory role of H2 receptors may potentially elicit a disinhibition of other systems, or even histamine receptor system itself. This disinhibitation may have an impact on the overall bladder compliance and voiding, becoming a contributing factor to OAB. Prolonged use of H2 receptor antagonists, such as cimetidine, may inform potential clinical use, where H2 antagonists may act as inverse agonists and upregulate H2 receptors (Smit et

al., 1996) or to be utulised for the treatment of bladder pain in IC/BPS (Dasgupta et al., 2001).

Based on the previous investigation on the histamine receptor system (Stromberga et al., 2019), the H3 and H4 receptor antagonist thioperamide exerted its function on other histamine receptor subtypes. As H3/H4 receptors have direct contractile involvement in smooth muscle contractions (Hill et al., 1997), it appears that this receptor agonist has a secondary effect on stimulating the H2 receptor in juvenile samples, thereby producing enhanced contractile responses. The involvement of this antagonist in enhancing contractions was ruled out using combination antagonist studies, where both H1 and H2 receptors were inhibited. It was determined that that thioperamide had no influence contractility of the U&LP or detrusor (Stromberga et al., 2019). In adult samples, no responses were observed to the stimulation or inhibition of the H2 receptor. Therefore, it is likely that thioperamide was acting on the H1 receptors and producing inhibited contractile responses.

Overall, the identification of a functional response to histamine agonists presents an interesting direction for future studies in histaminergic receptor signalling. The identification of which second-messenger systems are coupled to this response could provide more insight into the mechanisms underlying the contraction. In addition, future studies utilising immunocytochemical, immunohistochemical or radioligand binding assessments to determine the location, density, and prevalence of the histamine receptors would provide additional insights into this response.

6.7. Conclusions

The histamine receptor system may play an important role in the maintenance of bladder function or the stimulation of some contractile disease states in both juvenile and adult tissues. This study presents the possibility that a contributing factor to increased prevalence of bladder contractile disorders in an ageing population may be due to a general reduction in the responsiveness of histamine receptors, or the lack of response to H2 stimulation.

Chapter 7

Discussion

The overall aim of this study was to characterise the responses to histamine and the primary prostaglandins in U&LP and detrusor, as well as determine the specific receptor subtypes involved in mediating these responses. An additional outcome of this thesis was the investigation of age-associated contractile responses observed to histamine and prostaglandin E_2. Understanding the actions of these autacoid and inflammatory mediators, and the receptor systems involved may reveal future therapeutic targets for lower urinary tract dysfunction, as well as provide a greater understanding into the mechanisms underlying various forms of bladder dysfunction.

7.1. Inflammatory mediators in the urinary bladder

Inflammation is the immune system's defence mechanism against injury, pathogens, damaged cells and toxic compounds (Medzhitov, 2010, Netea et al., 2017). While the immune defence is vital to health, uncontrolled acute inflammation can become chronic (Isailovic et al., 2015) and contribute to a variety of inflammatory diseases (Zhou et al., 2016). Inflammation of the urinary bladder has often been observed in various lower urinary tract disorders, including neurogenic bladder overactivity (Compérat et al., 2006), overactive bladder (Loran et al., 2007, Apostolidis et al., 2008, Çulha et al., 2020) and benign prostatic hyperplasia (de Conti et al., 2020). Therefore, the role of inflammatory mediators and their influence on bladder function is an important venue to explore further.

There is a significant increase in the number of inflammatory mediators, specifically nerve growth factor, PGE_2 and $PGF_{2\alpha}$, present within the urine of patients with OAB (Cheung and Challis, 1989, Kim, 2005, Kim et al., 2006) and increased excretion of PGE_2 in IC/BPS

(Lynes et al., 1987). An elevated neutrophil-to-lymphocyte ratio has also been observed in the urine of OAB patients when compared to healthy controls (Çulha et al., 2020), further supporting the association of inflammation with OAB. An analysis of cytokine expression in patients with OAB has shown that there is an increased expression of monocyte chemoattractant protein 1, TARC (CCL17), PARC (CCL18) and Fas/TNFRSF6 (Ghoniem et al., 2011). These may have a role in mediating inflammation, by causing the migration of leukocytes including granulocytes and mast cells (Imai et al., 1997, Baggiolini, 1998, Mantovani, 1999).

Mast cells are tissue-based inflammatory cells that arise from $CD34^+$ progenitor cells found in the bone marrow and respond to signals of innate and adaptive immunity by releasing pro-inflammatory mediators (Forsythe, 2019). They reside in the lamina propria layer of the bladder and are triggered to degranulate via the secretions of stressed or damaged urothelial cells in response to interleukin-33 (Jang and Kim, 2015), ATP (Säve and Persson, 2010) and β-defensins (Soruri et al., 2007). Based on the pattern and distribution of neutral proteases present within the granules of mast cells, these cells can be classified as either MC_T (tryptase-positive, chymase-negative mast cell) or MC_{TC} (tryptase-positive, chymase-positive mast cell) (Irani and Schwartz, 1989). Generally, MC_T cells can be observed in nasal and bronchial mucosa, whereas MC_{TC} cells are found in the skin.

The involvement of mast cells in lower urinary tract dysfunction is supported by several observations suggesting their involvement in the pathogenesis (Aldenborg et al., 1986). Enhanced mast infiltration in all layers of the urinary bladder in people with IC/BPS was first reported in by Simmons (1961). Mast cells are present in all layers of the bladder, with the most significant accumulation noted in the detrusor smooth muscle using histological

analysis on the patient and healthy tissues (Malik et al., 2018). Increased detrusor mast cell count was also reported by Logadottir et al. (2014), with median counts of 52 cells/mm^2 compared to almost no mast cells present in healthy controls. In order to identify the types of mast cells present in the bladders of patients with IC/BPS, Yamada et al. (2000) used immunohistochemistry to analyse biopsy specimens. The authors found MC_{TC} to be the predominant mast cell type in both urothelium with lamina propria and detrusor layers in patients with IC/BPS, with a significant negative correlation observed between the number of MC_{TC} cells and bladder capacity.

The quantification of mast cells present in the bladder wall also has a diagnostic value for patients with suspected IC/BPS (Feltis et al., 1987). Larsen et al. (1982) defined ≥ 28 cells/$mm^{2,}$ and Kastrup et al. (1983) defined ≥ 20 cells/mm^2 as a diagnostic criterion for IC/BPS. Redistribution of mast cells into the urothelium and enhanced detrusor mast cell density distinguishes classic IC from non-ulcerative IC (Peeker et al., 2000). In detrusor of non-ulcerative IC, this increase was 6- to 8-fold higher compared with controls (Theoharides et al., 2001), whereas in ulcerative IC, mast cell infiltration was 2- to 3-fold higher than in non-ulcerative IC. Significantly increased infiltration of mast cells has also been observed in patients with OAB, reporting similar mean numbers of mast cells in the urothelium and lamina propria as the IC/BPS specimens (Liu et al., 2012). Furthermore, allergic inflammation with the involvement of mast cells was detected in the detrusor of patients with obstructive and idiopathic OAB (Loran et al., 2007).

Studies investigating the state of mast cells in IC/BPS using electron microscopy have reported large numbers of activated and degranulated cells (Letourneau et al., 1992, Pang et al., 1996), with reports indicating as high as 90% of patient samples exhibiting mast

activation to a various degree (Theoharides et al., 1995). Mast cells appear to be larger in the detrusor than in urothelium or lamina propria and tend to degranulate predominantly within the superficial layers of the bladder wall (Christmas and Rode, 1991). There are also reports of an increased histamine content within the bladder wall (Kastrup et al., 1983, Lynes et al., 1987) and elevated levels of urinary histamine,methylhistamine (Theoharides et al., 1990, El-Mansoury et al., 1994, Theoharides et al., 1995) and prostaglandin E_2 (Lynes et al., 1987) in patients with IC/BPS. Furthermore, mast cell tryptase, a chemical released along with histamine and other mediators during degranulation, has also been found in the urine (Boucher et al., 1995). In urine samples of women with IC, a significantly higher concentration of IL-6 and histamine, but not methylhistamine was observed when compared to control samples (Lamale et al., 2006), thereby suggesting its use as a specific marker for IC.

7.2. Histamine on bladder tonic contractions

Chapter 3 and Chapter 6 of this thesis focus on the effects of histamine on U&LP and detrusor using the single-concentration approach. This approach was chosen as preliminary experiments involving cumulative concentration-response curves to histamine showed no visible increases in tonic contractions in response to histamine (30 nM – 1 mM). This lack of response was attributed to a desensitisation of the histamine receptors. Previous research has shown that constant stimulation of G protein-coupled receptors, such as histamine receptors, can lead to a reduction of signalling events through receptor-regulatory processes, resulting in temporary or permanent removal from the cell membrane (Ferguson, 2001) independent of protein kinase A or C (Alonso et al., 2013). Specific histamine receptor

desensitisation has been reported in studies involving human uterine smooth muscle cells (Willets et al., 2008) and cultured Chinese hamster ovary-transfected cells (Alonso et al., 2013). As such, a single-concentration application was chosen and replicated in studies involving prostaglandins (Chapters 3 and 4). This methodology of a single concentration also allowed the measurement of the frequency and amplitude of phasic contractions. The changes in phasic activity (cycles per minute) cannot be calculated in studies involving cumulative concentration-response curves, as the time between two concentrations is commonly too short to get an accurate measurement.

The histamine receptor subtype involved in mediating responses to histamine in both U&LP and detrusor smooth muscle using two different age models, juvenile (Chapter 3) and adult (Chapter 6), was determined to be the H1 receptor. These findings agree with previous studies that utilised guinea pig (Khanna et al., 1977, Kondo et al., 1985) and human (Todd and Mack, 1969) isolated smooth muscle preparations and furthers our understanding of this receptor system in the urinary bladder by determining its involvement in U&LP contractions. Khanna et al. (1977) determined that the application of histamine causes strong contractile responses in the bladder body, moderate in the bladder base and slight in the proximal urethra that can be competitively antagonised by the H1 blocker methapyrilene. Via the use of alternative receptor antagonists, the authors further determined that the actions of histamine were not mediated by either cholinergic or adrenergic mechanisms. The responses to histamine in human detrusor preparations were also completely blocked by mepyramine and were unaffected by the presence of muscarinic or nicotinic receptor antagonists (Todd and Mack, 1969). Inhibited tonic contractions to histamine in the presence of pyrilamine, a competitive H1 receptor antagonist, have also been demonstrated in rabbit detrusor (Van Buren and Anderson, 1979) and cultured human detrusor cells (Neuhaus et al., 2006). These

findings were further replicated using two additional H1 receptor antagonists, cyproheptadine and fexofenadine (Chapter 3).

In most cases, there were remaining contractions after pyrilamine's addition, with this suspected to be due to incomplete antagonism, as only a small concentration was used (30 nM). Nonetheless, these increases in tonic contractions were completely inhibited in the presence of fexofenadine in both U&LP and detrusor. The effectiveness of fexofenadine in inhibiting increases in tonic contractions to histamine could be attributed to the fact that it is a second-generation antihistamine (Day, 1999) with a greater affinity for the H1 receptor, higher comparative concentration used (1 μM), slower dissociation rate and longer duration of action. Furthermore, subsequent experiments involving a combination of histamine receptor antagonists revealed that whenever the H1 antagonist was present in the antagonist combination, increases in tonic contractions were significantly inhibited. Based on findings in this thesis, the H1 receptor is responsible for increases in tonic contractions to histamine in both U&LP and detrusor, which are not influenced by ageing.

The involvement of H2 receptors in mediating relaxatory responses to histamine in U&LP preparations was highlighted in Chapter 3. In strips of U&LP, the addition of amthamine, which has previously been shown to be a potent and highly selective agonist for this receptor subtype (Poli et al., 1993), caused relaxation of baseline tension over 20 minutes. Furthermore, treatment with the H2 antagonist cimetidine resulted in a significant enhancement in tonic contractions in U&LP. Prolonged use of H2 receptor antagonists may inform potential clinical applications, where H2 antagonists may act as inverse agonists and upregulate H2 receptors (Smit et al., 1996) or to be utilised for the treatment of IC/BPS (Dasgupta et al., 2001). In later studies using preparations obtained from older animals

(Chapter 6), it was revealed that ageing eliminated these responses observed in U&LP. This thesis supports previous findings in detrusor tissue, with early research in the histamine responses using isolated smooth muscle strips of guinea pigs showing no activity on the H2 receptor (Khanna et al., 1977). Although H2 receptors are expressed on detrusor smooth muscle cells (Neuhaus et al., 2006), based on the findings in Chapter 3 of this thesis and previous literature, this receptor subtype is not functional or involved in the contractile response to histamine.

As neither H3 nor H4 receptors appeared to be associated with smooth muscle contractions (Nicoud et al., 2019), the histamine-induced increases to tonic contraction observed in this study are hypothesised to be due to secondary effects on the H2 receptor in juvenile animals. This response was further investigated by applying different combinations of histamine receptor antagonists (Chapter 3) and found no involvement of the H3 and H4 receptors in tonic contractions observed. In adult animals, in which the H2 receptor appears to be no longer functional, the inhibited tonic contractions are likely due to the stimulation of the H1 receptor. This lack of selectivity of thioperamide has also been documented in past literature (Lim et al., 2005, Venable et al., 2005) utilising cell culture of human SK-N-MC cell lines, which stably expresses the H3 and H4 receptors.

7.2.1. Effects of ageing on histamine responses

As lower urinary tract dysfunction is more common in an ageing population (Irwin et al., 2006), it was of great interest to explore whether ageing has an impact on the contractile responses exhibited in response to the inflammatory mediators, histamine and prostaglandin. Previous research has shown that contractile responses to noradrenaline, ATP and serotonin

are increased in ageing (Saito et al., 1993). It has also been noted that enhanced responses of detrusor smooth muscle to muscarinic and purinergic stimulation are associated with ageing (Daly et al., 2014).

In Chapter 6, it was observed that maximal tonic contraction to histamine in the detrusor smooth muscles of adult tissues was significantly smaller compared to those in juvenile samples. A similar age-associated decline in tonic contractions in detrusor has also been observed in response to stimulation using a muscarinic receptor agonist and potassium chloride in adult rat models (Munro and Wendt, 1993, Pagala et al., 2001). While the mechanisms underlying this decrease in contractility are unclear, greater deposition of collagen fibres has been observed in ageing bladders (Lepor et al., 1992, Nordling, 2002) and may contribute to the reduced distensibility of the bladder wall. Furthermore, previous research utilising mouse brains noted a significant reduction in the expression of H1, H2 and H3 receptors in ageing (Terao et al., 2004). Similarly, studies using the human brain had also shown age-related reductions in the prevalence of the H1 receptor subtype (Yanai et al., 1992). Therefore, reduced expression of the H1 receptor, as noted in the mouse (Terao et al., 2004) and human (Yanai et al., 1992) studies, is likely a contributor to the reduction in tonic contractions of detrusor to histamine.

This difference in maximal responses was only observed in the detrusor layer, as the maximal contraction to histamine in U&LP tissue (Figure 6-2) was the same across the two age groups. Muscularis mucosae tissue is thought to be the main contractile element present within the U&LP (Lee et al., 2016). As this tissue type also consists of smooth muscle cells, it is unclear as to why maximal tonic contractions remained the same across both age groups. Further investigation on the distribution and density of the H1 receptor involved in the

increases in tonic contractions in lamina propria and detrusor of both juvenile and adult bladders will provide greater insights into the responses observed.

Findings in this thesis have shown an exciting age-related change to H2 receptor activation. For example, treatment with the H2 agonist amthamine caused relaxation in the U&LP of juvenile preparations (Chapter 3) but not in adult preparations (Chapter 6). Similarly, antagonism of the H2 receptor significantly enhanced maximal tonic contractile responses to histamine in juvenile U&LP, but did not affect tonic contractions in adult tissues. It remains unclear as to why these responses to the stimulation of the H2 receptor are no longer observed in adult animals. There is a variety of possible reasons, including changes to receptor density, the number of receptors, internalisation of the receptors or coupling to G proteins. In support of the latter, reduction in H2 recepor activation has also been reported in studies involving human blood vessels (Bedarida et al., 1995). Decreased responses to the activation of the β-adrenergic receptor system, which also couples to adenylyl cyclase, have been noted in ageing rat urinary bladder (Derweesh et al., 2000). These are thought to occur from inhibition of adenylyl cyclase activity and changes in G-protein content or function.

7.3. Primary prostaglandins on bladder tonic contractions

The effect of the different primary prostaglandin agonists on tonic contractions was varied and dependent on concentrations used. Palea et al. (1993) outlined agonist potency when contracting detrusor muscle as $PGF_{2\alpha}$ > TXA_2 > PGE_2. As discussed in Chapter 4, prostaglandin E_2 had the most substantial effect on increasing tonic contractions when compared to other receptor agonists in both U&LP and detrusor tissue, followed by $PGF_{2\alpha}$

and TXA_2. There is a possibility that results vary between species, as historical data has found $PGF_{2\alpha}$ to have a more potent effect on the human detrusor contractile responses when compared to PGE_2, but repeated studies have not validated this claim (Abrams and Feneley, 1975, Andersson et al., 1977). Overall, the agonist potencies determined in this thesis (Chapter 5) across both tissue types is $PGE_2 > PGF_{2\alpha} > TXA_2 > PGD_2 > PGI_2$. Although with only two concentrations of prostanoids used, this is generally insufficient to be able to reliably measure pharmacological potency and warrants further research.

The potential for a prostaglandin E_2 agonist to activate other prostaglandin receptors or be converted into other metabolites upon tissue contact was considered. The conversion of prostaglandin E_2 to other prostaglandin metabolites has previously been documented (Canete Soler et al., 1987, Abadir and Siragy, 2015). For example, the presence of bladder carbonyl reductase 1 (CBR1) within the bladder wall may have caused the conversion of PGE_2 to $PGF_{2\alpha}$ (Forrest and Gonzalez, 2000), which would then become capable of activating the FP receptor. An important finding of this thesis was that when the FP receptor was antagonised, increases to contractile responses to PGE_2 were significantly inhibited, suggesting its partial involvement in mediating at least some of this response. This receptor subtype was also involved in mediating the increases in tonic contractions in response to $PGF_{2\alpha}$. However, the exact mechanisms involved in the remaining tonic contraction after the inhibition of the FP receptor inhibition remain unknown. There are also remains no literature documenting the expression and localisation of the FP receptor in either animal or human urinary bladder. As such, further research in the distribution and density of this receptor subtype would assist in a better understanding of the prostaglandin receptor system as a whole and provide further insights into this contractile response.

Both PGD_2 and PGI_2 (1 μM – 10 μM) had desmonstrated the smallest effect on tonic contractions in both U&LP and detrusor. These relatively minor increases in tonic contractions in responses to PGD_2 are thought to have occurred as a result of inhibitory effects on smooth muscle via DP receptor stimulation, previously reported by Guan et al. (2015). Further studies should examine the mechanisms of action of PGD_2 in the presence of DP receptor antagonists to confirm this observation. Although PGI_2 is the primary prostaglandin synthesised in the human bladder (Jeremy et al., 1987), its effects on tonic contractions in both U&LP and detrusor were minimal. Nonetheless, this finding should be interpreted with caution, as PGI_2 in aqueous solutions is chemically unstable with a relatively short half-life, depending on the buffer concentration (Stehle, 1982, Moncada, 1983) and further studies involving PGI_2 agonist analogues under tightly controlled conditions may be warranted.

After determining that the PGE_2 agonist is capable of eliciting clear increases in tonic contractions (Chapter 5), age-related changes in response to this agonist were explored (Figure 6-1). However, no changes in the increases in tonic contractions nor phasic contractions in response to PGE_2 were observed in either U&LP or detrusor preparations between the two age groups. Unlike histamine responses, which were affected by age in detrusor (Chapter 6), stimulation with PGE_2 remained consistent between juvenile and adult samples.

7.4. Phasic activity of the urinary bladder

In addition to the tonic contractile responses observed in this thesis, inflammatory mediators were also shown to influence the urinary bladder phasic activity. Detrusor smooth muscle exhibits spontaneous activity that can be regulated by neurotransmitters, such as acetylcholine and ATP, and by spontaneous action potentials through the activation of calcium channels (Andersson, 2010). Since the utilisation of porcine bladders in urology research, which are large enough for the separation of U&LP and detrusor, spontaneous phasic activity has also been noted in strips of U&LP (Sadananda et al., 2008, Moro et al., 2011, Vahabi et al., 2013). The origin of this activity in detrusor is not fully understood. There are several theories as to why these contractions occur in health and disease states, including actions of the nearby parasympathetic nerves, myogenic activity of the detrusor smooth muscle cells, pace-making activity from the interstitial cells, or as a result of interactions between the U&LP and detrusor (Wein and Rackley, 2006, McCarthy et al., 2019). Myogenic activity is not unique to the urinary bladder, and has also been observed in the musculature of the gastrointestinal tract (Huizinga and Chen, 2014) and uterus (Wray et al., 2001).

Optical imaging experiments revealed that spontaneous activity in rat bladders was generated within the lamina propria and then propagated to urothelium and underlying detrusor (Kanai, 2007), that can be enhanced via the stimulation of purinergic (P2Y) receptors on interstitial cells (Fry et al., 2012). The physiological role of the U&LP is not well understood; however, the spontaneous contractile activity that this tissue type develops

has been suggested to occur as a means to prevent the microvasculature from stretching during bladder distension (Lee et al., 2016, Drake et al., 2018).

7.4.1. Spontaneous phasic contractions in U&LP

In the absence of any stimulation, the U&LP is capable of exhibiting spontaneous phasic contractions (Moro et al., 2011). These spontaneous contractions are thought to be propagated by the actions of the muscularis mucosae cells found within the lamina propria (Fry and Vahabi, 2016, Drake et al., 2018, Mitsui et al., 2019). The distinction between muscularis mucosae and the underlying detrusor smooth muscle has been established using immunohistochemistry in guinea pig, pig and human bladders (Mitsui et al., 2019). The authors also noted that as the mice and rat bladders lack this tissue type within their lamina propria layer, phasic contractions that develop remain very small. As such, the porcine bladder provides a unique opportunity to study this tissue type in more detail. The development of spontaneous U&LP contractions was consistently observed within all studies from Chapters 3 to 6. As an interesting observation, the contractile frequencies remained constant over the 2 to 3-hour duration of each experiment.

The basal phasic contractions occurred between approximately 3.2 to 3.5 cpm, with an average amplitude of 0.5 g. Furthermore, there were no differences in the frequency or amplitude of phasic contractions between juvenile and adult tissue preparations. In previous research utilising porcine U&LP obtained from the same breed of pig as used throughout this study, the amplitude of phasic contractions in juvenile samples was found to be greater than in adults (Vahabi et al., 2013). The authors also noted a higher frequency in spontaneous activity in adult preparations when compared to juvenile preparations. The difference in

responses observed between the two porcine studies most likely occurred due to a variation in age for both juvenile and adult tissue samples. Throughout this thesis, juvenile pigs were considered to be aged approximately 6 months, with adult preparations obtained from 2-year-old pigs. Future studies involving a wider age group of animal samples would help establish a better understanding of how frequency and amplitude of U&LP phasic activity change across ageing.

Stimulation of M3 muscarinic receptors within the U&LP has previously shown to increase the frequency of spontaneous phasic contractions and reduce their amplitude (Moro et al., 2011, Vahabi et al., 2013). Similarly, the findings of this thesis demonstrated the ability of histamine (100 μM), PGE_2 (1 – 10 μM) and $PGF_{2\alpha}$ (1 – 10 μM) to enhance spontaneous phasic activity. The changes in this activity occurred as a direct result of the stimulation of inflammatory mediators. They are unaffected by the release of neurotransmitters from autonomic nerves, as shown in previous studies involving tetrodotoxin (Moro et al., 2011) which inhibits the firing of action potentials and subsequent muscle contraction. These findings are particularly noteworthy, as there is growing evidence suggesting that U&LP spontaneous activity is capable of modulating contractions in the underlying detrusor smooth muscle (Chakrabarty et al., 2019).

7.4.2. Phasic contractions in detrusor

In contrast to the urethra, in which phasic smooth muscle contractions are driven by pacemaker cells (Sergeant et al., 2000), detrusor cells are capable of developing spontaneous phasic contractions. As observed in this thesis, phasic contractions within detrusor preparations were extremely variable, even among the strips obtained from the same bladder.

Out of all bladders utilised, only 35% of all detrusor strips developed spontaneous phasic contractions in the absence of any stimulation. The frequency of these contractions was between 1.5 – 2 cpm, which is similar to previous findings reporting an average frequency of approximately 1.5 cpm (Buckner et al., 2002). In contrast, previous research that also utilised porcine detrusor strips of both juvenile and adult animals did not observe any basal phasic activity (Vahabi et al., 2013).

These contractions are thought to be myogenic, as they are not affected by atropine or tetrodotoxin (Levin et al., 1986). Myogenic activity is not unique to the urinary bladder and has also been observed in the musculature of the gastrointestinal tract (Huizinga and Chen, 2014) and uterus (Wray et al., 2001). Phasic contractions can be mediated by the actions of neurotransmitters, such as ATP and acetylcholine, and spontaneous action potentials through the activation of calcium channels (Andersson, 2010). The contractions can also be abolished by the use of ATP-sensitive potassium channel openers that hyperpolarise the smooth muscle cells (Buckner et al., 2002).

The majority of preparations that were quiescent during baseline developed phasic activity of varying frequency and amplitude after the stimulation by histamine and the five primary prostaglandins. The addition of histamine (100 μM) initiated spontaneous contractions in 54% of preparations, whereas the addition of all five primary prostaglandins commenced contractions in approximately 80% of otherwise quiescent tissues. Initiation of phasic contractions in detrusor strips has also been observed in response to muscarinic receptor agonist carbachol (Vahabi et al., 2013). These findings might have clinical implications associated with overactive bladder (Coolsaet et al., 1993, Brading and Turner, 1994), where heightened contractions of the smooth muscle occur spontaneously.

7.5. Future directions

This thesis has identified functional responses to histamine and prostaglandins occurring in porcine urinary bladder urothelium lamina propria and detrusor preparations. Furthermore, these contractile responses to histamine are altered between juvenile and adult samples, indicating that ageing plays a role in altering this receptor system. It is the first time that differently aged samples have been assessed in this way, presenting a range of new potential studies to investigate these findings further. In particular, research into the role of this receptor system as a future therapeutic target in the pharmaceutical management of bladder dysfunction.

Recommendations for future studies as an outcome of this thesis include assessments of the second-messenger systems related to histamine, PGE_2 and $PGF_{2\alpha}$, as well as the receptor densities and localisations within the urothelium and lamina propria. Although responses to PGE2 were not altered by ageing (Chapter 6), there remains the possibility that the responses to other prostaglandin agonists may be varied. As such, it would be beneficial to explore the effects of ageing on the contractility of U&LP and detrusor in response to $PGF_{2\alpha}$, TXA_2, PGD_2 and PGI_2 in future studies.

An additional investigation could be to conduct immunohistochemical analyses of histamine and prostaglandin receptors in the porcine model used throughout this thesis. The porcine tissue shares similar physiology and pharmacology to the human bladder, with a comparable resemblance in neural control as well as in urodynamic and structural characteristics (Sibley, 1984, Crowe and Burnstock, 1989, Parsons et al., 2012). However, validated antibodies for

this animal model are not readily available. Therefore, before undergoing further analyses of the receptor subtypes present within the bladder wall using antibodies, they have to be validated appropriately. Future investigations may further focus on the relationships between these systems in the normal and pathological states of the human bladder and identify the potential for these receptor systems to contribute to bladder dysfunction.

7.6. Concluding remarks

The findings presented in this thesis demonstrate that both histamine and prostaglandin receptor systems are capable of modulating both tonic and phasic contractions of urinary bladder detrusor and urothelium with attached lamina propria. Of all inflammatory mediators considered in this research, histamine, prostaglandin E_2 and $F_{2\alpha}$ exhibited the most significant increases to tonic contractions and phasic activities in both layers of the urinary bladder. This thesis proposes novel potential therapeutical targets for people suffering from lower urinary tract dysfunction.

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