Bladder spontaneous activity: influence of mild heating and inert injectables.

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A thesis submitted for the degree of Doctor of Philosophy

Faculty of Health and Medical Sciences

October 2015
Abstract.

Overactive bladder (OAB) syndrome is associated with increased spontaneous contractions of the bladder wall, potentially mediated by the release of chemical agents from the urothelium. Reduction of these spontaneous contractions offers a means to alleviate the symptoms of OAB. I tested two novel approaches using pig bladder in vitro or ex vivo preparations; bladder wall heating or injection of inert bulking agents.

In vitro, intact (mucosa + detrusor muscle) preparations were heated to 42, 46, or 50°C by a heating coil. Preparations of only detrusor muscle or mucosa were heated by changing superfusate temperature. Experiments were done to examine the role of heat-sensitive TRPV1 channels during heating by using TRPV1 antagonists. The effect of heating on urothelium ATP release was also measured. Possible changes to tissue structure were histologically assessed with haematoxylin & eosin or van Gieson staining. Inert bulking agents (Tyrode’s, polyethylene glycol or coaptite) were injected into the sub-mucosal space and their effect on spontaneous contractions also measured. Ex vivo experiments with perfused pig bladders recorded spontaneous pressure variations when perfusate temperature was increased to 42°C.

Spontaneous contractions in intact preparations were reversibly reduced when heated to 42, 46, or 50°C; TRPV1 antagonists had no effect. Heating to 42°C did not affect mucosa or detrusor-only preparations, but at 50°C contractions were abolished. Similar effects were seen in ex vivo experiments when heated to 42°C. No changes to tissue integrity were observed at 42 or 46°C. At all temperatures urothelial ATP release was increased. Spontaneous contractions were reduced by all bulking agents, coaptite was the most effective.

These novel findings suggest possible clinical approaches to treat the symptoms of OAB by reducing spontaneous contractions.
Author’s declaration.

This thesis and the work to which it refers are the results of my own efforts. Any ideas, data, images or text resulting from the work of others (whether published or unpublished) are fully identified as such within the work and attributed to their originator in the text, bibliography or in footnotes. This thesis has not been submitted in whole or in part for any other academic degree or professional qualification. I agree that the University has the right to submit my work to the plagiarism detection service Turnitin UK for originality checks. Whether or not drafts have been so-assessed, the University reserves the right to require an electronic version of the final document (as submitted) for assessment as above.

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Date………………………………………………………………………………………………
Acknowledgements.

First and foremost, I cannot give enough gratitude to my supervisors Professor Christopher Fry and Doctor Rita Jabr. They gave me the greatest opportunity and achievement I have endeavoured. Without their hard work, enthusiasm, and knowledge that they supported me with, I would not have submitted my Ph.D. Furthermore, I felt very welcoming from both Chris and Rita which has built not only a pleasant work relationship but a very good friendship.

I would also like to thank Bahareh Vahabi for teaching and assisting me with the whole perfused pig bladder. In addition, she has always supported me and helped keep sane with consistent laughter, enthusiasm, and an enjoyable work place. Also, many thanks to Linda McLatchie for her assistance and guidance for measuring ATP release, and Brian Parson’s for sending high quality images of the arterial perfusion of the pig bladder.

I would also like to thank my other work colleagues, Nobuyuki Nishikawa, Marcus Drake, Jon Crook, and Thelma Lovett (Bristol lab group), as well as, Fiona Hatch and Vadim Alexeenko (Surrey lab group) for general assistance and knowledge.

Obviously I cannot forget those closest friends and family who have consistently be in support. To start the list of many, I can’t thank enough, Manuela Ximenes for being part of my life and supporting me through my stressful last few months of my Ph.D. Her enthusiasm and support has been fantastic and heart-warming. Also, a huge thanks to Maxwell Roberts and James Clark (the boys, the lads) for being there throughout undergraduate and postgraduate. I think it’s safe to say we have all been there together when each of us have gone through the panic mode. In addition, who can forget Mike Lahaney, Josh Jackson, Ricky Pitt and Guy Herbertson for all the support and pub sessions. Cheers lads! Many thanks to my AW girls, Andria Siakalli, Ashanthie Tudugalle and Becky Clarke. We have had consistent laughs in and out of the office which will never be forgotten and your support has been amazing. I would further like to thank my closest friends in Bristol, Stefan Hirschberg, Dawid Walas, Dominika Bijos, and Robert Drake who have put up with me for my final year of Ph.D., and fantastic help throughout. Also my friends and family at home, Aaron Kitney and Leon Kitney for being amazing and inspirational brothers, as well as, Matt Porter, James Heard, Richard Heard, Philip Heard, Sarah Blake for all the laughs and good times. For all those other friends and family I have missed, you are not forgotten.

Finally, I would sincerely like to thank and dedicate this thesis to my loving parents, David Kitney and Veronica Kitney. I can’t even comprehend the love and support you both have given me not only through my Ph.D., but the progression that led me to this point. Hopefully one day I can return the favour. Thank you.
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## Abbreviations.

<table>
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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Ca(^{2+})](_i)</td>
<td>Intracellular calcium</td>
</tr>
<tr>
<td>ABMA</td>
<td>(\alpha,\beta)-methylene ATP</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AMG-9810</td>
<td>TRPV(_1) antagonist</td>
</tr>
<tr>
<td>ANG receptor</td>
<td>Angiotensin receptor</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>AUM</td>
<td>Unique asymmetric unit</td>
</tr>
<tr>
<td>BK channel</td>
<td>Large conductance Ca(^{2+}) -activated K(^+) Channel</td>
</tr>
<tr>
<td>BoTox</td>
<td>Botulinum toxin</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>Ca(^{2+})-CaM</td>
<td>Calcium-calmodulin complex</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge-coupled device camera</td>
</tr>
<tr>
<td>CCh</td>
<td>Carbachol</td>
</tr>
<tr>
<td>CICR</td>
<td>Calcium-induced calcium-release</td>
</tr>
<tr>
<td>CPZ</td>
<td>Capsazepine</td>
</tr>
<tr>
<td>Cx</td>
<td>Connexin</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>dH(_2)O</td>
<td>Deionised water</td>
</tr>
<tr>
<td>DO</td>
<td>Detrusor overactivity</td>
</tr>
<tr>
<td>DSM</td>
<td>Detrusor smooth muscle</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EFS</td>
<td>Electrical field stimulation</td>
</tr>
<tr>
<td>EP(_2) receptor</td>
<td>Prostanoid receptor</td>
</tr>
<tr>
<td>ET receptor</td>
<td>Endothelin receptor</td>
</tr>
<tr>
<td>FLIP</td>
<td>Flice-like inhibitory protein</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin &amp; eosin</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>IC</td>
<td>Interstitial cell</td>
</tr>
<tr>
<td>IP(_3)</td>
<td>Inositol-1,4,5-triphosphate</td>
</tr>
<tr>
<td>K(^+)</td>
<td>Potassium ion</td>
</tr>
<tr>
<td>LUT</td>
<td>Lower urinary tract</td>
</tr>
<tr>
<td>LUTD</td>
<td>Lower urinary tract dysfunction</td>
</tr>
<tr>
<td>LUTS</td>
<td>Lower urinary tract symptoms</td>
</tr>
<tr>
<td>M(_1,5) receptor</td>
<td>Muscarinic receptor family</td>
</tr>
<tr>
<td>MLC</td>
<td>Myosin light chain</td>
</tr>
<tr>
<td>MLCK</td>
<td>Myosin light chain kinase</td>
</tr>
<tr>
<td>MLCP</td>
<td>Myosin light chain phosphatase</td>
</tr>
<tr>
<td>NA</td>
<td>Noradrenaline</td>
</tr>
<tr>
<td>Na(^+)</td>
<td>Sodium ion</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>OAB</td>
<td>Overactive bladder</td>
</tr>
<tr>
<td>P1 or P2 receptor</td>
<td>Purinergic receptor</td>
</tr>
<tr>
<td>Pabd</td>
<td>Abdominal pressure</td>
</tr>
<tr>
<td>PAG</td>
<td>Periaqueductal grey</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Pdet</td>
<td>Detrusor pressure</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PG</td>
<td>Prostanoid</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphoinositides</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase-A</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PMC</td>
<td>Pontine micturition centre</td>
</tr>
<tr>
<td>Pves</td>
<td>Intravesical pressure</td>
</tr>
<tr>
<td>ROK</td>
<td>Rho-associated kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RTX</td>
<td>Resiniferatoxin</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine receptor</td>
</tr>
<tr>
<td>SK channel</td>
<td>Small conductance Ca(^{2+})-activated K(^+) Channel</td>
</tr>
<tr>
<td>SUI</td>
<td>Stress urinary incontinence</td>
</tr>
<tr>
<td>TE</td>
<td>Trypsin EDTA</td>
</tr>
<tr>
<td>TK receptor</td>
<td>Tachykinin receptor</td>
</tr>
<tr>
<td>TRP</td>
<td>Transient receptor potential</td>
</tr>
<tr>
<td>TRPA</td>
<td>Transient receptor potential ankyrin</td>
</tr>
<tr>
<td>TRPM</td>
<td>Transient receptor potential melastatin</td>
</tr>
<tr>
<td>TRPV</td>
<td>Transient receptor potential vanilloid</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine diphosphate</td>
</tr>
<tr>
<td>UP</td>
<td>Uroplakin</td>
</tr>
<tr>
<td>UTP</td>
<td>Uridine triphosphate</td>
</tr>
<tr>
<td>WGA</td>
<td>Wheat germ agglutinin</td>
</tr>
<tr>
<td>τ</td>
<td>Time constant</td>
</tr>
</tbody>
</table>
Chapter 1. General introduction.
1.1 Overview on the urinary tract system.

The urinary tract, comprising the upper urinary tract (kidneys and ureters) and the lower urinary tract (the bladder and urethra), is a highly organised system that requires the coordination of the upper and lower urinary tract to control storage of urine and occasional voiding (micturition). The lower urinary tract is crucial for the dual storage and voiding phases. The bladder in particular, being continuously active, finely controls storage and voiding by intimate communication between layers of the bladder wall, as well as transmitting signals to higher brain regions. Disturbances to the systems that coordinate storage and voiding will result in lower urinary tract disorders including overactive bladder (OAB) (Andersson and Arner, 2004, Chapple, 2011, GuhaSarkar and Banerjee, 2010).

OAB is a symptomatic disorder which has a huge economic burden and increases in prevalence with age. It is significantly associated with an increased spontaneous activity from isolated preparations of patients with OAB. Current therapeutic treatment tend to cause many side effects and therefore patients become uncompliant. Consequently it is essential for novel therapeutic approaches to be explored to minimise this elevated spontaneous activity and alleviate the symptoms associated with OAB. These approaches are, the use of heating the bladder or an injection of a bulking agent.
1.2 The anatomy of the lower urinary tract.

1.2.1 Urinary bladder.

1.2.1.1 Function and anatomy.

The urinary bladder acts as a reservoir for the storage and periodic voiding of urine and thus has two main functions:

1) To store urine from the kidneys to the bladder through the ureters under low pressure.
2) To expel the contents of the bladder, when socially acceptable under normal physiological conditions.

These functions are typically described as: 1) the storage phase; 2) the voiding phase, of the micturition cycle.

The bladder is located in the extraperitoneal space posterior to the pubic symphysis. The position and shape of the bladder tends to change from a flattened tetrahedron when empty, to a dome during filling. The apex of the bladder is positioned anteriorly towards the uppermost region of the symphysis pubis and is connected to the abdominal wall via the umbilicus. The superior surface of the bladder is lined with peritoneum, in which lie the colon and small intestine. Ureters expanding from the kidneys pierce the posterior wall above the bladder base to allow urine flow into the bladder. The bladder neck and trigone are located at the lowest point of the bladder. The trigone is a triangular region of the bladder wall, with the ureterovesical junctions and the bladder neck forming two apices. The bladder neck extends down towards the urethra and is located adjacent to the prostate in males and in the connective tissue of the anterior vaginal wall in females (Fletcher, 1996)(figure 1.1).
Figure 1.1. Schematic diagram of the lower urinary tract. Position of the bladder is located posterior to the public symphysis in both A) males and B) females (Mangera et al., 2013).
1.2.1.2 Histology of the bladder wall.

The bladder wall is composed of distinct layers which, from the outside to inside are the serosa, the detrusor smooth muscle (DSM), sub-urothelium, and the urothelium (figure 1.2) (GuhaSarkar and Banerjee, 2010, Lewis, 2000). The urothelium and sub-urothelium typically make up the mucosal layer of the bladder wall.

**Serosa.** The serosa is a thin membranous layer of cells covering the walls of the detrusor muscle. This thin layer contains a single layer of epithelial, or mesothelial, cells that produce serosal fluid. The serosa adheres to a layer of connective tissue, that aids in stabilising the bladder with respect to other structures and organs, as well as provides a support for vascular and nervous supplies to the bladder (Gabella and Uvelius, 1990) (figure 1.2).

**Detrusor smooth muscle (DSM).** The DSM contains fibres of muscle that run irregularly in human bladder, and in smaller species such as rabbit, tend to run in a more organised and parallel manner. DSM fibres in humans are more organised towards the internal urethral meatus forming three distinct layers; the inner and outer layers being longitudinal and the middle being circumferential (Zimmern et al., 1996, Gabella and Uvelius, 1990). DSM fibres contain bundles of muscle cells which are nucleated and spindle-shaped, varying in size, and contain actin and myosin contractile elements. These bundles of cells are surrounded by distinct layers of complex extracellular matrix, connective tissue, containing collagen (I, III, and IV) and elastin (Wilson et al., 1996). This matrix of elastic connective tissue is particularly important to ensure bladder compliance (Gabella and
Uvelius, 1990, Wilson et al., 1996). Also interspersed between the DSM fibres is a network of interstitial cells (ICs) (Andersson and Arner, 2004) (figure 1.2).

**Sub-urothelium.** The sub-urothelium is a complex of connective tissue that lies between the basement membrane and the detrusor muscle layer and is composed of an extracellular matrix (ECM) that surrounds many different cells types, vessels and nerves. The ECM is a structural scaffold composed of collagen I and III fibres that provide structural support, tensile strength, and compliance through their complex coiling (Gabella and Uvelius, 1990, McCloskey, 2010, Andersson and McCloskey, 2014). The assembly of these collagen molecules form fibrils in a loose meshwork lacking uniform orientation in an empty bladder. However, these fibrils become parallel to the urothelium as the bladder fills. Therefore, an elastic recoil of these fibres is essential for the bladder to return to its original shape during the transition between voiding and filling (Andersson and McCloskey, 2014, Chang et al., 1999). In addition this layer is interspersed by ICs, which act similar to, but are not necessary phenotypically the same as, myofibroblasts (Wiseman et al., 2003, McCloskey, 2010, Sui et al., 2002). The sub-urothelium has a rich plexus of blood and lymphatic vessels, smooth muscle fascicles, and nerve endings (McCloskey, 2010) (figure 1.2).

**Urothelium.** The urothelial layer is a stratified epithelium that acts as an impenetrable protective and physiologically active barrier lining the bladder lumen. The urothelium is multi-layered with three distinct groups of epithelial cells: a basal cell layer (10 µm diameter), intermediate layer (10-25 µm diameter), and an umbrella cell layer (25-250 µm
diameter). The umbrella cells of the epithelium tend to be long-lived (depending on urothelial damage) and are replaced via fusion by intermediate and basal cells (Lewis, 2000). The apical surface of umbrella cells has a unique asymmetric unit membrane (AUM) containing uroplakins (UP). Within the AUM, there are at least five UPs, UPIa, UPIb, UPII, UPIIIa, and UPIIIb. Each cell is surrounded by a tight, junctional ring containing multiple proteins such as occludins and claudins. The collection of these proteins together form a polygonal-shaped plaque which consists of 1000-3000 AUM particles. Individual plaques are surrounded by a hinge membrane (Lewis, 2000, Apodaca, 2004, Birder and Andersson, 2013). The plaques on the apical membrane of umbrella cells function to help maintain the permeability barrier (Apodaca, 2004). The urothelial layer rests on a basement membrane that acts as a scaffold for regrowth and regeneration of cells (Khandelwal et al., 2009, Birder and Andersson, 2013, Lewis, 2000) (figure 1.2). The luminal side of the urothelium is coated with a glycosaminoglycan layer called GAG (Lewis, 2000).

Nervous supply. Nerve endings from the parasympathetic nervous system are dispersed within the bladder wall. The DSM are innervated by a dense efferent nerve plexus allowing for muscle contraction and subsequent voiding of the bladder. Afferent nerve fibres lie in close proximity to ICs in the sub-urothelium and efferent nerves are interspersed between muscle bundles of the DSM. The urothelial layer has a rich afferent nerve plexus which is essential for the transition between the filling and voiding phases of the micturition cycle (Birder and Andersson, 2013, de Groat and Yoshimura, 2009).
Within the whole urinary bladder, the trigonal region contains the most dense nerve plexus.

**Figure 1.2. Cross sections of the LUT.**
(A) Structure of the lower urinary tract (LUT) comprising the bladder and outflow tract (Fulford, 2012). (B) Detailed view of the layers of the bladder wall showing the urothelium, sub-urothelium (or sub-mucosa), detrusor muscle and serosa (Birder and Andersson, 2013). (C) H&E staining of the bladder wall (Fulford, 2012).

### 1.2.2 Urethra.

The urethra, although not integral to this thesis, will be briefly discussed for completeness.

#### 1.2.2.1 Function and anatomy.

The urethra allows for the passage of urine during the voiding phase, and varies in length depending on sex. In males, the urethra tends to be around 20 cm separated into a shorter,
posterior urethra and longer anterior urethra. The posterior urethra passes antero-interiorly through the prostate. The anterior urethra can be divided into three distinct regions called the membranous urethra, the bulbar urethra, and the penile urethra. The membranous urethra expands out of the urogenital diaphragm and widens into a bulbar urethra at the lower edge of the perineal membrane. The bulbar urethra extends into the penile urethra at the root of the penis and continues through the corpus spongiosum where it opens to the tip of the glans penis passing urine via the external urethral meatus. In females, however, the urethra is only about 4 cm in length. Urine passes out through the sphincter urethrae out of the external urethral orifice (Gosling and Dixon, 1975, Fletcher, 1996).

1.2.2.2 Histology of the urethra.

The urethra wall has three distinct layers; the epithelial lining of the urethral lumen, the internal, and external urethral sphincter.

External sphincter. The external sphincter, or rhabdosphincter, is made up of circularly arranged striated muscle fibres and spans the membranous urethra in males, and extends the entire length of the urethra in females. This layer surrounds the internal sphincter (Karam et al., 2005) (figure 1.3).

Internal sphincter. The internal sphincter is made up of longitudinal smooth muscle. Surrounding the longitudinal smooth muscle is a layer of circular smooth muscle, continuous with the DSM (Karam et al., 2005) (figure 1.3).
**Epithelial layer.** Transitional epithelial cells line the vast majority of the urethra from the internal urethral orifice until the distal glans penis, which are stratified, squamous epithelium in males. In females, the proximal urethra is lined by transitional or pseudo-stratified epithelium. Stratified, squamous epithelium lines the distal region of the urethra. There are a number of mucous glands and follicles within the sub-mucosa between the urethral sphincter and epithelium (*figure 1.3*).

**Nervous supply.** The urethra is highly innervated by nervous input from both the autonomic and somatic nervous systems within the internal and external sphincters, respectively (*figure 1.3*).

*Figure 1.3. Nerve innervation of the urethral wall.* The internal urethral sphincter is innervated by the hypogastric and pelvic nerve from the sympathetic and parasympathetic branches, respectively. The rhabdosphincter is innervated by the pudendal nerve via the somatic branch (Thor, 2003).
1.3 The micturition reflex.

The micturition reflex is ‘the state of change of the bladder that switches from a prolonged storage phase to the temporary voiding of urine’. This cycle of events is controlled by two functional units of the LUT. The bladder, and the outflow tract (bladder neck and urethra). These functional units have neural input and outputs to control micturition reflexes. Neural control from the peripheral nerves are (Fowler et al., 2008, Birder et al., 2010, de Groat, 2006):

- Sacral parasympathetic (pelvic nerve): innervates the bladder wall and internal urethral sphincter.
- Thoracolumbar sympathetic (hypogastic nerve and sympathetic chain): innervates the bladder wall and internal urethral sphincter.
- Sacral somatic (pudendal nerve): innervates the external urethral sphincter.

1.3.1 Neural control of the LUT.

The transition between the storage and voiding phases is controlled by neural circuitry from the LUT to the central nervous system and back to the LUT. Typically storage of urine depends on a relay circuit governed by the lumbosacral reflex. Voiding of urine however depends on a spinobulbospinal reflex mediated by the pontine micturition centre (PMC). Afferent nerves convey information by relaying sensory inputs to reflex circuits stimulating efferent nerve outputs to control normal bladder storage and voiding (Fowler et al., 2008, Benaroch, 2010).
1.3.1.1 Filling phase.

During filling, bladder muscular tone remains relatively low, to maintain a low intravesical pressure and ensure the bladder is compliant. Afferent nerves are understood to play an important role in facilitating filling of the bladder by monitoring bladder volume, maintaining a relaxed bladder via the hypogastric nerve and a closed sphincter via the pudendal nerve to the urethra. Although the nature of the afferent innervation is not fully understood, it is known that small myelinated Aδ fibres emerge from a dense nerve plexus in the DSM and sub-urothelium that respond to changes in passive distension, and possibly active contractile tone of DSM. Unmyelinated C fibres have a higher threshold for activation and become more prominent than Aδ afferents in the transition between the filling and voiding phase (de Groat and Yoshimura, 2009, Gabella and Davis, 1998, Birder et al., 2010, Benarroch, 2010, Birder, 2013).

Distension of the bladder wall during filling, the urothelium in particular, releases chemical mediators, transmitters, and hormones such as ATP, NO, prostaglandins and neurotrophic factors that activate afferent myelinated Aδ, and/or unmyelinated C-fibres (Birder, 2013). Some evidence suggests that C-fibres are only activated during noxious stimuli i.e. chemical irritation, and therefore it is probable that the combination of both Aδ and C fibres projecting to the spinal cord and higher brain centres, including the periaqueductal grey (PAG; pontine storage centre), convey information about the bladder filling (Fowler et al., 2008).
During filling there is low-level firing of Aδ bladder afferent fibres from the bladder wall and internal sphincter (Benarroch, 2010, Birder, 2013), whilst parasympathetic efferents tend to be tonically inhibited, which maintain a reduced bladder tone. These afferent fibres project to the thoracolumbar (T10-L2) spinal cord where they can make spinal connections to sympathetic pre-ganglionic neurons originating in the intermediate grey matter in L1-L2 segments and the hypogastric ganglion. This in turn results in activation of descending sympathetic postganglionic nerve fibres to the bladder DSM and urethral smooth muscle, releasing noradrenaline (NA). Subsequently, released NA promotes relaxation of the bladder dome via β3-adrenergic receptors, and contraction of the urethra via α1-adrenergic receptor activation (Fowler et al., 2008, Vizzard, 2010, Andersson and Arner, 2004) (figure 1.4).

Furthermore, afferent activity conveys projections from the spinal cord to the PAG in the brainstem. This in turn inhibits activity of the PMC by inhibiting descending fibres to the sacral spinal cord that controls voiding – see below (Andersson and Arner, 2004, de Groat, 2006, de Groat and Yoshimura, 2006, Fowler et al., 2008). The PAG further projects to the mid-brain and prefrontal cortex to further suppress PMC activity. This enables higher control over initiation of voiding to suit emotional, social or other behavioural situations. Therefore suppression of PMC activity and increased sympathetic activity act together to reduce bladder tone in filling and maintain a high bladder compliance (figure 1.3).
Figure 1.4. Neural relay circuitry controlling the micturition reflex.
(A) Filling phase. Neuronal control of the bladder consists of inhibitory circuitry of parasympathetic fibres to higher brain regions and increased sympathetic output via low-level afferent firing. This involves spinal reflexes to relax the bladder and contract the rhabdosphincter via sympathetic innervation. Input from the pontine storage centre may further increase rhabdosphincter activity. (B) Voiding phase. Transition between filling and voiding occurs during intense afferent firing leads to excitation of parasympathetic and inhibition of sympathetic circuitry to higher brain regions. Afferent input to the spinal cord, relay to higher brain regions including the PAG and pontine micturition centre via spinobulbospinal reflexes (blue line). Subsequently this leads to activation of parasympathetic efferents innervating the bladder and internal urtheral sphincter (green line) and inhibition of sympathetic pathways to the bladder and rhabdospincter (red line) (Fowler et al., 2008).

1.3.1.1 Adrenergic receptor signalling cascade.

Sympathetic nerves innervating the DSM releases NA. NA binds to both α- and β-adrenergic receptors on DSM cells. β3-adrenergic receptors, being more abundant, plays
a greater role in maintaining relaxation of the DSM. The G-protein coupled receptor when activated will stimulate adenylyl cyclase for the formation of cAMP from ATP. cAMP relaxes smooth muscle and therefore contributes to DSM relaxation (Fry et al., 2010, Andersson and Arner, 2004, Fowler et al., 2008, Frazier et al., 2008).

1.3.1.1.2 Urethral control during filling.

Urethral contraction plays a substantial role in the filling phase to prevent leakage of urine, especially during sudden changes in intravesical pressure. Sudden changes in abdominal pressure that will transmit to the bladder lumen, arising for example during coughing, laughing, or sneezing, results in a sudden and temporarily dynamic contraction of the external urethral sphincter. This is known as the ‘guarding reflex’ (Benarroch, 2010, Thor, 2003, Vizzard, 2010). Typically the guarding reflex is tonically active during normal storage but changes to a dynamically more active state during such stresses on the bladder (Vizzard, 2010, Thor, 2003). The external urethral sphincter, being striated muscle, is controlled via a different route during urine storage than the internal urethral sphincter. Afferent nerve fibres, originating from the pelvic nerve projecting to the spinal cord, synapse via interneurons, to neurons within Onuf’s nucleus (S2-S4 segments) of the spinal cord (Thor, 2003). Onuf’s nucleus then projects somatic cholinergic motor nerves via the pudendal ganglia to innervate the external urethral sphincter (de Groat, 1995, Fowler et al., 2008, Benarroch, 2010). Cholinergic motoneurons release acetylcholine (ACh) that act on nicotinic ACh receptors on skeletal muscle cells of the rhabdosphincter to cause contraction (Benarroch, 2010, Fowler et al., 2008) (figure 1.3).
1.3.1.2 Voiding phase.

Transition between the filling and voiding phases is a result of a change of state of the interaction between the periaqueductal gray and pontine micturition centre in the brainstem (Fowler et al., 2008). The eventual result is activation of sacral (S2-S4) parasympathetic nerves to the bladder, inhibition of sympathetic fibre activity to the bladder and suppression of Onuf’s nucleus to allow relaxation of the rhabdosphincter. (figure 1.3). The switch from filling to voiding is due to a larger afferent input to the PAG, as well as activation of the PMC from higher centres when micturition would be appropriate. However, the precise nature of the switch of function over PMC function remains unclear.

The PMC is the main higher brain centre that regulates bladder contraction and urethral relaxation during micturition. Descending excitatory glutaminergic fibres project to the sacral (S2-S4) spinal cord and activate parasympathetic motor nuclei (Fowler et al., 2008, Benarroch, 2010, Yoshiyama and de Groat, 2005). In addition, inhibitory interneurons suppress Onuf’s nucleus to relax the external sphincter. In addition, descending fibres suppress thoraco-lumbar sympathetic fibres (Benarroch, 2010, Vizzard, 2010) (figure 1.3).

Parasympathetic pre-ganglionic neurons, originating in the sacral (S2–S4) spinal cord projects via the pelvic nerve to the pelvic ganglion located on the bladder base. Postganglionic fibres then innervate DSM resulting in the release of ACh and ATP from efferent nerve endings that bind to muscarinic and purinergic receptors, respectively. The
resultant downstream intracellular signalling cascades lead to DSM contraction and thus voiding (Andersson and Hedlund, 2002, Fowler et al., 2008, Fry et al., 2010, de Groat, 2006).

1.3.1.2.1 Muscarinic receptor signalling cascade.

Muscarinic receptors are three-dimensional seven transmembrane helices attached to a G-protein coupling system. In all there are five sub-types of muscarinic receptors (M₁–₅; see table 1.1 for localisation and mechanism of action). Of the five sub-types, the M₃ receptor is the functionally most important for normal detrusor contractions in numerous species, including humans, despite the fact that M₂ receptors are more abundant. During pathological conditions, the M₂ receptor, has been proposed to have an additional functional role (Fry et al., 2010, Braverman et al., 2006).
Table 1.1. Summary of muscarinic receptor subtypes including their localisation, 2nd messenger signalling pathway and subsequent physiological response.

<table>
<thead>
<tr>
<th></th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
<th>M5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Localisation</strong></td>
<td>Nerve fibres</td>
<td>Nerve fibres, urothelium, DSM</td>
<td>Urothelium, DSM</td>
<td>Nerve fibres</td>
<td>Substantia nigra</td>
</tr>
<tr>
<td><strong>Second messenger signalling pathway</strong></td>
<td><strong>G_q/11, PLC activation</strong></td>
<td><strong>G_i/o, inhibits adenylyl cyclase</strong></td>
<td><strong>G_q/11, PLC activation</strong></td>
<td><strong>G_i/o, inhibits adenylyl cyclase</strong></td>
<td><strong>G_q/11, PLC activation</strong></td>
</tr>
<tr>
<td><strong>Physiological response</strong></td>
<td>Suppression of transmitter release</td>
<td>Inhibits calcium channels, prevents cAMP formation.</td>
<td>Smooth muscle contraction.</td>
<td>Inhibits calcium channels, prevents cAMP formation, feedback inhibition of ACh.</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

In normal bladder conditions, the M₃ receptor is coupled to a G_q/11 protein and instigates a rise of intracellular Ca^{2+} ([Ca^{2+}]_i) through a G-protein second-messenger signalling pathway. Activation of the M₃ receptor results in a conformational change causing the dissociation of the trimeric G-protein to activate phospholipase Cβ (PLC) (Ferguson et al., 1986, Berridge, 1981). Once activated, PLC hydrolyses phosphoinositides (PIP₂) on the membrane into the intracellular second messengers inositol triphosphate (IP₃) and diacylglycerol (DAG). IP₃ acts by binding to IP₃ receptors on intracellular Ca^{2+} stores stimulating release into the sarcoplasm and initiating contraction. This release of Ca^{2+} localised to the surface of Ca^{2+} stores causes a further release of Ca^{2+} through ryanodine receptors (RyR) by a process known as Ca^{2+}-induced Ca^{2+} release (CICR) propagating a
further rise in \([\text{Ca}^{2+}]_i\) (Harriss et al., 1995, Wu et al., 2002, Streb et al., 1983). The rise of \([\text{Ca}^{2+}]_i\) mediates bladder contraction through the formation of \(\text{Ca}^{2+}\)-calmodulin complex that activates myosin light chain kinases (MLCK), catalysing the phosphorylation of myosin light chains (MLC) and resulting in DSM contraction (Braverman et al., 2006, Andersson and Arner, 2004). DAG may also play a role in maintaining bladder contraction by increasing the sensitivity of contractile proteins to \(\text{Ca}^{2+}\). This is achieved through DAG-activated protein kinase C, that in turn phosphorylates MLC phosphatase (MLCP) preventing MLCP from dephosphorylating MLCs, and thus maintaining the contractile state of the DSM (Mizuno et al., 2008, Wang et al., 2009). There is also evidence that activation of M3 receptors may activate rho-associated kinases (ROK) that would inhibit MLCP thus increasing the sensitivity of \(\text{Ca}^{2+}\) to contractile proteins (Wang et al., 2009, Andersson and Arner, 2004) (figure 1.5).
Figure 1.5. A schematic diagram of nerve-activated smooth muscle intracellular signalling pathways in the DSM leading to bladder contraction.

Acetylcholine (ACh) and ATP act on the surface receptors M3 and P2X1, respectively. The M3 receptor activates phospholipase C (PLC) that hydrolyses PIP2 into the second messengers DAG and IP3. IP3 acts on IP3 receptors on the SR membrane leading to Ca2+ release from intracellular Ca2+ stores. A rise in Ca2+ causes the formation of a Ca2+-calmodulin (Ca2+-CaM) complex that activates myosin light chain (MLC) kinase phosphorylating myosin that subsequently binds to actin filaments. Additionally, rho-kinase may be activated upon M3 receptor activation that increases the sensitivity of Ca2+ to contractile proteins through phosphorylating and reducing MLC phosphatase activity. P2X1 activation allows for the influx of cations, importantly Ca2+ and Na+, through the P2X1 receptor ion channel that leads to L-type Ca2+ channel and subsequent bladder contraction (Fry et al., 2010).
1.3.1.2.2 Purinergic receptor signalling cascade.

Purinergic receptors are divided into two families: P1 receptors that respond to the purine, adenosine, and P2 receptors that respond to both purines (ADP and ATP) and pyrimidines (UDP and UTP). The P2 receptors can be further sub-categorized into P2X and P2Y sub-families. The P2X sub-family are non-selective cation channels formed of two hydrophobic transmembrane motifs allowing for the channel gating of the ions Ca\(^{2+}\), Na\(^{+}\) and K\(^{+}\). There have been many sub-classes of P2X receptors identified in the bladder with each sub-class displaying different degrees of importance in terms of bladder physiology, whilst other sub-classes still remain unknown in their function. Conversely, the P2Y sub-family is a seven transmembrane-spanning G-protein coupled receptor. In addition, P2Y receptors also express multiple sub-types in the bladder wall (Andersson and Arner, 2004, Fry et al., 2010, Birder et al., 2004, Moore et al., 2001, Chopra et al., 2008, Ruan et al., 2006); see table 1.2 for location and mechanism of action).
Table 1.2. Summary of localisation, signalling pathway and physiological response to P2Y and P2X receptors.
(Andersson and Arner, 2004, Fry et al., 2010, Birder et al., 2004, Moore et al., 2001, Chopra et al., 2008, Ruan et al., 2006).

<table>
<thead>
<tr>
<th>P2 class</th>
<th>Localisation</th>
<th>Signalling pathway</th>
<th>Physiological response</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2Y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P2Y1</td>
<td>Mucosa, nerve fibres</td>
<td>$G_{q/11}$, PLC activation</td>
<td>Unknown bladder function. Possible DSM contraction and/or DSM relaxation possibly by cAMP-dependent protein kinase-A</td>
</tr>
<tr>
<td>P2Y2</td>
<td>Mucosa</td>
<td>$G_{q/11}$, PLC activation</td>
<td></td>
</tr>
<tr>
<td>P2Y4</td>
<td>Mucosa, nerve fibres</td>
<td>$G_{q/11}$, PLC activation or $G_i$</td>
<td></td>
</tr>
<tr>
<td>P2Y6</td>
<td>Mucosa</td>
<td>$G_{q/11}$, PLC activation</td>
<td></td>
</tr>
<tr>
<td>P2Y11</td>
<td>Unknown?</td>
<td>$G_{q/11}$, PLC activation or $G_s$</td>
<td></td>
</tr>
<tr>
<td>P2Y12</td>
<td>Unknown?</td>
<td>$G_i$, inhibits AC</td>
<td></td>
</tr>
<tr>
<td>P2Y13</td>
<td>Unknown?</td>
<td>$G_i/G_o$</td>
<td></td>
</tr>
<tr>
<td>P2X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P2X1</td>
<td>Mucosa, DSM</td>
<td>Non-selective cation channel, permeable to $Na^+$, $Ca^{2+}$, $K^+$.</td>
<td>DSM contraction</td>
</tr>
<tr>
<td>P2X2</td>
<td>Mucosa, nerve fibres</td>
<td></td>
<td>Alters afferent nerve firing</td>
</tr>
<tr>
<td>P2X3</td>
<td>Mucosa, nerve fibres</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P2X4</td>
<td>Mucosa</td>
<td></td>
<td>Unknown bladder function.</td>
</tr>
<tr>
<td>P2X5</td>
<td>Mucosa, nerve fibres</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P2X6</td>
<td>Mucosa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P2X7</td>
<td>Mucosa</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

During normal physiological conditions, bladder contraction is thought to be solely mediated by M₃ receptors in humans and old-world monkeys. All other species examined have a dual muscarinic and purinergic activation on parasympathetic nerve stimulation. However, in pathological conditions P2X receptors have a more profound role in bladder contraction, which may be due to insufficient breakdown of ATP in the synaptic cleft by extracellular ectoATPases, or an excess release of ATP from parasympathetic nerves (Fry et al., 2010).
Parasympathetic nerve endings also release ATP that activates P2X receptors, in particular, P2X₁ sub-type on DSM cells. Upon activation, the P2X₁ receptor allows the influx of Na⁺ and Ca²⁺ into the cell, producing an excitatory junction potential, depolarising the membrane. This activates L-type Ca²⁺ channels to further increase [Ca²⁺]ᵢ. This rise in [Ca²⁺]ᵢ can directly activate contractile proteins or more efficiently further raise [Ca²⁺]ᵢ via CICR from localised Ca²⁺ stores (Fry et al., 2010, Fowler et al., 2008, Andersson and Hedlund, 2002) (figure 1.5). Although, little is known about the functional role of P2Y receptors on the DSM, they could play a role in detrusor smooth muscle relaxation via cAMP-dependent protein kinase-A (PKA) activation. P2Y receptors in the mucosal layer and afferent nerve fibres within the bladder wall tends to exert more prolific physiological function.

1.4 The mucosa - not just a passive barrier.

1.4.1 Mucosal barrier properties.

As mentioned in section 1.2.1.2, the mucosa consists of an epithelial lining on the luminal surface of the bladder as well as a basement membrane and sub-urothelium. The uroepithelium was initially regarded as an impenetrable, passive barrier with the majority of literature focusing on its transport properties and its breakdown as a barrier in different pathological situations such as inflammatory cystitis. Nonetheless, this barrier property does have an integral part in bladder physiology and is reliant on many factors that control the movement of small molecules, ions and solutes between cells.
The structure of the urothelium is key for determining the permeability to ions, water, and solutes. In particular, tight junctions between urothelial so-called umbrella cells play a large part in maintaining reduced permeability. Umbrella cells are large, flat cells that form the luminal face of the urothelium. Tight junctions are highly specialized structures that connect to neighbouring cells and are specific to epithelial cells. They are composed mainly of a mixture of occludins and claudins that create lateral strands between adjacent cells, thus limiting the extracellular space (Ban et al., 2003). Recent studies have shown that changes in stress or mechanical stimuli affects barrier permeability through changes in the shape of umbrella cell tight junctions. Umbrella cells exposed to stretch show increased ion permeability through changes in tight junction shape (figure 1.6) (Carattino et al., 2013). Thus, the bladder responds during filling and voiding to altering ion flux across the urothelial wall that may affect sub-urothelial bladder afferents.

Figure 1.6. Urothelial lining immunostained for occludin (tight junction). Immunofluorescence of the urothelium showing tight junctions (green) and indicated by arrows. L = lumen (Spector et al., 2015).
In addition to tight junctions, gap junctions allow cytoplasmic communication between cells so that interchange of solutes and electrical signals is possible (Mese et al., 2007, Evans and Martin, 2002). Gap junctions are membrane-bound structures that form a pore or hemichannel, a connexon, formed of connexin proteins. When adjacent connexons from adjacent cells fuse they form an intercellular connection. Connexons are typically made of six connexin proteins (*figure 1.7*). There are many sub-types of connexin (Cx) proteins and their distribution within gap junctions determines the permeability properties of the latter. In the bladder wall, the expression profile of connexin subtypes shows that Cx26 is prominent in the urothelium, whilst in the suburothelium Cx40 is found in blood vessels and Cx43 on interstitial cells. This contrasts with Cx45 found between adjacent detrusor smooth muscle cells. The number associated with the Cx symbol is the molecular weight of the protein subtype (Neuhaus et al., 2005, Ikeda et al., 2007, Sui et al., 2003, Haefliger et al., 2004, Fry et al., 2007).
1.4.2 Distribution and function of receptors in the bladder wall.

There is a large distribution of receptors across the bladder wall which control cell functions associated with bladder filling and voiding. Some of these receptors may however be dormant under normal physiological conditions and become prominent during pathological conditions. Although this section is primarily concerned with the mucosa it is appropriate to discuss the distribution of receptors across the bladder wall as the mucosa and detrusor layers are functionally interconnected. Thus, receptor-mediated processes in one layer can have important consequence on another through the release of chemical
mediators.

**Muscarinic receptors.** All five muscarinic M₁-₅ receptors (see section 1.3.1.2.1) are expressed in the bladder wall. In the DSM, M₁,₂,₃ receptors are all expressed with M₂ the most abundant. However, M₃ plays the largest role in bladder contraction, but in pathological conditions, M₂ may also contribute (Mansfield et al., 2005, Chess-Williams et al., 2001, Wang et al., 1995, Braverman et al., 2007). In pre-junctional nerve endings, M₁,₂,₄ receptors have all shown to be active, though their functional role is still unknown. The mucosa also has a distribution of muscarinic receptors. All muscarinic sub-types (1-5) have been shown to be present in rat and human bladder urothelium (Mansfield et al., 2005, Tyagi et al., 2006, Giglio et al., 2005, Braverman et al., 2007). In sub-urothelium, M₁,₂,₃ were all expressed in human bladders (Datta et al., 2010), however, there is lacking evidence for expression of M₄,₅ in the sub-urothelium.

**Nicotinic receptors.** This receptor is also activated by ACh but is not known to be present in DSM. Nicotinic receptors are present in the urothelium and nerve fibres. The two receptor subtypes are α3* and α7. These receptors have opposing effects on bladder reflexes, whereby α3* have excitatory and α7 inhibitory on afferent nerves for controlling normal bladder reflexes (Beckel and Birder, 2012, Beckel et al., 2006).

**Adrenergic receptors.** The distribution and role of sympathetic receptors in the bladder has been much discussed. α-adrenergic receptors (α₁A and α₁D subtypes) are expressed in DSM at a low level, but do not have significant effects in regulating contraction (Rivera
More prominent are β adrenergic receptors within the DSM, in particular the β3 subtype, and mediates relaxation upon activation. Although expression of β1 and β2 subtypes has also been measured, these receptor subtypes do not contribute functional responses. In the mucosa, α1- and β-adrenergic receptors were all present at transcriptional level however their functionality is unclear (Moro et al., 2013, Propping et al., 2013, Levin and Wein, 1979, Rivera et al., 1991, Majewski et al., 1990).

**Purinergic receptors.** Purinergic receptors play an important role in controlling normal bladder function. The DSM expresses mainly the P2X1 receptor subtype but also shown expression of P2X7 in many animals and human bladders (Svennersten et al., 2015, Burnstock et al., 1978, Andersson, 2015, Lee et al., 2000, Vial and Evans, 2000). P2X1 receptor activation is important in nerve-mediated responses (see section 1.3.1.2.2) of animals and human in pathological conditions. P2X receptors have important functional roles at levels of the mucosa and nerve afferent fibres that innervate the mucosa. Nerve afferent fibres express P2X receptors, in particular, P2X2, P2X2/3, P2X3, and P2X4. These are likely to contribute to the sensation of bladder fullness mediated through stretch-induced ATP release from the urothelium (Ford and Cockayne, 2011, Cockayne et al., 2000, Andersson, 2015, Burnstock and Knight, 2004). The vast majority of P2X receptor sub-types (1-7) have been shown to be ubiquitously expressed on epithelial cells in the body including the urinary bladder. However, the major sub-types in the bladder urothelial and sub-urothelial cells are P2X1,2,3,7 (Birder et al., 2004, Lee et al., 2000, Burnstock and Knight, 2004, Vial and Evans, 2000, Ford and Cockayne, 2011, Andersson,
P2Y receptors are also present in the bladder wall. Although a lot of publications have confounding physiological functions, P2Y2,4 have all been shown to be expressed and/or be functionally active in feline, rat, and human urothelium as well as the sub-urothelial nerve fibres and detrusor muscle (Chopra et al., 2008, Birder et al., 2004, Shabir et al., 2013). However, the relative expression levels were far greater in the urothelium than the detrusor. P2Y1,6 have also been expressed in urothelial cells but these lack any supporting consensus (Birder et al., 2004, Sui et al., 2006).

**Transient receptor potential (TRP) channels.** To date, TRP vanilloid (TRPV), TRP melastatin (TRPM), TRP ankrin (TRPA) have all been expressed in the bladder. All TRP channels are highly expressed on afferent nerves that innervate the mucosa of the bladder wall. Some expression has also been shown on urothelial cells themselves. They play a large role in physical, thermal and chemical stress such as changes in shear stress, temperature, pH. Within each class of TRP channel there are subtypes which also varying in their physiological function. TRPV1,2,4 are non-selective cation channels with high selectivity to Ca\(^{2+}\). These tend to be known as ‘warm’ receptors due to their activation by noxious temperatures. These receptors become active at temperatures from around 27°C (TRPV4), 42°C (TRPV1), and ≥50°C (TRPV2). The other classes of TRP channels that are also activated by temperature are, TRPA1 and TRPM8. These are often known as ‘cold’ receptors, whereby they become active at temperatures from ≤26°C (TRPM8) to as low as 0°C (TRPA1). More details about their functional role in the bladder will be
discussed in section 1.7 and chapter 3, section 3.1 (McKemy et al., 2002, Avelino et al., 2013, Yu et al., 2011, Avelino et al., 2002, Nilius et al., 2007, Yamada et al., 2009, Andrade et al., 2006, Birder and Andersson, 2013).

**Neuropeptide receptors.** There are many neuropeptide receptors in the bladder that maybe involved in regulating bladder contractility. Vasoactive intestinal polypeptide receptors, VPAC$_1$ and VP$_2$ are expressed in DSM. Although their activity varies between species, they have been hypothesised to affect spontaneous activity (Uckert et al., 2002, Reubi, 2000, Sjogren et al., 1985). Endothelin (ET) receptors, ET$_A$ and ET$_B$ subtypes have both been shown present in many species, although their functional role is not fully understood (Uckert et al., 2002, Calvert et al., 2002, Okamoto-Koizumi et al., 1999, Traish et al., 1992). Tachykinin (TK) receptors are predominately expressed in afferent peripheral nerves. There are three types of TK receptors, TK1, TK2, and TK3 that have high potency to substance-P, neurokinin-A, and neurokinin-B, respectively. The release of NKS mediate neurogenic inflammatory responses (Maggi, 1995, Lecci and Maggi, 2001). Angiotensin (ANG) receptors have also been found in urinary bladder. ANG I and II are present in the DSM but recent findings show that ANG II is also present in the urothelium (Anderson et al., 1984). ANG I and II both present the ability to alter contractile function but their physiological importance is yet to be established (Tanabe et al., 1993, Andersson et al., 1992, Anderson et al., 1984).

**Prostaglandin receptors.** Prostaglandin receptors are activated by prostanoids present in the DSM as well as mucosa. Synthesis of prostanoids by COX-1 and 2 enzymes are
stimulated by stress conditions as well as bladder wall stretch, nerve stimulation, chemical influence (ATP), injury and inflammation. Of the prostanoids, PGE$_2$ is most common that act on EP$_{1-4}$ receptor subtypes throughout the bladder wall. Also expressed in the bladder are prostanoid receptors IP, DP, TP, and FP (Breyer et al., 2001, Rahnama'i et al., 2010, Coleman et al., 1994). Although the exact function of PGs are unknown in the bladder; studies have shown that PGs may influence the micturition reflex by altering afferent nerve activity to decrease the threshold stimuli initiating bladder contraction (Andersson et al., 1977, Park et al., 1999, Rahnama'i et al., 2010). However, the use of non-steroidal anti-inflammatory drugs (NSAIDs) showed no correlation between inverse of LUTS and NSAID use.

**Other receptors.** There are many other receptors that are likely to play some role in controlling normal bladder function but their functional role still lacks substantial evidence. Some of these include; protease-activated receptors, amiloride- and mechano-sensitive epithelial sodium channels, estrogen receptors, and transporters (glucose, vesicular ACh, ATP-binding cassette, polyspecific organic cation transporters) (Birder and Andersson, 2013, Iwatsuki et al., 2009, Reis et al., 2011, Smith et al., 1998, Teng et al., 2008). Many of these probably respond to changes of electrolytes, pH, and solutes in urine as well as physical and mechanical stress of the bladder wall to control normal bladder function.

**1.4.3 The sensory role of the mucosa.**

The mucosal layer has prominent nerve and blood plexuses which can control and support
normal bladder function. Function is also controlled by a complex cellular syncytium which spans the mucosal layer and the DSM. This syncytium could interact through direct cell-to-cell communication or by the release of chemical paracrine mediators that include neurotransmitters, peptides, and hormones. (Birder et al., 2012, Birder and Andersson, 2013). Moreover, the ability of the urothelium to regulate the flux of ions and large molecules across this barrier suggests that changes to the composition of urine, as well as physical stretch caused by filling, can control bladder function. Therefore it is becoming more evident that the urothelium is not just a passive barrier but actually has a fundamental role in controlling normal bladder function (Apodaca, 2004).

**Urothelium.** The urothelium in particular is an important sensory layer for changes in chemical and physical stress. The release of sensory chemicals/peptides/mediators such as ATP, ACh, nitric oxide (NO), prostaglandins and neuropeptides from urothelial cells can be a result from chemical and physical stresses on the bladder wall that activate various receptors on urothelial cells. These sensory molecules can therefore exert excitatory and inhibitory actions on the same cell, neighbouring cells, other underlining cells, afferent and efferent nerves, and also blood vessel, all of which can alter bladder contractility. Chemical and physical stresses include changes in urinary osmolarity or pH, leakage of K⁺ from the urine especially in inflammatory conditions, or release of chemicals/peptides/mediators from nerve and blood vessels or inflammatory cells and physical stresses including stretch or torsion of the bladder wall and alteration of transvesical pressure (figure 1.8).
Figure 1.8. Hypothetical model representing possible interactions between bladder afferent and efferent nerves, urothelial cells, smooth muscle cells and myofibroblasts (interstitial cells).

Urothelial, interstitial, detrusor muscle cells, and nerve fibres express many receptors. Urothelial cells in particular respond to many physical, mechanical and chemical changes. During distension, urothelial cells respond to release many chemical mediators such as ATP, NO, ACh. Subsequently these may act on neighbouring or distant cells to control the micturition cycle. For example, it is proposed that stretch-induced ATP release may act on P2X3 receptors on afferent nerves which could regulate spinal reflexes to promote bladder voiding. In addition, many other receptors are expressed on urothelial, interstitial cells and nerve fibres including TRP channels, muscarinic receptors, purinergic receptors, neurokinin receptors to name a few, that probably all regulate a fine control between filling and storage phases of the micturition cycle (Birder, 2010).

ATP has been regarded as a fundamental signaling molecular in the bladder wall during physical stresses such as stretching. Distension-evoked ATP release occurs from apical (luminal-facing) and basolateral surfaces of the urothelium, ATP from cytoplasmic stores could in principle be released from urothelial cells via vesicular exocytosis, transporters, channels or anion-selective channels (Wang et al., 2005, Birder and Andersson, 2013). More recent evidence has shown that shear stress of urothelial cells causes ATP release via multiple routes, including Cx or pannexin hemichannels, as well as vesicular
exocytosis (McLatchie and Fry, 2015). It is possible that the release of ATP during bladder distension may influence nearby afferent nerves, by binding to P2X₃ receptors to control afferent firing and thus maintaining normal micturition cycle (Vlaskovska et al., 2001). It is also possible that ATP may signal to adjacent cells i.e. DSM or interstitial cells (Sui et al., 2006, Young et al., 2012). Although the physiological relation between the release of ATP during different chemical and physical stresses, and bladder contractility is not fully understood, its initiation and/or contribution to spontaneous activity should not be excluded. In pathological bladders, including interstitial cystitis and overactive bladder, there is evidence that support an enhanced ATP release (Birder et al., 2003, Sugaya et al., 2009). This further emphasises the importance of ATP release and its role in controlling normal bladder function.

Although ATP plays an integral role in normal bladder function, it has also been shown that other chemical messengers are highly important in regulating bladder physiology. Some other important chemical mediators/peptides/hormones include; ACh, NO, and PGs. Non-neuronal release of ACh also occurs from the urothelium during physical stress such as stretching. This was confirmed as release of ACh was tetrodotoxin (TTX)-insensitive and was significantly enhanced with an intact urothelium (Yoshida et al., 2006). Stretch-induced ACh release was confirmed by Moro et al., which showed enhancement of spontaneous contractions during stretch were abolished with atropine (muscarinic antagonist) (Moro et al., 2011). ACh release is also important for controlling normal bladder function as there is significant correlation between increased age and ACh release. Thus may be another factor that could contribute to pathological bladders in
elderly.

**Sub-urothelium.** The sub-urothelium is an important layer within the bladder wall. It is populated by a network of ICs identified by antibodies to vimentin. A subpopulation of these cells also label to c-kit or PDGFRα (Koh et al., 2012). A subset of the IC population (not necessarily the same as those that label with c-kit and PDGFRα) have structural similarities to myofibroblasts, cells that have a contractile phenotype. The network of sub-urothelial ICs are connected by gap junctions (Cx43) and therefore may contribute to the spread of electrical signals, and possibly also chemical mediators throughout the bladder wall. This spread to activity could control spontaneous activity in the bladder. ICs are electrically active, in particular they exhibit large inward currents via Ca$^{2+}$-activated Cl$^{-}$ channels. These channels are activated by ATP and P2Y agonists such as ADP, UTP and UDP, as well as low pH and suggests they could mediate the spread of electrical signals initiated by ATP release from the urothelium (Zhu et al., 2009, Andersson and McCloskey, 2014, McCloskey, 2010, Fry et al., 2007). These inward currents induced by ATP were attenuated by capsaicin (a TRPV$_1$ receptor activator), suggesting that TRPV$_1$ channels are present on ICs and may also regulate or maintain Ca$^{2+}$ transients in ICs. This emphasizes that ICs probably regulate or initiate the spread of spontaneous activity across bladder wall by release of different chemicals such as ATP from the urothelium or sub-urothelium itself (Andersson and McCloskey, 2014) (*figure 1.9*).
Figure 1.9. Spontaneous Ca\textsuperscript{2+} transients recorded in interstitial cells (ICs).
A) Immunofluorescence of a pair of spindle shaped ICs stained for cKit in the bladder mucosa. B) Left picture shows a interstitial cell of cajal acting as a pacemaker cell from the GI tract by synchronising the spread of Ca\textsuperscript{2+} transients to a neighbouring myocyte. Right pictures are a series of frames (2 sec intervals) showing spontaneous Ca\textsuperscript{2+} transients from an interstitial cell which is asynchronatic to a neighbouring myocyte (Hashitani et al., 2004b).

1.5 Spontaneous contractions.

1.5.1 Generation of spontaneous contractions.

The bladder exhibits spontaneous activity which may contribute to maintain bladder wall tone. This allows individual muscle bundles to maintain similar resting lengths during filling of the bladder. Spontaneous contractions are of myogenic origin as they are abolished in low extracellular Ca\textsuperscript{2+} solution, but not nerve-mediated as they are insensitive
to neurotoxins such as TTX (Buckner et al., 2002). However, they do not necessarily originate from the detrusor smooth muscle itself. This was shown in isolated bladder preparation, where intact (mucosa & detrusor) preparations exhibits large spontaneous contractions compared to when the mucosa is removed (detrusor only preparations) (Fry et al., 2010, Kanai et al., 2007). However, detrusor only preparations lack any spontaneity demonstrating the need for communications between different layers of the bladder wall to control normal spontaneous activity (figure 1.10).

![Figure 1.10](image)

**Figure 1.10. Representative tracing of spontaneous contractions.** Spontaneous contractions are present in intact (top tracing) preparations and absent in detrusor-only preparations (bottom tracing). Their activity can be modulated in the presence of chemical or physical stimuli as shown by the induction of low extracellular pH (Kushida and Fry, 2015).

During bladder filling, spontaneous contractions remain small to ensure low intravesical pressure and a high bladder compliance. When the bladder becomes full, spontaneous contractions become greater, and if large enough may even cause significant transient increases of intravesical pressure. If these spontaneous contractions are large enough they may have pathological consequences: if co-ordinated across large regions of the bladder wall they manifest as ‘overactive’ bladder contractions that may even cause involuntary
loss of urine. If less co-ordinated they will lead to an increase of bladder tone and reduce bladder compliance (Coolsaet, 1985).

Spontaneous detrusor contractions are associated with oscillations of membrane potential that may themselves be accompanied by action potentials (Hashitani et al., 2004a). Depolarisation activates L-type Ca$^{2+}$ channels to permit influx of Ca$^{2+}$ that may further enhance the rise of the intracellular [Ca$^{2+}$] via CICR. Repolarisation results from activation of K$^+$ channels: in guinea-pig detrusor this is mediated importantly by small (SK) and large (BK) conductance Ca$^{2+}$-activated K$^+$ currents. BK channels are gated by a rise of intracellular Ca$^{2+}$; from CICR. SK channels are gated by the formation of Ca$^{2+}$-CaM complex (Fry et al., 2010, Hashitani and Brading, 2003, Wu et al., 2002). In contrast, in pig and human detrusor, ATP-sensitive K$^+$ channel have a more important role in repolarising the cell (Meng and Chi, 2009). Spontaneous electrical activity and contractions are able to spread within detrusor muscle bundles via Cx45 gap junctions between adjacent myocytes (Ikeda et al., 2007). What remains to be determined is how oscillations of membrane potential and hence contractions occur.

**1.5.2 The origin of spontaneous contractions.**

**1.5.2.1 Myogenic hypothesis.**

An increase of detrusor electrical excitability and enhanced coupling of detrusor myocytes could lead to increased spontaneous activity (Foon and Drake, 2010). Isolated human detrusor myocytes from overactive bladders have a greater rate of spontaneous action potential initiation that is consistent with this theory when compared to normal detrusor
smooth muscle that lack spontaneous activity unless the mucosa is left intact. By contrast, gap junction conductance is reduced in equivalent preparations, due to reduced expression of Cx45. The relative contribution these two opposing mechanisms contribute to an increase of myogenic activity is however unclear and remains unknown if the myogenic hypothesis is a significant origin of spontaneous contractions (Sui et al., 2009, Sui et al., 2003).

Interstitial cells are found between detrusor muscle bundles (McCloskey and Gurney, 2002) and it may be hypothesised that these generate action potentials that propagate to smooth muscle cells, in the same way the interstitial cells of Cajal initiate spontaneous activity in G-I tract smooth muscle (Hirst and Ward, 2003). Detrusor ICs generate spontaneous intracellular Ca$^{2+}$ transients however, they do not occur in synchrony with similar transients in adjacent smooth muscle cells (Hashitani, 2006). It has therefore been proposed that detrusor ICs do not initiate detrusor smooth muscle electrical activity as in the G-I tract, but they may synchronise activity between adjacent muscle bundles.

1.5.2.2 Urotheliogenic hypothesis.

The mucosal layer plays a profound role in enhancing or even initiating spontaneous contractions (figure 1.9). In addition, the mucosa is highly active and releases many chemical agents and transmits electrical activity through the bladder wall in response to physical, mechanical, and chemical stimuli (Fry et al., 2012). Chemical agents, in particular, ATP released from urothelial cells could affect smooth muscle function, either by direct diffusion to the smooth muscle layer, or mediated by cell-to-cell communication.
Diffusion of chemical agents can be tested by removal of the mucosa and subsequently ‘laying’ the mucosa back on top of the detrusor. If any spontaneous contractions generate once the mucosa is placed back onto the detrusor muscle then diffusion of transmitter release must be a means to initiate or enhance spontaneous contractions.

Cell-to-cell communication can be tested by examining electrical or chemical spread across from the mucosa to detrusor via gap junctions. Sub-urothelial interstitial cells have previously shown to generate spontaneous inward currents, accompanied by Ca\(^{2+}\) transients. These cells were responsive among stimulation with ATP via P2Y channels by causing an initial rise in [Ca\(^{2+}\)]\(_i\) followed by an inward current to cause membrane depolarisation. This current was augmented when two cells were in contact via the spread of electrical activity between cells via gap junctions (Fry et al., 2007). Further validation using optical imaging experiments, Ikeda and colleagues demonstrated the importance of gap junctions in the spread of spontaneous activity across the bladder wall (Ikeda et al., 2007). Therefore, it is quite possible that sub-urothelial ICs may be responsible for the depolarising effects on the DSM leading to spontaneous activity via gap junctions. This could be a means of electrical or chemical activity. For example, the accumulation of ATP in urothelial cells may spread through the mucosa via gap junctions and become released into the extracellular space binding to P2X\(_i\) in the DSM leading to the activation L-type Ca\(^{2+}\) channels. The influx of Ca\(^{2+}\) will result in oscillations in spontaneous activity (Fry et al., 2010, Foon and Drake, 2010).
1.5.2.3 Neurogenic hypothesis.

Increased nerve activity may initiate or permit the persistence of spontaneous contractions. Transmitter leakage from nerve terminals may result in activation of receptors such as P2X and muscarinic receptors on DSM cells. However, as spontaneous contractions are atropine and TTX-insensitive, this hypothesis is not supported (Foon and Drake, 2010, Yoshida et al., 2006, Moro et al., 2011, Young et al., 2008).

Overall evidence for the urotheliogenic hypothesis is most convincing, but it does not mean that the myogenic and neurogenic hypotheses may have some contribution. It is most likely that a multi-network of signals within the mucosal layer spreads electrical and chemical activity, via release of chemical mediators such as ATP, to DSM cells which initiate and control spontaneous contractility (Fry et al., 2010, Ruan et al., 2006, Hashitani et al., 2004b, Andrade et al., 2006, Birder et al., 2012, Nilius et al., 2007, Yamada et al., 2009). Control of spontaneous contractions is critical to avoid detrusor overactivity and hence the development of an overactive bladder (OAB).

1.6 Bladder pathologies.

The lower urinary tract is susceptible to several benign conditions (lower urinary tract disorders, LUTDs) that manifest in patients as lower urinary tract symptoms (LUTS). A summary of some of the more common conditions is shown in table 1.3.

Other LUTDs with associated LUTS include neurogenic bladder dysfunction, urinary tract infections, and in men chronic prostatitis and benign prostatic hyperplasia. In addition,
bladder tumours and urinary tract stones can cause irritation to the bladder with symptoms that can include LUTS. Of the many LUTDs, overactive bladder is most common and one of the most problematic to treat.

**Table 1.3. Some more common bladder pathologies with their symptoms, clinical management and prevalence.**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Symptoms</th>
<th>Management options</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overactive bladder</td>
<td>Increased urinary urgency and frequency, nocturia, ±urinary incontinence.</td>
<td>Anti-muscarinics, β-adrenergic agonists, PDE antagonists, botulinum toxin, Others</td>
<td>11.8% of total population with similar rates among sex, significantly rising with age.</td>
</tr>
<tr>
<td>Underactive bladder</td>
<td>Hesitancy, straining, incomplete emptying (without obstruction).</td>
<td>α-adrenergic antagonists, muscarinic agonists, acetylcholinesterases</td>
<td>Males: 9-28% of &lt;50 years old to up to 48% &gt;70 years old. Females: 12-45% &gt;70 years old.</td>
</tr>
<tr>
<td>Interstitial cystitis (bladder pain syndrome)</td>
<td>Pelvic pain and pressure, with at least one other urinary symptom.</td>
<td>Self-management (pelvic floor exercises), botulinum toxin, surgery i.e. cytoscopy</td>
<td>~0.5% of the total population.</td>
</tr>
</tbody>
</table>

1.6.1 **Overactive bladder (OAB).**

OAB is a symptom-based condition characterized by storage or voiding symptoms and has a standardised definition from the International Continence Society as, “urgency, with or without urge incontinence, usually with frequency and nocturia.” (Abrams et al., 2003). Typically there are two types of OAB, neurogenic OAB and idiopathic OAB. In addition, bladder outflow obstruction, due for example to benign prostatic hyperplasia in men, is associated with OAB symptoms. Neurogenic OAB is defined as overactive bladder symptoms associated with neurogenic disorders, such as spinal cord injury or spina bifida.
Idiopathic OAB is a much more prevalent condition, and as the name suggests the causes and origin are unknown. OAB is often associated with detrusor overactivity (DO), characterized by involuntary detrusor contractions during the filling phase (Fry et al., 2010, Brading, 1997).

1.6.1.1 Detrusor overactivity (DO).

DO is an entity of OAB in patients and is diagnosed using filling urodynamics as ‘a urodynamic observation characterized by involuntary detrusor contractions during the filling phase, which may be spontaneous or provoked’ (Abrams, 2003). However, although DO is heavily linked to OAB problems, this overactivity does not necessarily cause symptoms of OAB. It is therefore important to understand and reduce these involuntary detrusor contractions in order to treat the symptoms of OAB.

1.6.1.2 Urodynamics.

Intravesical bladder pressure, the force exerted on a unit area of bladder wall, is measured during artificial filling of the bladder. In practice, urodynamic testing measures both intravesical bladder pressure (Pves) and abdominal pressure (Pabd), as changes to the latter (e.g. with standing, coughing) also transmit into the bladder lumen. Pves is measured by a catheter inserted via the urethra into the bladder lumen; Pabd is measured by a catheter in the rectum. Changes to intravesical pressure from bladder activity alone, Pdet, is defined as: Pdet = Pves – Pabd. Examples of urodynamic traces from patients with a normal, stable bladder (left) and from one showing DO are shown in figure 1.10. The stable bladder shows a slow rise of Pdet with filling until a voiding contraction is
evoked, associated with flow (lower trace). The overactive bladder shows several contractions during filling that are not necessarily associated with flow (Gray, 2010) (figure 1.1). Although DO is heavily linked to OAB problems, it does not necessarily cause symptoms of OAB. It is therefore important to understand and reduce these involuntary detrusor contractions in order to treat the symptoms of OAB in many patients.
Figure 1.11. Urodynamic tracings.
Recordings of urodynamics from two patients showing the detrusor pressure (Pdet, top tracings) and urine flow (bottom tracings). A) normal bladder shows a high pressure during flow; B) overactive bladder showing OAB pressure movements without flow. Bristol urological institute, unpublished data.
1.6.1.3 Therapeutic treatment for OAB.

Anti-muscarinic agents are the current mainstay for therapeutic treatment of OAB symptoms. However, these drugs tend to lack efficacy and along with side-effects (e.g. dry mouth) many patients become incompliant after several months. More recently other drugs have been introduced, such as β-adrenergic agonists, PDE antagonists. They have fewer unacceptable side-effects, but their relative novelty means that their efficacy has not yet been fully evaluated. Another recent and effective treatment is the injection of botulinum toxin (BoTox) into the bladder wall. The mechanism of action of BoTox in the bladder is not fully understood. Patients need repeat injections after 9-12 months under general anaesthesia, so is not available to many especially elderly patients, and may also result in urinary retention requiring self-catheterisation (Granese et al., 2012, Hesch, 2007). Therefore, there is a huge demand for new treatment for OAB syndrome, that is effective, easy to apply, and lacks significant side-effects.

1.7 Novel approaches to manage OAB?

1.7.1 The use of heating to reduce bladder contractility.

An increase of spontaneous contractile activity is significantly associated with OAB in patients. Therefore therapeutic approaches that reduce spontaneous activity could be devised to treat the symptoms associated with OAB. One possible approach is the use of heating to depress spontaneous contractions.

Heating is used as a therapeutic treatment in other organs, such as the heart. The application of heat, by means of a raised body temperature to 42°C for 15 minutes,
protected the heart against ischaemia by inducing the expression of heat shock proteins as a protective role against further stress (Yellon et al., 1992). In addition, heating has been shown to decrease the contractile function of many different muscle types including smooth muscle (Everett et al., 2001, Mustafa et al., 2004). However, there are no studies with the urinary bladder and by contrast much emphasis has been put on cooling to alter bladder contractile function. Rapid cooling of guinea-pig bladder altered electrically-stimulated contractions (Kurihara et al., 1974, Turner and Weetman, 1977). Cooling to 30ºC produced a significant enhancement of activity, attributed in part to increased ATP release from efferent nerves (Ziganshin et al., 2000, Ziganshin et al., 2002). Furthermore, sustained cooling to 4ºC (4 hour/day for 21 days) led to a significant down-regulation of the carbachol-induced muscarinic receptor activated pathway (Demir et al., 2007).

One pathway whereby heat changes can alter bladder contractility is via activation of temperature sensitive TRP channels. TRP channels are activated during noxious stimuli including changes in temperature and different TRP channel subtypes respond over different temperature ranges. For example, TRPV1 channels respond to temperatures ≥41ºC; TRPV2 channels, on the other hand, are more responsive at temperatures ≥50ºC. TRPV4 receptors are less responsive to high temperatures and become more active at lower temperatures, ≤27ºC. Therefore, it is likely that heating to temperatures beyond physiological conditions i.e. ≥37ºC, will activate TRPV1 and TRPV2, thus these subtypes could play an important role in modulating bladder contractility (Fry et al., 2010, Nilius et al., 2007, Yamada et al., 2009, Venkatachalam and Montell, 2007, Avelino et al., 2013, Birder and Andersson, 2013). More details will be discussed in chapter 3, section 3.1.
Nerve conduction velocity, the rate at which electrical signals propagate along a nerve fibre, is also temperature-dependent. Typically, raising temperature will increase conduction velocity and as a consequence can increase neurotransmitter release including ATP, ACh, NO, and/or noradrenaline (Burnstock, 1958, Madsen et al., 2012). Temperature-dependent mediator release has also been shown in erythrocytes whereby ATP release is increased when raising the temperature to 42°C (Kalsi and Gonzalez-Alonso, 2012).

1.7.2 The use of inert bulking agents to reduce bladder contractility.

The interaction of the mucosa and DSM is highly important for the enhancement of spontaneous contractions. The separation of the mucosa from DSM greatly dampens spontaneous contractions when compared to intact preparations (figure 1.10). DSM-only preparations lack any spontaneity whereas mucosa-only preparations do spontaneously contract. This highlights the importance of communication between the mucosa and DSM layers. It is likely the release of diffusible chemical agents from urothelial and/or interstitial cells can alter the contractile function of the bladder. Thus reducing this communication may also offer a means to reduce spontaneous contractions. Another approach to reducing the unprovoked spontaneous activity in OAB patients could therefore be the use of inert bulking agents to increase the spacing between the mucosa and underlying muscle.
A bulking agent is a substance used to increase the spacing between a given material i.e. increased spacing between the urothelium and underlining detrusor. These agents should be hypoallergenic, biocompatible, and non-immunological. For commercial use and treatment against pathological conditions such as OAB, these compounds should be in current medical use. Some ideal bulking agents would include, collagen-based compound, autologous fats, carbon-coated beads, silicon, polyethylene oxides, calcium hydroxylapatite, hyaluronic acid, and injectable microballoons. For clinical approaches to treat OAB, the bulking agent therefore should be easy to inject, low cost, high stability and low mobility to conserve its volume over time (Khullar et al., 1997, Olson et al., 1998, Lightner et al., 2001, Herschorn, 2005).

1.8 Hypotheses.

1 Heating.
Progressive heating from 37 to 42, 46, and 50°C leads to a continuous decrease of spontaneous activity, through a TRPV1 channel activated pathway mediating a decrease of urothelium ATP release.

2 Injectables.
Sub-mucosal injection of bulking agents reduces spontaneous activity by means of increased separation between the urothelium and underlying detrusor muscle.
1.9 Aims.

To test the proposed hypotheses, the following aims will be followed:

1 Heating.

1. To determine the effect of increasing temperature (42, 46, 50°C) on spontaneous contractile variables in intact bladder preparation.
2. To determine the optimal temperature to alter bladder contractility.
3. To characterise the best suitable model to quantify the effect of heating on bladder contractility by using a whole perfused pig’s bladder.
4. To measure the release of ATP from the bladder during heating.
5. To describe changes in tissue integrity during heating.

2 Injectables.

1. To determine the effect of a sub-mucosal injection on bladder spontaneous contractions.
2. To locate the site of injection in the bladder wall.

1.10 Objectives.

1. *In vitro* contractility – this technique will be used to measure changes in bladder contractility.
2. *Ex vivo* whole perfused bladder – this technique is used to measure intravesical pressure changes.
3. ATP-assay – the release of ATP from bladder multicellular preparations and urothelial cells will be measured with a luciferin-luciferase ATP-assay.
4. Histology – examine tissue integrity with vital stains.
Chapter 2. General materials and methods.
2.1 Chemicals and solutions.

2.1.1 Tyrode’s and Krebs’ solution.

Tyrode’s solution was prepared and used as a tissue superfusate. An initial 10-times concentrated batch was made and diluted for experimentation. Krebs’ solution was made as a 10 l batch and stored at room temperature. All solid reagents were weighed out on a calibrated balance and added to deionised, distilled water (dH₂O) (table 2.1). All liquid reagents were measured using a calibrated, variable volumetric pipette. All physiological buffers were gassed with 95% O₂ : 5% CO₂, pH 7.54.

2.1.2 Drugs.

Most drugs were made into concentrated stock solutions using dH₂O and stored at 4°C or -20°C. Some drugs were made up on the day of experiments from the original stock. Prior to the experiment, drug solutions were made and dissolved into Tyrode’s solution to yield their respective final concentration. Some chemicals were not soluble in water and dissolved in appropriate solvents, table 2.2.
Table 2.1. Composition of the physiological, Tyrode’s, Krebs’ and HEPES buffer solution including reagents, final concentrations and manufacturing company.

<table>
<thead>
<tr>
<th>Tyrode’s solution</th>
<th>Concentration (mM)</th>
<th>Manufacture</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>118</td>
<td>Sigma-Aldrich Company Ltd. (UK)</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>24</td>
<td>VWR (UK)</td>
</tr>
<tr>
<td>KCl</td>
<td>4</td>
<td>VWR (UK)</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1</td>
<td>VWR (UK)</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>0.4</td>
<td>VWR (UK)</td>
</tr>
<tr>
<td>Glucose</td>
<td>6.1</td>
<td>VWR (UK)</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>5</td>
<td>Sigma-Aldrich Company Ltd. (UK)</td>
</tr>
<tr>
<td>CaCl₂.6H₂O</td>
<td>1.8</td>
<td>VWR (UK)</td>
</tr>
</tbody>
</table>

**HEPES buffered solution**

To the above reagents add:

| HEPES          | 5 or 10           | Sigma-Aldrich Company Ltd. (UK) |

<table>
<thead>
<tr>
<th>Krebs’ solution</th>
<th>Concentration (mM)</th>
<th>Manufacture</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>118.3</td>
<td>Sigma-Aldrich Company Ltd. (UK)</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>24.9</td>
<td>VWR (UK)</td>
</tr>
<tr>
<td>KCl</td>
<td>4.7</td>
<td>VWR (UK)</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>1.15</td>
<td>VWR (UK)</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.15</td>
<td>VWR (UK)</td>
</tr>
<tr>
<td>D-glucose</td>
<td>11.7</td>
<td>VWR (UK)</td>
</tr>
<tr>
<td>CaCl₂.6H₂O</td>
<td>1.8</td>
<td>VWR (UK)</td>
</tr>
</tbody>
</table>
Table 2.2. Preparation and final concentration of drugs and chemicals used for each experiments. N/A represents no dilution needed.

<table>
<thead>
<tr>
<th>Chemical/drug name</th>
<th>Experimental use</th>
<th>Solvent</th>
<th>Final concentration</th>
<th>Manufacture</th>
</tr>
</thead>
<tbody>
<tr>
<td>4’,6-diamidino-2-phenylindole (DAPI)</td>
<td>Nuclear fluorescent stain</td>
<td>PBS</td>
<td>0.1 μg/ml</td>
<td>Sigma-Aldrich Company Ltd. (UK)</td>
</tr>
<tr>
<td>α,β-Methyleneadenosine 5’-triphosphate lithium salt (ABMA)</td>
<td>P2X₁ desensitiser</td>
<td>dH₂O</td>
<td>3 μM</td>
<td>Sigma-Aldrich Company Ltd. (UK)</td>
</tr>
<tr>
<td>Adenosine diphosphate (ADP)</td>
<td>P2Y agonist</td>
<td>dH₂O</td>
<td>30 μM</td>
<td>Sigma-Aldrich Company Ltd. (UK)</td>
</tr>
<tr>
<td>Adenosine 5’-triphosphate disodium salt hydrate (ATP)</td>
<td>Standard curve for ATP measurements</td>
<td>dH₂O</td>
<td>10 pM - 0.1 μM</td>
<td>Sigma-Aldrich Company Ltd. (UK)</td>
</tr>
<tr>
<td>ATP assay mix containing luciferin and luciferase</td>
<td>Quantifying ATP samples</td>
<td>Sterile H₂O</td>
<td>4 or 10 times dilution</td>
<td>Sigma-Aldrich Company Ltd. (UK)</td>
</tr>
<tr>
<td>Chemical Description</td>
<td>Description</td>
<td>溶剂</td>
<td>Concentration</td>
<td>Supplier</td>
</tr>
<tr>
<td>-------------------------------------------------------------------------------------</td>
<td>----------------------------------</td>
<td>---------</td>
<td>---------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>(E)-3-(4-t-butylphenyl)-N-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl) acrylamide (AMG9810)</td>
<td>TRPV&lt;sub&gt;1&lt;/sub&gt; antagonist</td>
<td>DMSO</td>
<td>0.3 μM</td>
<td>Sigma-Aldrich Company Ltd. (UK)</td>
</tr>
<tr>
<td>Capsazepine (CPZ)</td>
<td>TRPV&lt;sub&gt;1&lt;/sub&gt; antagonist</td>
<td>Methanol</td>
<td>3 μM</td>
<td>Sigma-Aldrich Company Ltd. (UK)</td>
</tr>
<tr>
<td>Carbamoylcholine chloride (CCh)</td>
<td>M&lt;sub&gt;1.5&lt;/sub&gt; agonist</td>
<td>dH&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>1 μM</td>
<td>Sigma-Aldrich Company Ltd. (UK)</td>
</tr>
<tr>
<td>Coaptite</td>
<td>Inert bulking agent</td>
<td>N/A</td>
<td>N/A</td>
<td>Boston scientific</td>
</tr>
<tr>
<td>DPX mountant</td>
<td>Histology</td>
<td>Distyrene and plasticiser dissolved in toluene and xylene</td>
<td>N/A</td>
<td>Sigma-Aldrich Company Ltd. (UK)</td>
</tr>
<tr>
<td>Eosin</td>
<td>Histology</td>
<td>Dissolved in dH&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>1%</td>
<td>Sigma-Aldrich Company Ltd. (UK)</td>
</tr>
<tr>
<td></td>
<td>Application</td>
<td>Dissolved in</td>
<td>Concentration</td>
<td>Supplier</td>
</tr>
<tr>
<td>----------------</td>
<td>--------------------------------------</td>
<td>-----------------------</td>
<td>---------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Histology</td>
<td>dH₂O</td>
<td>50, 70, 85, 90, 96, 100%</td>
<td>Sigma-Aldrich Company Ltd. (UK)</td>
</tr>
<tr>
<td>Formalin</td>
<td>Tissue preservative</td>
<td>Neutral buffered with 4% formaldehyde</td>
<td>10%</td>
<td>Sigma-Aldrich Company Ltd. (UK)</td>
</tr>
<tr>
<td>Ehrlich’s haematoxylin</td>
<td>Histology</td>
<td>Dissolved in H₂O, ethanol, glycerol, glacial acetic acid</td>
<td>7 g/l</td>
<td>Sigma-Aldrich Company Ltd. (UK)</td>
</tr>
<tr>
<td>Hydrochloric acid (HCl)</td>
<td>Histology</td>
<td>Dissolved in dH₂O</td>
<td>1%</td>
<td>Sigma-Aldrich Company Ltd. (UK)</td>
</tr>
<tr>
<td>Paraffin wax</td>
<td>Histology</td>
<td>N/A</td>
<td>N/A</td>
<td>VWR international Ltd. (UK)</td>
</tr>
<tr>
<td>Phosphate buffered saline (PBS)</td>
<td>Urothelial cell isolation, Immunohistochemistry</td>
<td>Dissolved in dH₂O</td>
<td>10%</td>
<td>Oxoid (UK)</td>
</tr>
<tr>
<td>Polyethylene glycol (PEG) grade 600</td>
<td>Inert bulking agent</td>
<td>Melted in warm H₂O</td>
<td>N/A</td>
<td>Sigma-Aldrich Company Ltd. (UK)</td>
</tr>
<tr>
<td><strong>Scott’s tap water</strong></td>
<td><strong>Histology</strong></td>
<td><strong>Diluted in dH₂O</strong></td>
<td><strong>10x dilution</strong></td>
<td><strong>Sigma-Aldrich Company Ltd. (UK)</strong></td>
</tr>
<tr>
<td>----------------------</td>
<td>--------------</td>
<td>---------------------</td>
<td>------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td><strong>Toluene</strong></td>
<td><strong>Histology</strong></td>
<td><strong>N/A</strong></td>
<td><strong>N/A</strong></td>
<td><strong>VWR international Ltd. (UK)</strong></td>
</tr>
<tr>
<td><strong>Triton x-100</strong></td>
<td><strong>Immunohistochemistry</strong></td>
<td><strong>dH₂O</strong></td>
<td><strong>0.05%</strong></td>
<td><strong>Sigma-Aldrich Company Ltd. (UK)</strong></td>
</tr>
<tr>
<td><strong>Trypan blue</strong></td>
<td><strong>Urothelial cell isolation</strong></td>
<td><strong>N/A</strong></td>
<td><strong>N/A</strong></td>
<td><strong>Sigma-Aldrich Company Ltd. (UK)</strong></td>
</tr>
<tr>
<td><strong>Trypsin EDTA</strong></td>
<td><strong>Urothelial cell isolation</strong></td>
<td><strong>Dissolved in dH₂O</strong></td>
<td><strong>10%</strong></td>
<td><strong>Sigma-Aldrich Company Ltd. (UK)</strong></td>
</tr>
<tr>
<td><strong>Wheat germ agglutinin</strong></td>
<td><strong>Cell membrane fluorescent stain</strong></td>
<td><strong>PBS</strong></td>
<td><strong>0.2 μg/ml</strong></td>
<td><strong>Life Technologies (UK)</strong></td>
</tr>
<tr>
<td><strong>Xylene</strong></td>
<td><strong>Histology</strong></td>
<td><strong>N/A</strong></td>
<td><strong>N/A</strong></td>
<td><strong>Fisher Scientific (UK)</strong></td>
</tr>
</tbody>
</table>
2.2 *In vitro* contractility.

2.2.1 Animal tissue preparation.

For *in vitro* contractility experiments, male or female pig bladders, weighing 45 ± 12 g were obtained from a local abattoir and transported for ~45 minutes in ice-cold gassed Tyrode’s back to the laboratory. The bladder was prepared by dissecting sagitally through the ventral face of the bladder wall and pinned out on a Sylgard dissection dish (*figure 2.1*). The trigone of the bladder was removed and discarded. The bladder dome was used in all *in vitro* experiments. The bladder was stored in fresh cold (4°C) gassed Tyrode’s solution for no longer than 28 hours during experimental use.

Isolated bladder strips were prepared as intact (mucosa and detrusor), mucosal, or detrusor strips. For intact preparations, longitudinal sheets were dissected from the bladder wall (10 x 20 mm). For mucosal and detrusor strips (2 x 5 mm), the detrusor and mucosa of the bladder wall was removed respectively using a microscope (Nikon SMZ1, Japan). Loops of suture were made at both ends of the mucosal or detrusor strip.
Figure 2.1. Photograph of an opened pig bladder (not to scale). The bladder shows the urothelial face of the bladder wall in a sylgard dish of Tyrode’s solution. Segments of the bladder wall were dissected from the bladder dome (above dotted line).

2.2.2 Tissue bath.

Tissue preparations were mounted in a horizontal tissue bath superfused with gassed Tyrode’s solution (~37°C). For intact bladder sheets, preparations were mounted in a large tissue bath by using clamps which held ~50 ml solution. One end of the tissue was attached to a Grass force-displacement transducer FT03 (Quincy, MA, USA) and the other to a fixed clamp on the tissue bath itself (figure 2.2). Preparations were superfused by a constant flow of physiological solution using a peristaltic pump (12.5 ml.min⁻¹). Superfusate was kept in a heated water bath (45.5 ± 0.5°C) to obtain a tissue bath temperature of 36.8 ± 0.8°C.
Figure 2.2. Photograph of the tissue bath used for intact bladder sheets (not to scale).
Wide view of the horizontal tension setup. The constant flow of physiological solution from the inlet was allowed to drain freely through a drainage system at the end of the tissue bath. A ceramic block was placed at the end of the tissue bath to allow the solution to rise to the desired volume in order to cover the whole tissue. The force transducer is attached to a clamp that measures isometric contractions from the bladder sheet.

For mucosal and detrusor strips, each end of the tissue strip was attached to hooks on the tissue bath. One end was attached to a Grass force-displacement transducer FT03 (Quincy, MA, USA) and the other to a static fixation hook using pre-tied loops. Superfusate was kept in a heated water bath (39.5 ± 0.5°C) to obtain a tissue bath temperature of 37.2 ± 0.4°C (figure 2.3).
Figure 2.3. Photograph of the tissue bath used for mucosal and detrusor strips (not to scale). Bladder strips were hooked with the trough of the tissue bath superfused continuously with Tyrode’s solution (heated in a water bath) and drained at the end of the tissue bath. Isometric force was measured by the force transducer, conditioned with a bridge amplifier and digitised before stored and displayed on a computer interface. Electrical stimulation was generated through platinum electrodes either side of the tissue strip.

For both setups, the tubing of the superfusate was enclosed with a circulating water-jacket at a temperature the same as the water bath.

2.2.3 Measuring isometric force.

Isometric force generated by bladder preparations was measured with a force transducer. The signal was transmitted to a WPI transbridge 4M bridge amplifier (Sarasota, FL, USA), and digitised (10 kHz) using an analogue-to-digital converter (Axon instruments digidata 1200 or Powerlab/800). Digital representations of bladder force was displayed on a
personal computer using either AxoScope version 9.0.2.03 (Axon instruments, Inc. USA) or Labchart (Chart4). Signals were filtered via a low-pass filter (corner frequency 5 Hz) to attenuate any higher frequency background noise.

Figure 2.4. Block diagram of in vitro contractility set up.
Force detected by the force transducer is signalled and amplified by a bridge amplifier. This analogue signal is digitised and displayed as a digital signal on a computer.

2.2.4 Calibration of force transducer.
For the above system, a voltage reflection was obtained from known weights (g) suspended from the force transducer to generate (figure 2.5).
Figure 2.5. Calibration curve of tension. Voltage output of the bridge amplifier plotted against known weights (grams) to obtain a calibration of force of known gram weight.

For experiments using LabChart, calibration was obtained by a voltage reflection of a known weight (g wt). All measurements of force was expressed as Newtons from the relationship, \( F=mg \) (\( g=9.8 \text{ m.s}^{-2} \)). Calibration was obtained prior to each experiment in LabChart.

2.2.4 Calibration of thermistor probes.

Thermistor probes (Omega engineering. Hypodermic needle probe specifications: needle diameter, 0.2 mm. Thermocouple: Chromega®-Alomega®, 33 gage, 25 mm length, continuous temperature rating of 200°C) were used to record temperature in the superfusate and in the suburothelial space.
The principle of measurements is the same as that used with force transducers where the probe is a variable resistor in a bridge amplifier (figure 2.6). The voltage output of the thermistor bridge amplifier was obtained for known temperatures of dH$_2$O to generate a calibration curve.

![Figure 2.6. Calibration curve of thermistor probes.](image)

Voltage output from thermistor bridge amplifier as a function of water temperature.

Thermistors were routinely calibrated prior to each experiment with a two-point measurement between 37 and 50ºC to check the calibration slope.

2.2.5 Modalities of bladder contraction.

Bladder contractions were elicited by three different physiological mechanisms:

1. Spontaneous contractions
2. Agonist-induced contractions
3. Electrically-stimulated contractions (EFS-induced contractions)
2.2.5.1 Spontaneous contractions.

All bladder preparations were stabilised for 60 minutes before any interventions. Before this stabilisation period, strips were firstly pre-stretched (9.8 or 49.0 mN; denuded/mucosal or intact preparations) three times to generate a resting baseline tension and pre-stimulate the tissue. Preparations were then exposed to pre-treatment of CCh (1 μM) for 10 minutes unless otherwise stated. CCh was given to enhance spontaneous contractions as some preparations lacked any generation of spontaneous activity. CCh was washed out after 10 minutes and then allowed to stabilise for 60 minutes. Spontaneous contractions were measured as oscillations of force around a baseline value.

2.2.5.2 Agonist-induced contractions.

CCh was used to increase smooth muscle contractions, as a rise in baseline tension. A test concentration of 1 μM CCh was used for every experiment, and is a submaximal concentration (Sadananda et al., 2008). A maximal concentration of about 10 μM was not used to ensure the tissue remained unfatigued and this also reduced washout times.

2.2.5.3 Electrically-stimulated contractions.

Electrical field stimulation (EFS) was used to induce nerved-mediated contractions. EFS was achieved using a Grass S44F stimulator (Quincy, Mass, USA) coupled to a NeuroLog system (Digitimer, Welwyn Garden City, UK) and generated through a pair of platinum electrodes at either side of the tissue bath. Stimulation was carried out using 0.10 ms 40 V square wave pulses at 10 Hz for 3 seconds periods every 90 seconds. EFS was administered throughout the whole experiment when used.
2.2.6 Methods of heating the preparation.

For intact preparations, a heating coil was placed immediately above the preparation and used to radiate localised heat. The heating coil was made from nichrome wire (diameter 0.4 mm; Coils: 7; Resistance: 3.2Ω/ft) attached to a variable voltage output. Temperature was measured using thermistor probes; one probe was placed in the sub-urothelial layer of the preparation and the other at the end of the tissue bath (**figure 2.7**).

**Figure 2.7. Close-up of the method of heating (heating coil).**
The heating coil is placed immediately above the urothelial surface of the bladder. A thermistor probe is inserted into the sub-urothelial layer of the bladder immediately below the heating coil.

For isolated mucosal and detrusor preparations in the horizontal tissue bath (**figure 2.3**), heating was induced by raising the superfusate temperature to reach a tissue bath temperature of up to 50°C. The superfusate temperature was measured using a hand-held temperature probe, placed immediately before the tissue strip.
2.2.7 Changes to superfusate pH during heating.

Preliminary experiments were done to determine any changes to superfusate pH when it was heated or exposed to radiant heat. At higher temperatures (50°C) there was an increase of 0.28 ± 0.03 in pH from the initial controlled Tyrode’s solution in the water bath at 37°C, using either methods of heating. The use of a HEPES-buffered Tyrode’s solution did not prevent changes to pH during heating. Therefore, control experiments when the pH of Tyrode’s solution was increased by 0.3 pH units were carried out by titrating gassed Tyrode’s solution with 1M NaOH at 37°C.

2.2.8 Temperature gradient across the bladder wall during heating.

Monitoring of the temperature gradient across the bladder wall was examined to determine the degree of heating across the bladder wall (figure 2.8).

Figure 2.8. Measurement of temperature gradient.
Numbers indicate temperature (°C) recorded using thermistor probes at specific regions within the bladder wall.
2.2.9 Measurements of bladder contractions.

All bladder contractions were measured during an offline recording using customised algorithms with Clampfit (molecular devices, USA) or LabChart (AD Instruments, UK) – see below.

2.2.9.1 Spontaneous contractions.

For spontaneous contractions, individual contractile variables measured were: area under the curve (AUC), amplitude, and duration. The frequency of contractions was measured over a 10-minute period by counting the number of distinct contractions (figure 2.9).

Figure 2.9. Spontaneous contractile variables.
Data analysis of spontaneous contractions showing the frequency of contractions over 10 minutes, and averaged area under the curve (AUC), amplitude, and contraction duration over the same time interval.
2.2.9.2 Agonist-induced contractions.

For agonist-induced contractions, the amplitude of contraction was measured at the maximal point during a 10 minute exposure to the agent (figure 2.10).

![Figure 2.10. CCh-induced contraction.](image)

Data analysis of the amplitude of contraction from baseline.

2.2.9.3 EFS-induced contractions.

For EFS-induced contractions, 5 contractions were analysed for the AUC, amplitude, and 50% relaxation of individual contractions. For the AUC and amplitude, a computer-based algorithm was used (figure 2.11).
Figure 2.11. EFS-induced contraction.
Data analysis for the AUC and amplitude of an EFS-contraction.

To estimate the time to 50% relaxation, the decline of force was modelled as an exponential decline. Tension values were transformed to the natural logarithm (ln) of their values (figure 2.12).

Figure 2.12. EFS-induced contraction.
Data analysis for the contracture to reach 50% relaxation.
The exponential phase of tension decline, \( T \), over time, \( t \), is modelled as: 
\[
T(t) = A . e^{-t/\tau},
\]
where \( A \) is the amplitude of the contraction and \( \tau \) is a characteristic time constant, that describes the time taken to decline from \( \approx 63\% \) of its original value (figure 2.13).

\[
T(t) = A . e^{-t/\tau} + c
\]

**Figure 2.13. Schematic curve of an exponential decline of tension showing the time to 50\% relaxation and the value of the time constant, \( \tau \).**

The equation to the right of the plot describes the decline of tension as a function of time. The constant \( c \) represents the final tension at large values of time.

The time-dependent fraction of tension decline can be linearised by transforming the above equation to: 
\[
\ln \frac{T(t)}{A} = -\frac{t}{\tau}.
\]

The function is one of a straight-line with slope \( -\frac{1}{\tau} \) (figure 2.14).
Therefore, to obtain the time to reach 50% relaxation:

\[
\ln 0.5 = \frac{t}{\tau}
\]

\[
t = \ln 0.5 \times \tau
\]

2.2.10 TRPV\textsubscript{1} antagonists.

The TRPV\textsubscript{1} channel antagonists, CPZ and AMG9810, were used at 3 µM and 0.3 µM, respectively. These concentrations were used for complete inhibition of TRPV\textsubscript{1} channels based previous literature. CPZ is a competitive TRPV\textsubscript{1} channel antagonist against the agonist capsaicin. AMG9810 was also used to confirm any effects from CPZ. More details about use of TRPV\textsubscript{1} antagonists will be discussed in chapter 3.
2.3 Ex vivo: The whole perfused pig’s’ bladder.

2.3.1 Whole pig bladder preparation.

For *ex vivo* experiments, female pig (*Sus scrofa domestica*, ~6 months old) bladders were obtained from the local abattoir immediately *post mortem*. At the abattoir, the bladder, distal caudal aorta and associated branch were carefully excised and stored on ice-cold Krebs’ solution (*figure 2.15*). Upon arrival at the laboratory, the distal aorta was cannulated using a 14-gauge needle (BD Vialon, UK) and perfused using a peristaltic pump (Watson Marlow 101U/R) at 10 ml.min\(^{-1}\). All redundant branches of the aorta were ligated.

![Figure 2.15. Photograph of the arterial supply to the bladder from the distal end of the pig abdomen.](image)

1) caudal vena cava; 2) caudal aorta; 3) left external iliac artery; 4) right external iliac artery; 5) internal iliac trunk; 6) left umbilical artery; 7) right umbilical artery; 8) bladder. B. Parsons, unpublished data.
A 14 Ch Foley catheter (Bard, UK) was passed into the bladder through the urethra and tied off to fill the bladder lumen with physiological solution. In addition, a 6 Ch urodynamic double-lumen catheter (Mediplus, UK) was introduced into the bladder via the left ureter and secured using a vicryl ligature (company and location); the right ureter was tied off to ensure isovolumic conditions. The double-lumen catheter (Mediplus, UK) was attached to a pressure transducer (MX960, Medex Inc., UK) to measure intravesical pressure changes. The bladder, once prepared, was enclosed in a heated water jacket set at the temperature the same as the water bath (figure 2.16).

**Figure 2.16.** Photographic image showing the *ex vivo* set up of whole perfused pig bladder. A) A custom-made organ bath for whole organ experiments; B) Close-up of whole perfused pig bladder.
2.3.2 Perfusion of the whole bladder.

For perfusion of the whole bladder, the blood vessel(s) branching from the distal aorta were perfused with heated, gassed Krebs’ solution. Vahabi et al 2012, validated the distribution of perfusate in the whole bladder through the arterial branches by the use of methylene blue dye (figure 2.17)(Parsons et al., 2012).

![Image of perfusion showing A) serosal staining and B) internal staining, and C) urothelium and sub-urothelium; D) detrusor smooth muscle](image)

**Figure 2.17. Distribution of methylene blue from the perfusate through the arterial supply.** Top tracings are photographs of the whole pig bladder showing, A) serosal staining and B) internal staining. Bottom tracings are H&E stained sections showing the spread of dye throughout the bladder wall: C) urothelium and sub-urothelium; D) detrusor smooth muscle (Parsons et al., 2012).

During interventions, the bladder lumen was filled with 150 ml Krebs’ solution (30 ml.min⁻¹) using a 60 ml syringe via the catheter placed through the urethra. Subsequent drainage of solution was via the same route.
2.3.3 Calibration of pressure transducer.

Fluid-filled catheters, connected to an external pressure transducer, were fixed at a pre-determined reference height. Signals were converted to a digital signal via an analogue-to-digital converter (Powerlab 8/30, AD Instruments, UK) and hydrostatic pressure was calibrated to atmospheric pressure prior to experimentation using LabChart (figure 2.18).

![Figure 2.18. Calibration of pressure transducer.](image)

A two-point calibration of hydrostatic pressure ranging from 0 – 10 cmH$_2$O.

2.3.4 Calibration of thermistor probes.

Temperature was recorded using thermistor probes (see section 2.2.6 for details of thermistor probes). These were placed in the organ chamber, the serosal layer of the bladder, and through the urethra into the lumen of the bladder. Calibration was obtained by a voltage deflection of known water-based temperatures and converted to °C. Calibration was done prior to each experiment.

2.3.5 Method of heating the bladder.

Heating was induced by changing the temperature of the perfusate. Maintenance of the temperature change was facilitated by additional small changes (up to 1°C) of water bath
temperature. Therefore, both the perfusate supplying the bladder vasculature and lumen were heated to ensure a stable temperature gradient across the bladder wall. For changes during the heating period, the perfusate was heated to reach \(42 \pm 1\degree C\) (bladder lumen) to maintain temperatures of \(42 \pm 0.5\degree C\) in the organ chamber itself and \(42 \pm 0.5\degree C\) as measured in the bladder serosa.

### 2.3.6 Measurement of intravesical pressure changes.

Intravesical pressure changes were monitored via a calibrated pressure transducer and data were digitised and represented in LabChart. All bladder contractions were measured during an offline recording using a LabChart algorithm. Pressure values were recorded in units of cmH\(_2\)O. For spontaneous pressure changes, individual contractile variables of AUC, duration, and amplitude were all measured. The frequency of contractions was measured over a 10 minute period (figure 2.19).
Figure 2.19. Data analysis of spontaneous intravesical pressure changes showing different contractile variables.
A) averaged AUC; B) amplitude; C) contraction duration of individual contractions; and D) frequency of contractions over 10 minute intervals.

2.5 Measurement of extracellular ATP.

ATP release from the urothelium was measured either in superfusate samples from the tissue bath or from the suspension medium containing isolated urothelial cells.
2.5.1 Isolation of urothelial cells.

2.5.1.1 Cell trypsinisation.

Bladder wall sections (10 x 20 mm) of the pig bladder was dissected and the mucosa carefully excised from the DSM for preparation isolating urothelial cells. The mucosa was fully stretched onto a Sylgard dish containing 5 ml of phosphate-buffered saline (PBS) with trypsin EDTA (TE) medium: 10% PBS, 10% TE, 80% dH₂O (PBS/TE). The mucosal layer containing PBS/TE was incubated for 40 minutes in a water bath set at 37°C.

2.5.1.2 Cell isolation and incubation.

Urothelial cells were dissociated from the mucosal surface using a 10 ml plastic Pasteur pipette to gently blow off the cells into a PBS/TE medium. The medium containing cells was extracted using the Pasteur pipette, placed in 10 ml falcon tubes and incubated for a further five minutes at 37°C. The mucosal layer was again digested with fresh 2.5 ml PBS/TE, incubated for five min and the cells isolated as above, this procedure was repeated twice. Aliquots of the cell suspension were pipetted into 1.5 ml Eppendorf tubes and centrifuged (Thermoscientific Heraeus Fresco 17, UK) five times for at least five minutes (five 1-minute spins at 500 g. Rotor: max. 21,100 xg; 14,800 rpm, 530 mm), or until a visible pellet of suspended cells is formed. The supernatant was removed from the Eppendorfs, leaving only the pellet of cells, and re-suspended with gassed, Tyrode’s solution into a single 1.5 ml Eppendorf. Resuspension was done in Tyrode’s, with or without 1.8 mM CaCl₂ according to the experimental protocol (see Results, section 3.2.5.5). The suspension was centrifuged a further two times at the same speed, replacing the supernatant with fresh Tyrode’s solution each time. Finally, a known volume (1.0-1.5
ml) of Tyrode’s solution was added to the Eppendorf containing the cells pellet and gently mixed to form a uniform suspension. Portions of the suspension (30 µl) were pipetted into 1.5 ml Eppendorf tubes and incubated for a minimum of 1.5 hours at 37°C prior to any interventions.

2.5.1.3 Cell counting.
Cells were counted at the end of the experiment using a bright-line improved Neubauer haemocytometer stage (Hausser Scientific, USA). The suspension of cells was mixed with trypan blue (20 µl trypan blue : 20 µl cell suspension). The haemocytometer was cleaned with 70% ethanol and a glass coverslip placed over the gridline. A portion (20 µl) of the suspension mixed in trypan blue was pipetted under the coverslip and examined through a 10x objective microscope (Motic AE30, Hong Kong). The total number of cells was counted: dead cells were evident as filled with trypan blue stain and were excluded to generate a final live cell count; the percentage of live cells was calculated.

2.5.2 Collection of superfusate from isolated bladder preparations.
Superfusate samples (100 µl) were taken directly above the tissue preparation from the urothelial surface (~2.5 mm from the surface). The point of sampling did not change and was always taken at the end of the tissue sheet in direction of the superfusate flow. This sample point minimised any biomechanical disturbances from the pipette but ensured sufficient released ATP could be collected (see Figure 2.20).
2.5.3 ATP concentration curve.

ATP samples of known concentrations, diluted in Tyrode’s solution, were used to produce a concentration curve. Nine concentrations were used in a logarithmic scale from 1 pM to 10 nM. Each concentration was measured three to four times and the result expressed as an average. The method to assay ATP is described below (see section 2.5.4). ATP values of samples were estimated from the calibration curve and expressed either as pmoles per gram dry tissue weight for contractility experiments (figure 2.21) and nmoles per cell for isolated-cell experiments (figure 2.22).
Figure 2.21. Algorithm plot of an ATP concentration curve in four time’s diluted luciferin-luciferase.


ATP concentration can be extracted from the ATP standard curve and by calculating the logarithm of [ATP] (nmoles/l) as a function of luminescence.

\[
\log(\text{luminescence}) = 0.656 (\log[\text{ATP}]) + 5.187
\]

Rearranging the formula will yield the [ATP].

\[
\frac{\log(\text{luminescence}) - 5.187}{0.656} = \log[\text{ATP}] (z)
\]

Thus, using inverse functions, [ATP] = $10^z$ nmoles/l
Figure 2.22. Algorithm plot of an ATP concentration curve in ten time’s diluted luciferin-luciferase.

2.5.4 ATP measurements: Luciferin-luciferase bioassay.

An ATP assay mix containing luciferin and luciferase was used to catalyse the breakdown of ATP: hydrolysis of each ATP molecule produced a photon of light. ATP samples containing luciferin-luciferase were measured using a GloMax 20/20 Luminometer (promega, UK). An ATP standard curve of known ATP concentrations was measured using 4 or 10 times diluted luciferin-luciferase assay mix, reconstituted in dH₂O. ATP values during experiments were converted to known concentration using an ATP standard curve.

2.6 Injection of inert bulking agents.

The in vitro experimental system described in section 2.2.6 was used to measure changes to bladder wall contractility with or without injectate of an inert agent below the mucosa.
The injectate was used as an external barrier between the urothelium and underlying cells to minimise communication between layers of the bladder wall.

2.6.1 Compounds used as bulking agents.

Two compounds were chosen as bulking agents for their low toxicity and inert structure: Polyethylene glycol (PEG) and coaptite®. Both substances have been certified for medical use. PEG is a polymer which varies in the chain length of its repeating unit, thus altering its physical properties, including its viscosity. PEG600 was used due to its low viscosity (10.8 cSt at 100°C) and ease of injection (liquid at 15-25°C). This compound exists as a chain of 600 ethylene glycol repeated structures (figure 2.23).

![Schematic structure of PEG600](figure 2.23. Schematic structure of PEG600. A polymer of ethylene glycol.)

Coaptite is an inert agent made of spherical particles of synthetic calcium hydroxyapatite, a compound naturally found in teeth and bone, in an aqueous carrier gel. This gel-like texture makes the injectate ideal for injections to provide a long-lasting static bulking agent as shown in a previous study (Hague and Fry, unpublished data).

2.6.2 Method of injecting into the sub-urothelium.

The bulking agents were injected into the sub-urothelium using a 23 gauge rigid needle on a 1 (coaptite) or 10 (PEG and Tyrode’s) ml syringe (figure 2.24). Tissue sheets were
taken from the superfusion bath and placed onto a Sylgard dish containing warm Tyrode’s solution (37°C). Identification of the layers of the bladder wall was by naked-eye visualisation which allowed injection direct into the sub-urothelium. After injection or no injection (null-injection), the tissue sheet was clamped backed to the tissue bath.

Figure 2.24. Coaptite injectate into the sub-urothelium. Injectate (0.2 ml) was delivered using a 23 gauge deflux needle through the sub-urothelial layer. A bleb of injectate was visible from the urothelial surface.

2.6.3 Confirmation of sub-urothelial injection.

Successful injection of the inert agents into the sub-mucosal layer was confirmed with haematoxylin & eosin staining. Injected agents were mixed with undiluted methylene blue particles for visual confirmation under a light microscope.

2.7 Immunohistochemistry, H&E and van Gieson techniques.

2.7.1 Tissue sample sectioning.

Bladder preparations (intact or detrusor) were fixed in 10% neutral-buffered formalin, containing 4% formaldehyde dissolved in dH2O solution for at least three days before
tissue processing. Bladder wall preparations were dehydrated in 70, 90 ethanol 96 and 100% absolute alcohol followed by a clearing process in toluene as a translucent material in replace of the dehydrant. This process allows the tissue to be miscible with paraffin wax. The sample is then impregnated in paraffin wax overnight, all of which is run through an automatic tissue processor (Shandon Citadel, Thermo Electron Corporation, UK). After the processing of fixed tissue, the preparation was embedded in molten wax and cooled to embed the tissue in a wax block before tissue sectioning. The wax block was cut using a microtome (Reichert Jung 2040 Autocut) into 5 μm sections (figure 2.25). Sections of the bladder wall were floated onto a warm water bath (40-50°C) and individually placed and dried upright on superfrost plus (VWR, UK) microscope slides before tissue staining.

Figure 2.25. Region of section cutting.
Intact bladder preparations were cut inward from the clamp indent. This region was exposed to the temperature of interest.
2.7.2 Deparafinisation and rehydration of tissue sections.

Wax embedded bladder sections were deparaffinised in histoclear solution twice for 30 minute exposures each. Following deparaffinisation, a rehydration process was achieved which consisted of exposing sections to decreasing concentration of alcohol. For immunofluorescence sections were rehydrated in 1 x 5 minutes (100% ethanol) followed by successive 3-minute rehydration in 95, and 70 ethanol, respectively until dH$_2$O. Bladder sections were left in distilled water to prevent the sections drying out. For histological staining, sections were rehydrated decreasing alcohol solutions (100, 70, 50%) and then dH$_2$O, for 1-minute intervals.

2.7.3 Haematoxylin & Eosin staining.

H&E staining was used to examine the structural integrity of the bladder wall. For tissue staining, haematoxylin stains the nucleic acid enclosed in the nucleus a blue colour, whilst eosin stains the cytoplasm and extracellular matrix a red colour. Once sections were rehydrated, slides were transferred and incubated in Ehrlich’s haematoxylin solution for 10-15 minutes. The sections were gently rinsed under running tap water (1 minute) and differentiated in 1% HCl : 70% alcohol for 5-10 seconds. The sections were washed in Scott’s tap water for 15 seconds to remove excess haematoxylin. Successful staining was examined under a light microscope to ensure efficient washing of haematoxylin from cellular compartments other than the nuclei using a light microscope (Vickers M17, Vickers instruments Ltd, UK). Slides were then counterstained with 1% eosin solution (Sigma) for 2 minutes, rinsed briefly under tap water (1 minute) and dehydrated in incremental alcohol concentration (85, 100, 100%) for 30 seconds each. The sections were then cleared in two absolute xylene solutions for 30 seconds each and a coverslip
placed over the section using DPX mountant (company: Sigma. Composition: distyrene, a plasticiser, and xylene mixture) to preserve the stained section. Sections were left in xylene to prevent tissue drying out prior to mounting of the coverslips. Sections were left to dry and photographed with a charge-coupled device camera (CCD) camera-attached wide-field microscope.

2.7.4 van Gieson staining.

van Gieson staining was used to examine the collagen and elastin components of the bladder wall. Collagen are stained as bright red, muscle and elastin (red blood cells) are stained as yellow, and the nuclei is blue. Prior to any staining, sections were deparaffinised and rehydrated to water (see section 2.7.3). Sections were stained in haematoxylin solution for 8-10 minutes and washed in running tap water for 5 minutes. Slides were then stained in van Gieson stain for 5 minutes where they were dehydrated, cleared and mounted with a cover slip as in section 2.7.3. Sections were left to dry and photographed with a CCD camera-attached wide-field microscope.

2.7.5 Immunohistochemistry.

Immunohistochemistry was used to examine cell death as a result to heating. Before any immunostaining, paraffin embedded samples were deparaffinized and rehydrated for successful staining (see section 2.7.2).

Immunostaining. Sections were stained with the fluorescently tagged antibody wheat germ agglutinin (WGA) that recognises and binds to sialic acid and N-acetylglucosaminyl residues, commonly found on cell membranes. 4',6-diamidino-2-phenylindole (DAPI)
was used as a fluorescent stain that strongly binds to adenine-thymine rich base pairs regions in DNA. Fluorescent tags were excited at different wavelengths and fluorescence emitted which is later detected using a laser scanning inverted microscope.

WGA (0.2 μg/ml) was prepared in 0.01 M PBS (composition: NaCl 8.0 g/l, KCl 0.2 g/l, Na₂HPO₄ 1.15 g/l, KH₂PO₄ 0.2 g/l) containing 1% bovine serum albumin and 0.05% Triton x-100. Sections were incubated with 200 ml of WGA containing PBS composite for 1 hour in a slide-staining glass trough. After incubation, slides were washed briefly (1-2 minutes) in PBS (0.01 M) prior to DAPI staining. DAPI (0.1 μg/ml) was diluted in 200 ml PBS (0.01 M) and incubated for 5 minutes. Post-DAPI staining, sections were thoroughly washed in PBS three times for 5, 10, and 10 minutes. All staining was done in darkness using aluminum foil. Slides were removed from PBS solution and dried using a pre-heated oven (≤37°C) for no longer than 10 minutes. Cover slips were mounted onto the slides using fluoroSave solution (Merck Millipore, UK). Sections were left to dry in a dark room covered in foil until examined on a confocal microscope (see section 2.7.6).

2.7.6 Microscopy.

**H&E and van Gieson.** Colour photographs were taken using a wide-field microscope (Leica Microsystems DM LB2) at 5, 20, or 63x objective using a CCD camera (Leica DFC450C: 1280 x 960 pixels). Image brightness and saturation were manually adjusted using Leica control software and images taken with ~25% overlapping between images. Single images were merged to yield an individual photograph of the bladder wall using Photoshop (Adobe Creative Suite 5, CS5 for Macintosh). Additional adjustments to the overall photograph brightness and saturation was done in Photoshop ensuring the overall
quality of the bladder integrity was not lost. Final figures were put together using Illustrator (Adobe CS5 for Macintosh) to ensure no disruption in scaling or loss of picture quality. Scale bars for bladder sections were done in ImageJ (Fiji for Macintosh) using a calibration scale (pixels/μm) (figure 2.26-2.28).

Figure 2.26. 0.1 mm calibration scale at 5x objective.

Figure 2.27. 0.05 mm calibration scale at 20x objective.

Figure 2.28. 0.01 mm calibration scale at 63x objective.
**Immunofluorescence.** Confocal laser scanning microscopy (Leica Microsystems LAS AF – TCS SP5) was used to detect fluorescence emitted as a result of excitation of fluorophores. A laser beam (350-700 nm) is shone through the aperture of the optical system and reflected by a dichroic mirror onto the specimen to excite the fluorophore. Fluorescent light of higher wavelength light is transmitted through a pin-hole to eliminate out-of-focus light rays and collected by a photomultiplier tube (PMT) to amplify the fluorescent light. The analogue output of the PMT was digitised and recorded on the computer.

DAPI has a maximum excitation wavelength at 358 nm and fluoresces at a maximum wavelength of 461 nm, in the visible blue region. WGA was conjugated with Alexa Fluor® 594 which has a maximum excitation wavelength at 590 nm and fluoresces at a maximum wavelength of 617 nm, in the visible red region.

For all images, a z-stack was used to obtain the optimum section, using different focal distances. From this the minimum and maximum depths of each section was obtained and the intermediate region of the z-stack taken for analysis for every position along the bladder wall. With intact preparations, the middle section was always taken, whereas with detrusor preparations, a region completely surrounded by smooth muscle was taken. Images were taken by a CCD camera between 300-500 µm in unit area.
2.7.7 Analysis of DAPI staining.

For intact preparations, the urothelium, sub-urothelium and detrusor smooth muscle were analysed to examine tissue integrity and changes in the nuclei count. For all analytical processes, the threshold of each image was altered for optimal nuclei imaging using ImageJ (Fiji for Macintosh OS X and Microsoft Windows). Images were not over-exposed to prevent false positive readings of nuclei.

For detrusor preparations, segments of detrusor smooth muscle were analysed in the same unit area.

2.7.7.1 Examination of urothelial integrity.

Three methods for changes in urothelial integrity were examined:

- Urothelial length
- Urothelial width
- Nucleus size

Urothelial length. The total length of the urothelium was recorded against the total intact length of the urothelium (figure 2.29). An intact urothelium was regarded as obvious lining of the bladder wall of 2 or more cells. Difficulty in differentiation between intact and non-intact urothelium was excluded from the final intact count.
Figure 2.29. Urothelial length analysis.
Analysis of the total length of intact urothelium (yellow line).

Urothelial width. The urothelial width was measured in four segments of the urothelium.
Only regions that showed an intact urothelium were analysed. Those sections that did not show an intact urothelial surface were counted as zero (figure 2.30).

Figure 2.30. Urothelial width analysis.
Analysis of the width of intact urothelium was taken at different regions along the surface (yellow line).
Nuclei size. The nuclei size of the urothelium were measured. Six nuclei were counted in the region of intact urothelium (figure 2.31).

Figure 2.31. Nuclei size analysis.
Analysis of the area of six nuclei are shown. Yellow lines indicate the position of analysis.

2.7.7.1 Sub-urothelium and detrusor nuclei count.

For sub-urothelial and detrusor nuclei count, individual counting of the nucleus was done. Automatic counting could not be achieved due to inaccurate differentiation between overlapping nuclei. The area of the selected regions of interest were the same (1518 x 1152 pixels).
2.9 Data analysis.

2.9.1 IVC.

All data analyses, including graphical presentations of the data and mathematical functions, were performed on a personal computer using pClamp (2003, version 9 for Windows), LabChart (2014, version 8.0 for Windows), Microsoft Excel (2013, professional for Windows) and Graphpad Prism (2014 for Windows).

2.9.2 Spontaneous contractions.

Isometric contractions elicited by spontaneous contractions were measured at the end of each intervention in 10-minute time frames, as described above (see section 2.2.9.1). Data sets were all normalised to their respective pre/post-control average values unless otherwise stated.

For all data analyses, the basal tone (tonic contraction that results in changes in the baseline of a physiological tracings) was recorded as a separate variable.

2.9.3 EFS-induced contractions.

The average of five EFS-induced contractions were all normalised to pre/post-control average values. For recovery experiments, the time constant ($\tau$) was used to denote a time over which contractile strength will recover to 63% of steady-state tension. The following equation was used, where $T$ is tension and $t$ is time (figure 2.32):
Figure 2.32. Schematic curve plot of the recovery of EFS post-heating.

The equation to the right of the plot was used to extract a time constant for the recovery of EFS over a given time. Firstly to obtain a value, the equation is extrapolated into a straight line plot using logarithms.

To rearrange in the format where the slope = -1/τ:

\[
\frac{y(t)}{A} = (1 - e^{-\frac{t}{\tau}})
\]

\[
(1 - \frac{y(t)}{A}) = e^{-\frac{t}{\tau}}
\]
Thus the natural logarithm of the above equation to produce a linear plot (figure 2.33):

\[
\ln \left(1 - \left(\frac{y(t)}{A}\right)\right) = -\frac{t}{\tau}
\]

Figure 2.33. Linear plot extrapolated from the curve plot using algorithms. The natural log of a curve plot was used to extract a time constant (slope) for the recovery of EFS.

The slope of the linear plot will therefore be \(-1/\tau\).

2.9.4 CCh-induced contractions.

For CCh-induced contractions, data sets were normalised to the initial pre-control (before interventions).

2.9.5 ATP analysis.

2.9.5.1 Superfusate.

Superfusate samples were taken at different time points throughout the sampling protocols (see Heating, section 3.2.5). Each sample was read in the luminometer three times and averaged to obtain a single light emission value (section 2.5.4). These values were converted to ATP concentrations from the calibration curves (section 2.5.3).
experimental interventions, three consecutive samples from the superfusate were taken and averaged as a single pre-control value. Subsequent samples were all normalised to the pre-control value.

2.9.5.2 Isolated cell suspensions.
Suspension samples (20 µl) were taken from each cell suspension after incubation with interventions or control medium. Samples were read at least three times and averaged to generate a single ATP value.

2.9.6 Fluorescent studies.
For fluorescent studies, the urothelial, sub-urothelial, and detrusor smooth muscle integrity was analysed. Urothelial integrity was analysed as:

1. % of intact urothelium
2. % urothelial width against control
3. % nuclei size against control

Sub-urothelial and detrusor smooth muscle were analysed as:

4. Nuclei count per unit pixel
2.10 Statistical analysis.

Comparison of data sets used non-parametric statistical comparisons as some data sets were not normally distributed. Data sets are thus described as median values [25%, 75% interquartiles]. Data sets were compared using one-way ANOVA. Post-hoc comparison of pairs of data sets used Wilcoxon (for rank comparison) or Dunns (for repeated measures) tests for paired comparisons. Mann-Whitney U-tests was used for any unpaired comparisons. The association between two variables were tested using Spearman’s rank correlation test. The null hypothesis was rejected at p<0.05 and significance denoted by * p<0.05, ** p<0.01, *** p<0.001, with appropriate symbols as indicated.
Chapter 3. Heating from 37°C to 50°C and pig bladder contractility.
3.1 Introduction

Heating is a potential therapeutic paradigm to reduce spontaneous bladder contractions as evidenced from its ability to alter and preserve muscle contractile function (Everett et al., 2001, Mustafa et al., 2004, Yellon et al., 1992)(see Introduction, section 1.7.1). However, there is little published data regarding the effects of heating on changes to bladder contractility. It is well-established that some TRP channel subtypes (see Introduction, section 1.7.1) are activated during heating. Therefore, heating the bladder wall may alter contractility via TRP channel activation.

3.1.1 Heat-sensitive TRP channels

Of the many TRP channel subtypes the TRPV sub-family, in particular TRPV₁, is activated during heating to 42-50°C. Activation of TRPV₁ channels permits influx of Ca²⁺ in cells (see Introduction, section 1.4.2) that may modulate muscle contractility. In addition, TRPV₁ channels can evoke the release of mediators such as NO and ATP (Birder et al., 2012, Nilius et al., 2007). The production of NO via TRPV₁ channels was shown by an increased eNOS activity. This activity is largely dependent on free [Ca²⁺], but also direct actions from TRPV₁ channels itself (Fleming et al., 1997, Ching et al., 2011). The increased production of NO can lead to relaxation via a cGMP-dependent mechanism activating MLCP. Another possible role for these mediators in the bladder is to activate afferent nerves, that could in turn modulate efferent nerve function (Andersson and Arner, 2004, Fry et al., 2010, Nilius et al., 2007, Yamada et al., 2009, Venkatachalam and Montell, 2007, Birder and Andersson, 2013, Avelino et al., 2013). In addition, these mediators may diffuse directly to the detrusor smooth muscle to alter contractile function.
TRPV channels play a role in various bladder disorders, in particular they have been implicated as a therapeutic target against neurogenic bladder disorders, including DO. Capsaicin-activated TRPV$_1$ channels modulate afferent nerve firing; repeated exposure to capsaicin desensitized the receptor which subsequently abolished afferent firing (Fowler et al., 1992). Capsaicin however has been withdrawn from clinical trials due to intense abdominal pain generated during the initial excitation of afferent firing. An analogue of capsaicin, resiniferatoxin (RTX), was designed as an alternative agent and was shown to be effective in reducing pain in patients with bladder pain syndrome, including interstitial cystitis (Caterina et al., 1997). In addition, RTX also desensitised TRPV$_1$ receptors thus making it a potentially useful therapeutic against neurogenic DO (Fowler et al., 1992, Caterina et al., 1997, Brady et al., 2004, Apostolidis et al., 2005). However, RTX was also discontinued due to difficulty in its administration to patients; it was subsequently found that it was absorbed strongly by the materials in infusion catheters.

3.1.2 The induction of cell death during heating

Thermal stimulation of tissue or cells can lead to apoptotic, or in extreme cases, necrotic responses. Hyperthermic conditions severely affect protein structure and function and even small increases in temperature to 40-45°C can lead to their unfolding and exposure of hydrophobic groups, that can form protein aggregates. This effect is reversible due to the presence of chaperones including heat shock proteins (HSP) (Stege et al., 1994, Kampinga et al., 1994, Roti Roti, 2008, Sapareto et al., 1978). Therefore, mild thermal stimulation may lead to temporary alterations in protein folding, including contractile proteins. At temperatures $\geq$46°C there may be a more detrimental effect on cells and
tissues, when apoptosis will predominate (Majno and Joris, 1995, Roti Roti, 2008). At even higher temperatures (≥50°C) cellular necrosis will be more profound, as there is denaturation of proteins and DNA (Roti Roti, 2008, Lewis, 1977, Dewey et al., 1971, Sapareto et al., 1978).

3.1.3 Objectives

The objectives for characterising the effects of heating on the reduction of spontaneous contractions are:

1. In vitro experiments:
   a. To determine and explore the reduction of spontaneous contraction during heating to 42, 46, and 50°C.
   b. To determine whether heating is dependent through TRPV$_1$ channels activation.
   c. To measure the release of ATP from the mucosa or isolated urothelial cells during heating.

2. Ex vivo experiments:
   a. To characterise the most suitable model for reducing spontaneous contractions (whole perfused pigs bladder).

3. Histological experiments:
   a. To examine changes to tissue integrity during heating.
3.2 Methods.

A full description of the methods is in chapter 2. Here is a description of the experimental designs.

3.2.1 Heating as a means to modulate bladder spontaneous contractions.

All experiments were pre-treated with 1 µM CCh unless otherwise stated. After the stabilisation period and generation of spontaneous contractions, the experimental protocol could proceed.

3.2.1.1 Protocol 1: Effect of 42, 46, and 50°C on spontaneous contractions.

This protocol was designed to examine the effect of varying temperatures from 37°C to up to 50°C on spontaneous contraction variables (figure 3.1).

![Figure 3.1](image)

**Figure 3.1. Heating from 37°C to 42, 46, 50°C on spontaneous contractions.**

Protocol design to examine the effect of varying temperature on spontaneous contraction variables. After an initial 60 minute control period (CCh pre-treatment), the sub-mucosal temperature was raised to 42°C for 15 minutes followed by a 45 minutes recovery period. This heating and recovery cycle was then repeated for both 46°C and 50°C.
3.2.1.2 Protocol 2: Time control (no heating).

This protocol was designed to quantify any changes in contractile activity over the same time course of protocol 1 (section 3.2.1.1) (figure 3.2).

![Diagram](image)

Figure 3.2. Time control (no heating).
Protocol design to validate any contractile activity changes assessed in protocol 1. Following an initial 60 min control period bladder sheets were left over the same time course as in protocol 1. No heating was given throughout this time course.

3.2.1.3 Protocol 3: The effect of raised superfusate pH.

Changes to superfusate pH were measured during heating of the superfusate (see chapter 2, section 2.2.7). Therefore, another control experiment was to examine the effects of raised pH (+0.3) on bladder contractile activity (figure 3.3).

![Diagram](image)

Figure 3.3. The effect of raised superfusate pH.
Tissue preparations followed the same stabilisation as all other experiments. An increase of pH (+0.3) was superfused for 15 minutes. High pH was removed and controlled pH was left for 45 minutes.
3.2.1.4 Protocol 4: Changes to the order of heating (42-50°C) to changes of contractile activity.

This protocol was designed to validate changes in contractile activity during varied temperatures (figure 3.4).

Figure 3.4. Changes to the order of heating to confirm changes in contractility induced by varied heating.
After the initial stabilisation period, the tissue was exposed to an altered order of heating changes (50, 42, 46°C) from protocol 1 and allowed to recover for 45 minutes between heating periods.

3.2.1.5 Protocol 5: Reproducibility of changes to contractile activity at 42°C.

This protocol was designed to determine if the changes to contractile activity are reproducible (figure 3.5).

Figure 3.5. Repeated exposure of heating (42°C) on bladder spontaneous contractions.
After stabilisation of the bladder, strips were exposed to two consecutive periods of heating to 42°C. 45 minutes was given to allow recovery of the tissue between heating interventions.

3.2.1.6 Protocol 6: Changes to contractile activity during long exposures to heating.

This protocol was designed to determine the effects of long exposure of heating on the changes of contractile activity (figure 3.6).
Figure 3.6. Long exposure of heating (42°C) on bladder spontaneous contractions. 
Post-stabilisation, bladder strips were exposed to heating for an hour at 42°C. The tissue was allowed to recover for 45 minutes thereafter.

3.2.1.7 Protocol 7: The recovery from heating (42°C) over a prolonged period.

This protocol was designed to determine the recovery of spontaneous contractions, post exposure to heating at 42°C (figure 3.7).

Figure 3.7. Recovery of spontaneous contractions after exposure to heating (42°C).
Bladder preparations were exposed to an initial heating period at 42°C after stabilisation and allowed to recover at 37°C for at least 1 hour 30 minutes. This was to determine the time course of changes to contractile activity post-heating.

3.2.1.8 Protocol 8: Alterations to bladder contractility on heating in the presence of ADP or high KCl.

This protocol was designed to examine the effects of heating when superfused with ADP or KCl rather than carbachol (figure 3.8).
Figure 3.8. Heating from 37°C to 42, 46, 50°C on spontaneous contractions superfused with ADP (A) or high KCl (B). During stabilisation of the tissue, bladders were exposed to either 30 μM ADP or 10 mM KCl, which was sustained throughout the protocol. After the 60 minutes of stabilisation, the submucosal temperature was raised to 42°C for 15 minutes, followed by a 45 minutes recovery period. This heating and recovery cycle was then repeated for both 46°C and 50°C.

3.2.2 The effect of heating on contractile activity in the superfused pig bladder

The whole perfused pig model was used as an alternative to isolated bladder wall preparations. For all experiments, the perfused bladder was allowed to stabilise for at least 60 minutes after starting perfusion and filling the lumen with Kreb’s solution. This allowed generation and stabilisation of intravesical pressure changes. During this time temperature was maintained at 37°C.

3.2.2.1 Protocol 9: The effect of heating (42°C) on spontaneous pressure changes in the perfused pig bladder.

This protocol was designed to examine effects of heating on spontaneous pressure changes (figure 3.9).
Figure 3.9. The effect of heating (42°C) on spontaneous pressure changes.
After the whole bladder preparation was established and calibrated, 150 ml of Krebs’ solution was perfused into the bladder at 37°C for 30 minutes. This was subsequently drained and refilled at 37°C as a time control. When the bladder was again drained, the water bath and perfusate temperature was raised to 42°C and the bladder also filled with the heated solution for a further 30 minutes and drained thereafter. The temperatures were lowered to 37°C and allowed to recover for 30 minutes.

3.2.3 The effect of TRPV1 channel modulators on the effects of heating.
TRPV1 channels were an initial target mechanism due to their activation by temperature in the range of 41-43°C. Other TRPV channels were not considered at this time, in particular TRPV4, as their temperature threshold is ≤50°C. The effects of TRPV1 channel antagonists, capsazepine (CPZ, 3 µM) and AMG9810 (0.3 µM) (see chapter 2 Methods section 2.1.2 for more details) on altering the effect of heating on spontaneous contractile activity, were measured using isolated bladder wall preparations.

3.2.3.1 Protocol 10: Changes to contractile activity on heating in the presence of TRPV1 antagonists.
This protocol was designed to determine if changes in contractile activity during heating was mediated through TRPV1 channels (figure 3.10).
Figure 3.10. Changes to contractile activity on heating in the presence of TRPV1 antagonists. An initial heating period was administered followed by superfusion of a TRPV1 antagonist, CPZ or AMG9810, for 30 minutes. A final heating period with the TRPV1 antagonist was given for 15 minutes, followed by a final recovery for a further 30 minutes.

3.2.4 Where does heating effect the bladder wall? Mucosa or detrusor.

Isolated strips of detrusor or mucosa were prepared as described in chapter 2.2.

3.2.4.1 Protocol 11: Effect of heating on carbachol contractions of detrusor strips.

This protocol was designed to determine if heating altered smooth muscle contractility (figure 3.11).

Figure 3.11. Heating from 37°C to 42, 46, 50°C on carbachol (CCh)-induced contractions. Strips were allowed to equilibrate at least 30 minutes prior to the first CCh (1 µM) addition as a pre-control. CCh was superfused in the presence and absence of heating to 42, 46, and 50°C. Between each heating intervention a recovery period (with no heating) was given and CCh superfused (post-controls). 30 minutes was left between each administration of CCh. CCh was superfused for 10 minutes at all interventions.
3.2.4.2 Protocol 12: CCh-induced contractions with no heating (control).

This protocol was designed to quantify any time-dependent changes to contractility over the same time course as protocol 11 (figure 3.12).

![Diagram](image)

Figure 3.12. Time control for the effect of heating on CCh-induced contractions. Strips were allowed to equilibrate at least 30 minutes prior to the first CCh addition as a pre-control. Strips were then exposed to CCh using the same protocol as in Fig 3.11, but at 37°C throughout.

3.2.4.3 Protocol 13: Variation of the order of temperature changes (42-50°C) on CCh contractures.

This protocol was designed to validate any changes to smooth muscle contractile activity during varied temperatures (figure 3.13).

![Diagram](image)

Figure 3.13. Heating from 37°C to 50, 46, 42°C on CCh-induced contractions. Strips were allowed to equilibrate at least 30 minutes prior to the first CCh addition as a pre-control. CCh was superfused in the presence and absence of heating, reversed to protocol 3.2.4.1. Between each heating intervention a recovery period was allowed and CCh superfused. 30 minutes was left between each administration of CCh. CCh was superfused for a maximum of 10 minutes at all interventions.
3.2.4.4 Protocol 14: Effect of heating on nerve-mediated contractions.

This protocol was designed to determine if heating could influence nerve-mediated contractions generated by EFS (figure 3.14).

![Figure 3.14](image)

**Figure 3.14.** Heating from 37°C to 42, 46, 50°C on EFS-induced contractions. Preparations were pre-stretched and exposed to CCh pre-treatment. DSM strips were electrically stimulated (10 Hz) for 60 minutes. The superfusate was raised to temperatures of 42, 46, and 50°C for 15 minutes with 45 minutes recovery to 37°C between each heating period.

3.2.4.5 Protocol 15: EFS-induced contractions with no heating (control).

This protocol was designed to quantify any time-dependent changes to EFS contractions over the same time course as protocol 14 (figure 3.15).

![Figure 3.15](image)

**Figure 3.15.** Time control (no heating) on EFS-induced contractions. Preparations were exposed to CCh pre-treatment post-stretching. DSM strips were exposed to electrical stimulation (10 Hz) for 60 minutes (stabilisation). Electrical stimulation was left as a time control for protocol 14 (section 3.2.4.4) at 37°C.
3.2.4.6 Protocol 16: Variation of the order of temperature changes (42-50°C) on EFS contractures.

This protocol was designed to validate changes to EFS contractions during heating (figure 3.16).

![Figure 3.16](image)

**Figure 3.16. Heating from 37°C to 50, 46, 42°C on EFS-induced contractions.** Preparations were pre-stretched and exposed to electrical stimulation. The superfusate was raised and reversed to the temperatures 50, 46, and 42°C with 45 minutes recovery between each heating period once temperature was decreased back to control (37°C).

3.2.4.7 Protocol 17: Recovery of nerve-mediated responses after heating to 42-46°C.

This protocol was designed to examine the extent of recovery of EFS-induced contractions post-heating to 46°C (figure 3.17).

![Figure 3.17](image)

**Figure 3.17. Do nerve-mediated responses recover after heating to 46°C?** Post-stabilisation, electrically stimulated preparations were exposed to heating at 42 and 46°C with 45 minutes recovery between interventions at 37°C. Upon removal of heating at 46°C tissue strips were allowed to recover for 90 minutes at 37°C.
3.2.4.8 Protocol 18: Changes to mucosa spontaneous contractions during heating (42-50°C).

This protocol was designed to measure the effects of heating on mucosa spontaneous contractions and determine if changes to contractile activity in intact preparations are influenced by the presence of mucosa (figure 3.18).

![Diagram of Protocol 18](image)

**Figure 3.18. Heating from 37°C to 42, 46, 50°C on mucosal spontaneous contractions.** Following an initial 60 minute control period (CCh pre-treatment), the superfusate temperature was heated to 42°C for 15 minutes and allowed to recover for 45 minutes (recovery period). This heating and recovery cycle was then repeated for both 46°C and 50°C.

3.2.4.9 Protocol 19: Time control (no heating) for mucosal spontaneous contractions.

This protocol was designed to quantify any changes to contractile activity over the same time course of protocol 18 (section 3.2.1.1)(figure 3.19).

![Diagram of Protocol 19](image)

**Figure 3.19. Mucosa control (no heating).** Protocol design to validate any changes to contractile activity measured during protocol 18. Following an initial 60 min control period mucosal strips were left over the same time course as in protocol 18. No heating was given.
3.2.4.10 Protocol 20: Variation of the order of temperature changes (42-50°C) on mucosa contractile activity.

This protocol was designed to validate changes to mucosa contractile activity during heating to 42-50°C (figure 3.20).

**Figure 3.20. Confirmation of changes to mucosa contractile activity induced by varied heating.**

After the initial stabilisation period, the experiment was exposed to randomised heating (50, 42, 46°C) from protocol 18 and allowed to recover for 45 minutes between heating periods.

3.2.5 The effect of heating on ATP release from the mucosa.

ATP extraction and measurement from the superfusate is explained in detail in chapter 2.5.2.

3.2.5.1 Protocol 21: Effect of 42, 46, and 50°C on ATP release from the mucosa.

This protocol was designed to determine the effects of heating at varying temperature on ATP release (figure 3.21).

**Figure 3.21. Effect of 42, 46, and 50°C on ATP release from the mucosa.**

ATP samples were taken at specific time points throughout the protocol. During heating samples were taken immediately after a specified temperature is reached and 15 minutes thereafter. Heating was exposed in a step-wise increase from 42, 46, and 50°C, with 45 minutes recovery between each heat exposure. Arrows represent the point of sampling and numbers above represent time after the first sample (minutes).
3.2.5.2 Protocol 22: Variation of the order of temperature changes (42-50°C) on mucosa ATP release.

This protocol was designed to validate the effects of heating at varying temperature on ATP release (figure 3.22).

![Figure 3.22](image)

**Figure 3.22. Effect of randomising heating to 50, 42, and 46°C on ATP release from the urothelium.**

ATP samples were taken at specific time points throughout the protocol. During heating, samples were taken immediately after a specified temperature is reached and 15 minutes thereafter. Heating was randomised to confirm ATP release from protocol 21. Arrows represent the point of sampling and numbers above represent time after the first sample (minutes).

3.2.5.3 Protocol 23: Prolonged heating to 42°C on ATP release.

This protocol was designed to determine the time-dependence that heating to 42°C may have on ATP release (figure 3.23).

![Figure 3.23](image)

**Figure 3.23. Effect of long exposure of heating (1 hour at 42°C) on ATP release from the urothelium.**

ATP samples were taken at specific time points throughout the protocol. During heating, samples were taken immediately after a specified temperature is reached and various times during heating thereafter. Arrows represent the point of sampling and numbers above represent time after the first sample (minutes).
3.2.5.4 Protocol 24: The effect of TRPV<sub>1</sub> antagonists on mucosa ATP release.

This protocol was designed to determine if the TRPV<sub>1</sub> antagonist AMG9810 modulated any heat-dependent effect on ATP release (figure 3.24).

![Figure 3.24](image)

**Figure 3.24. Effect of heating in the presence and absence of TRPV<sub>1</sub> antagonist, AMG9810, on ATP release from the urothelium.**

ATP samples were taken at specific time points throughout the protocol. A pre-heating intervention was administered followed by superfusion of TRPV<sub>1</sub> antagonist. Heating was repeated and ATP samples taken at specific time points during the protocol. Arrows represent the point of sampling and numbers above represent time after the first sample (minutes).

3.2.5.5 Protocol 25: Heating and ATP release from isolated urothelial cells.

The effects of heating (42°C) of ATP release from urothelial cells was studied in the presence and absence of extracellular Ca<sup>2+</sup>. After cell isolation (see Methods, section 2.5.1.2), cells were suspended in Tyrode’s solution with or without CaCl<sub>2</sub>. After an initial incubation period (1.5 hours), suspensions were gently moved to heated water baths at either 37°C or 42°C for 15 minutes, when samples of suspension medium were analysed for ATP. Data were presented as released ATP quantity per cell.

3.2.6 Heating and tissue integrity.

3.2.6.1 Bladder wall integrity.

**Visualising bladder integrity.** Visual integrity of the mucosa, collagen fibres, and DSM were examined using H&E (urothelial cells) and van Gieson (collagen) staining of tissues.
exposed to temperatures from 37°C to 42, 46, and 50°C. For intact bladder preparations, 15 minutes of heating at different temperatures was done using a heating coil (see Methods, section 2.2.6). For detrusor-only preparations, the Tyrode’s solution was heated at varying temperature as previously discussed (see Methods, section 2.2.6). Following heating interventions, preparations were immediately stored in formalin (see Methods, section 2.7.1).

**Analysis of tissue integrity.** The measurements of tissue integrity was inaccurate using H&E and van Gieson staining due to contrasts in colour of images obtained using a wide-field microscope. Therefore, analytical processes in tissue integrity for intact and detrusor preparations was examined by fluorescent microscopy using DAPI and WGA staining (see Methods, section 2.7.4). The integrity of the urothelium (intact preparations) was examined as;

1. % of intact urothelium – this was regarded as a urothelial lining at least two cells thick
2. % width of urothelium – the urothelial thickness was measured at four different positions for any intact urothelium.
3. % size of nuclei

For sub-urothelial and DSM layers (intact preparations) and DSM only preparations, a nuclei count was obtained and converted to nuclei count per unit pixel. 37°C and compared to heated bladder at 42, 46, and 50°C. Tissue preparations were exposed to either control conditions (37°C) or heated to 42, 46, or 50°C and compared.
3.3 Results.

3.3.1 Bladder wall heating and spontaneous contractions.

3.3.1.1 Protocol 1a: Effect of 42, 46, and 50°C on spontaneous contractions.

The area under the curve (AUC) of spontaneous contractions was measured at control (37°C) and with raised temperatures (42, 46, and 50°C): data are normalised to the pre-control values. At all raised temperatures (42, 46, 50°C), the AUC was significantly reduced (42°C: 36.7% [21.4%, 67.8%], p<0.01; 46°C: 44.8% [19.8%, 71.2%], p<0.05; 50°C: 38.6% [12.1%, 58.3%], p<0.01, n=10) when compared to the pre-control. The reduction at higher temperatures (46 and 50°C) was even greater when data were normalised to the immediately previous control at 37°C (46°C: 35.5% [19.5%, 80.7%], p<0.01; 50°C: 30.7% [21.9%, 52.4%], p<0.01, n=10) due to the trend of 37°C values to increase throughout. No significant difference was observed between the different increased temperatures (figure 3.25, n=10).

Figure 3.25. The effects of varying temperature (42-50°C) on the AUC of spontaneous contractions normalised to the pre-control.

All interventions were normalised to the pre-control (black bar and shaded bars) and expressed as % medians [25%, 75%], n=10, compared using one-way ANOVA with Wilcoxon post-hoc tests. Symbols; * vs. pre-control (37°C), # vs. previous control (37°C). The number of symbols represents the p value; * p<0.05, ** p<0.01, *** p<0.001.
3.3.1.2 Protocol 2: Time control (no heating).

Changes to AUC were measured over the same time course as protocol 1 (section 3.3.1.1) and showed a significant decline over 175 minutes ($r = -0.857$, $p<0.05$, $n=7$) (figure 3.26).

![Figure 3.26](image)

**Figure 3.26. The effect of no heating on bladder spontaneous contractions.**

All interventions were normalised to the pre-control (before heating) and expressed as % medians [25%, 75%], $n=7$, the correlation between data sets compared using a Spearman’s rank, ‘$r$’.

Due to the progressive decline in AUC over time, data sets were now normalised to the average pre/post-heating control values.

Figure 3.27 shows that the AUC for varying temperature was significantly decreased when interventions (42, 46, 50°C) were normalised to pre/post-heating (42°C: 38.8% [26.2%, 52.8%], $p<0.01$; 46°C: 37.9% [21.7%, 56.1%], $p<0.01$; 50°C: 24.1% [15.0%, 43.6%], $p<0.01$, $n=10$) (figure 3.27).
3.3.1.3 Protocol 3: The effect of extracellular pH changes on AUC at 37°C.

An increase of superfusate pH had no significant change on AUC at constant temperature (37°C: pH 7.7: 87.7% [75.5%, 120.7%], n=6) when normalised to the pre-control value alone. AUC was also not significantly different from pre-control upon return to normal pH (pH 7.4: 87.4% [74.3%, 174.7%], n=6). Sample traces of recordings are shown in figure 3.28 with summarised data in figure 3.29.
Figure 3.28. Tracings show the effect of heating, control (no heating), and pH control on spontaneous contractions.
Tracings were taken in the final 10 minutes of each intervention. Top plot (green), shows the effect of heating (42°C) and post-control to the pre-control. Middle plot (blue), shows the time control. Bottom plot (red), compares the effect of increasing pH and post-pH.
Figure 3.29. Comparison of the effect of heating, control (no heating), and pH control on the AUC of spontaneous contractions.
All data points were normalised to their pre-control value and expressed as % medians. Each line represents an individual paired experiment. Left plot (green), compares the effect of heating (42°C) and post-control to the pre-control, n=10. Middle plot (blue), the time control in relation to the left plot, n=8. Right plot (red), compares the effect of increasing pH and post-pH to the pre-control, n=6. Data sets were compared using Wilcoxon rank tests. Symbol; * vs. pre-control.

3.3.1.4 Protocol 1b: The effect of varying temperature (42-50°C) on bladder spontaneous contraction variables.
Changes to the AUC of spontaneous contractions can be due to changes of several component; duration or amplitude (figure 3.30 and 3.31A-C). The amplitude and duration both significantly decreased at any temperature.
Amplitude: 42°C: 69.1% [51.0%, 80.5%], p<0.05;
46°C: 77.3% [48.5%, 100.5%], p<0.05;
50°C: 69.3% [54.6%, 88.6%], p<0.05, n=10 (figure 3.31A).
Duration: 42°C: 60.2% [49.0%, 66.2%], p<0.05;
46°C: 53.9% [41.5%, 67.6%], p<0.05;
50°C: 49.2% [35.3%, 59.5%], p<0.05, n=10 (figure 3.31B).
The frequency of spontaneous contractions, however, significantly increased.
Frequency: 42°C: 143.0% [129.4%, 182.7%], p<0.05; 46°C: 170.8% [133.2%, 192.7%], p<0.05; 50°C: 190.1% [181.4%, 202.6%], p<0.05, n=10 (figure 3.31C).

There was no significant difference between temperatures for any of the above contractile variables.

There was no change in baseline tension at any temperature (figure 3.31D, n=10).

Figure 3.30. Tracings showing the effect of heating (42, 46, and 50°C) on bladder spontaneous contractions.
Segments of tracings are shown to compare contractile changes during heating to 42°C (red), 46°C (blue), and 50°C (purple) against pre-control (black).
Figure 3.31. The effect of heating (42-50°C) on spontaneous contractile variables.
All temperatures were normalised to their respective averaged pre/post-heating value and expressed as % medians [25%, 75%], n=10. Contractile variables are; A) amplitude, B) duration, C) frequency, D) baseline. Data sets were compared using one-way ANOVA with Wilcoxon post-hoc tests. Symbol, * vs. pre/post-heating.
3.3.1.4 Protocol 4. Changes to the order of heating (42-50°C) to changes of contractile activity.

The order of heating from 37°C to up to 50°C was altered to determine if changes to contractions with raised temperature might be influenced by earlier heating episodes (section 3.3.1.1 and 3.3.1.4).

Heating to both 50°C and 46°C significantly reduced AUC of spontaneous contractions, whilst the value at 42°C failed to reach significance.

AUC: 50°C: 15.1% [47.3%, 14.7%], p<0.05; 42°C: 54.7% [48.1%, 74.4%]; 46°C: 26.8% [20.0%, 57.0%], p<0.05, n=7 (figure 3.32A).

A similar pattern was seen with amplitude although of lesser magnitude.

Amplitude: 50°C: 35.9% [33.9%, 83.6%]; 42°C: 78.3% [70.0%, 116.3%]; 46°C: 53.9% [38.7%, 97.9%], n=7 (figure 3.32B).

But duration was significantly reduced at all temperatures.

Duration: 50°C: 55.1% [46.2%, 61.2%], p<0.05; 42°C: 70.3% [62.3%, 74.1%], p<0.05; 46°C: 59.0% [39.1%, 62.5%], p<0.05, n=7 (figure 3.32C).

The frequency remained elevated at all temperatures (figure 3.32D, n=7). No significant difference was seen between any temperatures for all contractile variables. The baseline did show a slight enhancement at 50°C (122.4% [120.4%, 143.1%], p=0.0781, n=7) but was unchanged at both 42°C and 46°C (figure 3.32E).
Figure 3.32. The effect of altering the heating protocol (50, 42, 46°C) on bladder spontaneous contractile variables.
Data at all test temperatures were normalised to their respective averaged pre/post-heating value and expressed as % medians [25%, 75%], n=7. Contractile variables are; A) AUC, B) amplitude, C) duration, D) frequency, E) baseline. Data sets were compared using one-way ANOVA with Wilcoxon post-hoc tests. Symbol, * vs. pre/post-heating.
From these results (section 3.3.1.1-3.3.1.4), any temperature is effective at reducing bladder spontaneous contractions in intact preparations. Henceforth, the effect of heating to 42°C was considered.

### 3.3.1.5 Protocol 5: Reproducibility of changes to contractile activity at 42°C.

Heating to 42°C significantly reduced the AUC of spontaneous contractions (figure 3.33A).

- AUC: 1\textsuperscript{st} 42°C: 30.5% [24.6%, 37.9%], \( p<0.01 \);
- 2\textsuperscript{nd} 42°C: 32.1% [26.3%, 44.0%], \( p<0.01 \), \( n=8 \).

There was a small decrease in amplitude but a larger decrease in the duration (figure 3.33B-C, \( n=8 \)). The frequency was increased during heating (figure 3.33D, \( n=8 \)). This pattern was mirrored when exposed to heating for a second time. The baseline remained unchanged throughout (figure 3.33E, \( n=8 \)).
Figure 3.33. The effect of repeated heating (42°C) on bladder spontaneous contractile variables.

Both heating interventions were normalised to their respective averaged pre/post-heating value and expressed as % medians [25%, 75%], n=8. Contractile variables are; A) AUC, B) amplitude, C) duration, D) frequency, E) baseline. Data sets were compared using one-way ANOVA with Wilcoxon post-hoc tests. Symbol, * vs. pre/post-heating.
3.2.1.6 Protocol 6: Changes to contractile activity during long exposures to heating.

Preparations were exposed to a continuous, one-hour exposure of heating to 42°C. The AUC of spontaneous contractions remained significantly reduced throughout the exposure.

AUC: 15 minutes at 42°C: 35.3% [15.0%, 69.7%], p<0.01;

30 minutes at 42°C: 37.0% [23.4%, 66.4%], p<0.01;

60 minutes at 42°C: 37.6% [30.6%, 67.4%], p<0.01, n=9 (figure 3.34A).

This was a combination of a small reduction of amplitude and a significantly greater reduction in duration (figure 3.34B-C, n=9). The frequency of contractions also remained elevated throughout heating (figure 3.34D, n=9). There was no significant difference at any time point during heating for all contractile variables. After one hour of heating, the baseline was significantly increased (figure 3.34E, n=9).
Figure 3.34. The effect of a long exposure (1 hour) of heating to 42°C on spontaneous contractile variables.

Heating was normalised to the averaged pre/post-heating value and expressed as % medians [25%, 75%], n=9. Contractile variables are; A) AUC, B) amplitude, C) duration, D) frequency, E) baseline. Data sets were compared using one-way ANOVA with Wilcoxon post-hoc tests. Numbers indicate p value. Symbol, * vs. pre/post-heating.
3.2.1.7 Protocol 7: The recovery from heating (42°C) over a prolonged period.

The effect of recovery of spontaneous contractions after heating was examined. Data sets are normalised to the pre-control (before heating). A reduction of the AUC, albeit not significant, was observed during heating to 42°C (figure 3.35A, n=6). This was a consequence of decreased amplitude, duration, and elevated frequency (figure 3.35B, C, D, n=6). After removal of heating, spontaneous contractions returned to a near pre-control value at 30, 45, 60 and 90 minutes. The median baseline value gradually increased post-heating, but this was not significantly different to the pre-control (figure 3.35E, n=6).
Figure 3.35. The effects of post-heating on bladder spontaneous contractions.
Data sets were all normalised to the pre-control value and expressed as % medians [25%, 75%], n=6. Contractile variables are; A) AUC, B) amplitude, C) duration, D) frequency, E) baseline. Data sets were compared using one-way ANOVA with Wilcoxon post-hoc tests. Symbol, * vs. pre-control, # vs. heating (42°C).
3.2.1.8 Protocol 8: Alterations to bladder contractility on heating in the presence of ADP or high KCl.

Heating to any temperature (42, 46, or 50°C) significantly reduced the AUC of spontaneous contractions when superfused with ADP throughout (figure 3.36A). The reduction in amplitude however was temperature-dependent, i.e. at 42°C it was significantly reduced but this was not evident at higher temperatures.

Amplitude: 42°C: 59.0% [47.6%, 76.6%], p<0.05;
46°C: 82.5% [71.5%, 91.1%];
50°C: 91.5% [53.5%, 127.9%], n=10 (figure 3.36B).

The duration however was significantly reduced at all temperatures.

Duration: 42°C: 55.2% [43.5%, 59.4%], p<0.01;
46°C: 71.1% [43.8%, 79.5%], p<0.01;
50°C: 70.5% [37.7%, 80.2%], p<0.01, n=10 (figure 3.36C).

The frequency of contractions remained increased at all temperatures (figure 3.36D, n=10). No baseline change was observed at any temperature (figure 3.36E, n=10).

When superfused with high-KCl solution throughout, the AUC of bladder spontaneous contractions were reduced when exposed to heating. Higher temperatures showed the greatest suppression. This effect was seen for both the amplitude and duration of contractions. The frequency of contractions remained elevated at all temperatures and no change was seen for the baseline tension (figure 3.37A-E, n=5).
Figure 3.36. The effects of varied heating (42, 46, and 50°C) on bladder spontaneous contractions with ADP superfusion.
Data sets were all normalised to the pre-control value and expressed as % medians [25%, 75%], n=10. Contractile variables are; A) AUC, B) amplitude, C) duration, D) frequency, E) baseline. Data sets were compared using one-way ANOVA with Wilcoxon post-hoc tests. Symbol, * vs. pre-control, # vs. heating (42°C).
Figure 3.37. The effects of heating (42, 46, and 50°C) on spontaneous contractions with KCl superfusion.

Data sets were all normalised to the pre-control value and expressed as % medians [25%, 75%], n=5. Contractile variables are: A) AUC, B) amplitude, C) duration, D) frequency, E) baseline. Data sets were compared using one-way ANOVA with Wilcoxon post-hoc tests. Symbol, * vs. pre-control, # vs. heating (42°C).
**3.2.2.1 Protocol 9: The effect of heating (42°C) on spontaneous pressure changes in the perfused pig bladder.**

Intravesical pressure fluctuations were not significantly altered during the time control for any contractile variable at 37°C; 60 minutes post-stabilisation.

Time control: AUC: 94.1% [81.6%, 94.6%), n=5;

   Amplitude: 91.6% [86.5%, 127.2%), n=5;

   Duration: 81.7% [80.9%, 100.6%), n=5;

   Frequency 105.8% [103.3%, 130.0%), n=5 (*figure 3.38 and 3.39A-D*).

Upon exposure to heating (42°C) the AUC was profoundly reduced when compared to the pre/post-control (*figure 3.39A*). This was a consequence of reduced amplitude and duration of pressure changes (*figure 3.39B-C*). The frequency was remained enhanced during heating (*figure 3.39D*).

42°C: AUC: 50.1% [26.1%, 67.3%), n=5;

   Amplitude: 66.7% [48.7%, 93.5%), n=5;

   Duration: 61.0% [50.6%, 64.8%), n=5;

   Frequency 155.5% [144.7%, 202.8%), n=5.

<table>
<thead>
<tr>
<th>Control (37°C)</th>
<th>Time control (37°C)</th>
<th>42°C</th>
<th>Post-control (37°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.75 cmH₂O</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Figure 3.38. Traces of spontaneous changes to intravesical pressure on heating to 42°C in the perfused pig bladder.*

Tracings were taken in the final 10 minutes of each intervention.
Figure 3.39. The effects of heating (42°C) on intravesical pressure changes. The time control, pre/post-control and 42°C were all normalised to control. Data sets are expressed as % medians [25%, 75%], n=5. Contractile variables are: A) AUC, B) amplitude, C) duration, D) frequency. Data sets were compared using one-way ANOVA with Wilcoxon post-hoc tests.
3.3.3 The effect of TRPV₁ channel modulators on the effects of heating.

3.3.3.1 Protocol 10: Changes to contractile activity on heating in the presence of TRPV₁ antagonists.

Heating to 42°C significantly reduces the AUC as a consequence of reduced amplitude and duration. Addition of the TRPV₁ antagonist, CPZ (3 µM), had no significant effect on the reduction of AUC by heating to 42°C (53.7% [29.7%, 66.9%] vs 61.7% [42.6%, 74.7%]; with and without CPZ, respectively, n=8).

This was mirrored by equivalent reductions of contraction duration (64.3% [51.8%, 66.8%] vs 66.4% [54.7%, 73.4%]; with and without CPZ, respectively, n=8) (figure 3.40A-C). There were no significant effects on amplitude.

The frequency of contractions were similarly increased at 42°C with and without CPZ (175.1% [145.9%, 223.1%] vs 147.9% [137.0%, 176.8%], respectively, n=8) (figure 3.40D). Baseline tension was similar during heating with or without CPZ (figure 3.40E).
Figure 3.40. The effect of CPZ on spontaneous contractions when heating to 42°C.
Data sets were all normalised to the pre/post-control value and expressed as % medians [25%, 75%], n=8. Contractile variables are; A) AUC, B) amplitude, C) duration, D) frequency, E) baseline. Data sets were compared using one-way ANOVA with Wilcoxon post-hoc tests. Symbol, * vs. pre/post-control.
Similar experiments were carried out with a second TRPV$_1$ antagonist, AMG9810. The AUC remained decreased (figure 3.41A).

**AUC:**
- $42^\circ$C without AMG9810: 33.5% [25.4%, 55.6%];
- $42^\circ$C with AMG9810: 37.9% [31.4%, 66.5%], n=7.

Changes of AUC were dependent on both a reduced amplitude and duration when compared to the pre/post-control whilst frequency was similarly increased in the absence and presence of AMG9810 (figure 3.41B-D).

**Amplitude:**
- $42^\circ$C without AMG9810: 62.4% [57.3%, 73.0%], p<0.05;
- $42^\circ$C with AMG9810: 62.2% [59.9%, 82.2%], p<0.05, n=7 (figure 3.41B).

**Duration:**
- $42^\circ$C without AMG9810: 67.5% [51.1%, 77.7%], p<0.05;
- $42^\circ$C with AMG9810: 60.2% [57.1%, 88.4%], n=7 (figure 3.41C).

**Frequency:**
- $42^\circ$C without AMG9810: 149.4% [143.1%, 166.5%], p<0.05;
- $42^\circ$C with AMG9810: 161.7% [127.6%, 186.4%], p<0.05, n=7 (figure 3.42D).

Baseline tension was no different during heating with or without AMG9810 (figure 3.41E, n=7).
Figure 3.41. The effect of AMG9810 on spontaneous contractions when heating to 42°C.

Data sets are expressed as individual experiments and compared as heating with or without AMG9810, n=7. Contractile variables are; A) AUC, B) amplitude, C) duration, D) frequency, E) baseline. Data sets were compared using one-way ANOVA with Wilcoxon post-hoc tests. Symbol, * vs. pre/post-control.
3.3.4 Where does heating effect contractile function of the bladder wall? Mucosa or detrusor

The magnitude of spontaneous activity is dependent on the segment of the bladder wall. Intact bladder wall preparations (mucosa + detrusor) produced large, spontaneous contractions. Mucosa-only preparations also generated smaller spontaneous contractions but detrusor-only preparations produced virtually no spontaneous contractions (*figure 3.42*).

*Figure 3.42. Tracings of spontaneous contractions in different segments of the bladder wall.* Top tracing, detrusor-only strips. Middle tracing, mucosa-only strips. Bottom tracing, intact strips (detrusor and mucosa).
3.3.4.1 Protocol 11: Effect of heating on carbachol contractions of detrusor strips.

Heating to 42°C had no significant effect on the amplitude of the CCh contracture 42°C:

92.0% [91.4%, 103.1%], n=6.

Heating to 46 and 50°C had progressive effects on amplitude when compared to the pre/post-control (figure 3.43-3.44).

46°C: 67.0% [61.1%, 75.2%], p=0.0625, n=6;

50°C: 5.3% [2.0%, 9.3%], p<0.05, n=6.

Figure 3.43. Tracings of CCh-induced contractions in the presence and absence of heating (42, 46, 50°C).

Tracings of whole experiments showing the effects of CCh, A) in the presence of heating, or B) control (no heating). Lines above tracing indicates exposure to heating. Tracing without lines above are at 37°C. Arrows indicate CCh contracture.
3.3.4.2 Protocol 12: CCh-induced contractions with no heating (control).

The peak amplitude of CCh contractures did not change over time when normalised to the pre/post-control (figure 3.45).

42°C: 92.5% [87.1%, 97.3%), n=6;

46°C: 103.6% [98.1%, 107.4%), n=6;

50°C: 98.0% [97.4%, 99.9%), n=6.
Figure 3.45. Time control of CCh-induced contractions without heating.
Data sets were all normalised to the pre/post-control value and expressed as % medians [25%, 75%], n=6. Contractile variable is the peak amplitude CCh contracture. Data sets were compared using one-way ANOVA with Wilcoxon post-hoc tests. Number denotes p value vs. pre/post-control.

3.3.4.3 Protocol 13: Variation of the order of temperature changes (42-50°C) on CCh contractures.

Variation of the heating changes caused complete inhibition of CCh contracture for both 50 and 46°C. Albeit heating to 46°C was not significant, little recovery of CCh contractures was observed after 50°C. Three out of six experiments even showed no recovery after heating to 50°C. CCh contractures remained reduced even when heated to 42°C but not completely inhibited (figure 3.46, n=6).
Figure 3.46. The effect of varying the heating interventions (50, 42, 46°C) on CCh-induced contractions.
Data sets were all normalised to the pre/post-control value and expressed as % medians [25%, 75%], n=6. Contractile variable is the peak amplitude CCh contracture. Data sets were compared using one-way ANOVA with Wilcoxon post-hoc tests. Number denotes p value. Symbol, * vs. pre/post-control.

3.3.4.4 Protocol 14: Effect of heating on nerve-mediated contractions.
Heating to 42°C did not affect the AUC nor amplitude of nerve-mediated contractions but did significantly reduce the 50% relaxation time (figure 3.47A-C).

AUC: 42°C: 97.6% [81.4%, 124.6%], n=10.
Amplitude: 42°C: 99.9% [92.4%, 106.9%], n=10.
50% relaxation: 42°C: 80.4% [65.8%, 100.3%], p<0.05, n=10.

Higher heating to 46 and 50°C had contrasting effects.
At 46°C median AUC, amplitude and 50% relaxation were increased, but the large variability ensured that these changes were not significant (figure 3.47A-C).

AUC: 46°C: 135.8% [102.9%, 266.5%], n=10.
Amplitude: 46°C: 110.5% [81.3%, 199.2%], n=10.

50% relaxation: 46°C: 103.4% [78.8%, 133.8%], n=10.

At 50°C, EFS-induced contractions were completely inhibited (figure 3.47A-C, n=5).

The baseline tension was increased at all temperatures (figure 3.47D).

Baseline: 42°C: 108.4% [106.4%, 143.6%], p<0.01, n=10;
  46°C: 223.8% [163.6%, 273.2%], p<0.01, n=10;
  50°C: 217.5% [183.2%, 229.8%], n=5.

3.3.4.5 Protocol 15: EFS-induced contractions with no heating (control).

Changes to EFS-induced contractions were measured over the same time course as protocol 14 (section 3.3.4.4). The AUC and amplitude of EFS-induced contractions showed a significant positive increase with time (AUC: r=0.91, p<0.001; amplitude: r=0.97, p<0.001, n=6). No correlation was observed for both the 50% relaxation of contractions and baseline tension (50% relaxation: r=0.59; baseline: 0.79, n=6) (figure 3.48A-D).
Figure 3.47. The effect of varied heating on nerve-mediated contractions.
Data sets were all normalised to the pre/post-control value and expressed as % medians [25%, 75%]. Contractile variables are, A) AUC, B) amplitude, C) 50% relaxation, D) baseline. Data sets were compared using one-way ANOVA with Wilcoxon post-hoc tests. Numbers in bars represents ‘n’. Numbers above bars denotes p value. Symbols; * vs. pre/post-control.
Figure 3.48. Time-control of nerve-mediated contractions without heating.
Data sets were all normalised to the pre/post-control value and expressed as % medians [25%, 75%], n=6. Contractile variables are, A) AUC, B) amplitude, C) 50% relaxation, D) baseline. The correlation between data sets compared using a Spearman’s rank, ‘r’.

3.3.4.6 Protocol 16: Variation of the order of temperature changes (42-50°C) on nerve-mediated contractures.

Heating to 50°C completely inhibited nerve-mediated contractions, which did not recover. Therefore no contractions were generated at subsequent exposures to post-control and 46 or 42°C. There were no changes to baseline tension during heating (50, 46, and 42°C) (figure 3.49A-D, n=6).
Figure 3.49. The effect of varying the heating protocol (50, 42, 46°C) on EFS-induced contractions.

Temperatures were varied from protocol 14, normalised to their respective averaged pre/post-heating value and expressed as % medians [25%, 75%], n=6. Only 50°C is shown as EFS-induced contractions did not recover. Contractile variables are; A) AUC, B) amplitude, C) 50% relaxation, D) baseline. Data sets were compared using one-way ANOVA with Wilcoxon post-hoc tests.

3.3.4.7 Protocol 17: Recovery of nerve-mediated responses after heating to 42-46°C.

Nerve-mediated contractions were transiently reduced after heating to 46°C. The time (τ) and rate (1/τ) constants of recovery from heating were measured, in particular the AUC, amplitude, and 50% relaxation time. Table 3.1 shows that the AUC has a mean τ of 90.42
minutes, and the amplitude a mean $\tau$ of 23.77 minutes. The 50% relaxation was unaffected during and after heating (figure 3.50 and 3.51A-C, n=5).

Table 3.1. Time and rate constants of the recovery of nerve-mediated contractions after heating to 46°C.
Data are shown as the rate ($1/\tau$) and the time constant, $\tau$ of five experiments. No recovery: responses did not return to control after the decline of force on return to 37°C. Colours represent different preparations.

<table>
<thead>
<tr>
<th>Amplitude</th>
<th>Mean ± SEM</th>
</tr>
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<tr>
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<td>$1/\tau$ (min$^{-1}$)</td>
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<table>
<thead>
<tr>
<th>AUC</th>
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</tr>
<tr>
<td>$1/\tau$ (min$^{-1}$)</td>
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<table>
<thead>
<tr>
<th>50% relaxation</th>
<th>Mean ± SEM</th>
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<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>$\tau$ (min)</td>
<td>No recovery</td>
</tr>
<tr>
<td>$1/\tau$ (min$^{-1}$)</td>
<td>No recovery</td>
</tr>
</tbody>
</table>
Figure 3.50. Tracings of the recovery of nerve-induced contractions. After heating to 46°C, nerve-induced contractions were allowed to recover.

Figure 3.51. Recovery of nerve-mediated contractions after heating to 46°C. The rate constant (1/τ) is equivalent to –slope of the curve, which allowed calculation of the time constant, τ. The lines are fitted by least-squared regression. A) AUC; B) Amplitude data. N=5.

3.2.4.8 Protocol 18: Changes to mucosa spontaneous contractions during heating (42-50°C).

Heating to 42°C did not significantly change the AUC or amplitude of spontaneous contractions but did reduce their duration and increase frequency (figure 3.52A-D).
42°C: AUC: 91.8% [69.6%, 106.7%], n=9;
Amplitude: 143.9% [91.2%, 206.9%], n=9;
Duration: 52.6% [42.4%, 95.1%], p<0.05, n=9;
Frequency: 233.3% [132.4%, 291.6%], p<0.05, n=9.

Heating to 46°C significantly increased the AUC, which was a consequence of a large enhancement of amplitude rather than changes in duration or frequency (figure 3.52A-D).

46°C: AUC: 605.8% [402.8%, 700.0%], p<0.01, n=9;
Amplitude: 912.4% [486.2%, 959.8%], p<0.01, n=9;
Duration: 81.7% [73.6%, 180.5%], n=9;
Frequency: 141.3% [112.5%, 200.0%], n=9.

Higher heating to 50°C greatly reduced the median values of AUC, amplitude, duration, and frequency of contractions. However, because of the large variability the changes were not significant. In addition, in two out of nine experiments completely inhibited spontaneous contractions after 50°C heating (figure 3.52A-D).

50°C: AUC: 16.5% [0.0%, 267.8%], n=9;
Amplitude: 40.0% [0.0%, 74.4%], n=9;
Duration: 9.1% [0.0%, 75.7%], n=9;
Frequency: 0.0% [0.0%, 53.6%], n=9.

The baseline tension remained unchanged at 42 and 46°C but did significantly increase at the higher temperature of 50°C (figure 3.52E).

Baseline: 42°C: 94.6% [93.9%, 95.9%], n=9;
46°C: 98.8% [95.6%, 116.8%], n=9;
50°C: 150.9% [128.0%, 204.5%], p<0.05, n=9.
3.3.4.9 Protocol 19: Time control (no heating) for mucosal spontaneous contractions.

Changes to mucosal spontaneous contractions were measured over the same time course as protocol 18 (section 3.3.4.8) but with no heating. The AUC, amplitude and frequency of spontaneous contractions showed a significant decline over time (AUC: $r=-0.921$, $p<0.01$; amplitude: $r=-0.978$, $p<0.001$; frequency: $r=-0.926$, $p=0.001$, $n=6$) (figure 3.53A, B, D). However, duration did not change ($r=0.889$, $n=6$) (figure 3.53C). Baseline tension also significantly declined ($r=-0.983$, $p=0.001$) (figure 3.53E).

3.3.4.10 Protocol 20: Variation of the order of temperature changes (42-50°C) on mucosa contractile activity.

When the order of heating was changed from 50°C to 46, and 42°C, mucosal spontaneous contractions are completely inhibited during and after the initial exposure to 50°C, with little recovery from all experiments after 50°C heating. A significant decrease of baseline tension was observed at 50°C and a slight increase at 46 and 42°C (figure 3.54E).
Figure 3.52. Changes to mucosal spontaneous contractions during heating (42-50°C).
Data sets were all normalised to the pre/post-control value and expressed as % medians [25%, 75%], n=9. Contractile variable are, A) AUC, B) amplitude, C) duration, D) frequency, E) baseline. Data sets were compared using one-way ANOVA with Wilcoxon post-hoc tests. Symbol, * vs. pre/post-control.
Figure 3.53. Time control of mucosal spontaneous contractions without heating. Data sets were all normalised to the pre/post-control value and expressed as % medians [25%, 75%], n=6. Contractile variable are, A) AUC, B) amplitude, C) duration, D) frequency, E) baseline. The correlation between data sets compared using a Spearman’s rank, ‘r’.
Figure 3.54. The effect of varying the order of heating (50, 42, 46°C) on mucosal spontaneous contractions.
Temperatures were varied from protocol 18, normalised to their respective averaged pre/post-heating value and expressed as % medians [25%, 75%], n=6. Only 50°C is shown as mucosal spontaneous contractions did not recover. Contractile variables are; A) AUC, B) amplitude, C) duration, D) frequency, E) baseline. Data sets were compared using one-way ANOVA with Wilcoxon post-hoc tests.
3.3.5 The effect of heating on ATP release from the mucosa.

3.3.5.1 Protocol 21: Effect of 42, 46, and 50°C on ATP release from the mucosa.

Heating to any temperature (42, 46, and 50°C) caused a large significant increase in ATP release 15 minutes after heat exposure.

Pre-control: 37°C: 34.1 [23.3, 51.9] pmol.g⁻¹ (dry weight), n=11.

15 minutes after heating: 42°C: 125.13 [96.4, 204.3] pmol.g⁻¹, p<0.01, n=11;
46°C: 185.9 [140.7, 242.3] pmol.g⁻¹, p<0.001, n=11;
50°C: 181.4 [141.6, 244.8] pmol.g⁻¹, p<0.001, n=11.

This increase was also significant when compared to their respective previous controls (before heating) for both 46 and 50°C. On return to 37°C, ATP release was reduced to a similar value as the pre-control in each case. The increase at 42°C was not significantly different from that at 46 and 50°C (figure 3.55).
Figure 3.55. The effect of heating (42, 46, 50°C) on mucosa ATP release.
Data points are normalised as pmol.g\(^{-1}\) dry tissue weight and expressed as medians [25, 75] pmol.g\(^{-1}\), n=11. Data sets were compared using repeated measures one-way Friedman ANOVA with Dunns post-hoc tests. Symbol, * vs. pre-control, # vs. previous control.

### 3.3.5.2 Protocol 22: Variation of the order of temperature changes (42-50°C) on mucosa ATP release.

Varying the order of heating interventions showed that at initial heating to 50°C again generated a large ATP release. However, on subsequent exposure to 42°C release was still evident but smaller. Final exposure to 46°C generated a large release, not significantly different from that at 50°C. Values were,

Pre-control:  
37°C: 49.7 [36.9, 132.1] pmol.g\(^{-1}\), n=7.

15 minutes after heating.  
50°C: 157.5 [150.1, 296.1] pmol.g\(^{-1}\), p<0.05, n=7.
42°C: 86.5 [62.4, 151.5] pmol.g\(^{-1}\), p<0.05, n=7.
On return to 37°C after each heating episode, ATP release was reduced to a similar value as the pre-control (before heating) (figure 3.56).

Figure 3.56. The effect of varying the order of heating (50, 42, 46°C) on ATP release. Data points are normalised as pmol.g⁻¹ dry tissue weight and expressed as medians [25, 75] pmol.g⁻¹, n=7. Data sets were compared using repeated measures one-way Friedman ANOVA with Dunns post-hoc tests. Symbol, * vs. pre-control, # vs. previous control.

3.3.5.3 Protocol 23: Prolonged heating to 42°C on ATP release.

ATP release was increased during the initial rise of temperature to 42°C. However, over the one-hour period the increase was not sustained declining to a level not significantly different from the post-control values at 37°C (figure 3.57).
Pre-control (37°C): 52.1 [18.4, 98.7] pmol.g⁻¹, n=4.

42°C:
- t=0 min: 58.5 [57.3, 136.0] pmol.g⁻¹, n=4;
- t=5 min: 185.8 [77.0, 189.7] pmol.g⁻¹, n=4;
- t=10 min: 123.3 [89.5, 224.8] pmol.g⁻¹, n=4;
- t=15 min: 121.2 [111.5, 130.6] pmol.g⁻¹, n=4;
- t=30 min: 106.9 [88.4, 107.3] pmol.g⁻¹, n=4;
- t=45 min: 86.1 [74.2, 98.8] pmol.g⁻¹, n=4;
- t=60 min: 104.9 [57.5, 111.2] pmol.g⁻¹, n=4.

Peak release of ATP was therefore measured within the first 5 minutes of heating and declined thereafter.

**Figure 3.57. The effect of a prolonged exposure (1 hour) to 42°C on ATP release.**
Data points are normalised as pmol.g⁻¹ dry tissue weight and expressed as medians [25, 75] pmol.g⁻¹, n=4. Data sets were compared using repeated measures one-way Friedman ANOVA with Dunns post-hoc tests.
3.3.5.4 Protocol 24: The effect of TRPV\textsubscript{1} antagonists on mucosa ATP release.

Heating to 42°C significantly increased ATP release and exposure to the TRPV\textsubscript{1} antagonist, AMG9810, did not significantly alter ATP release. (figure 3.58).

without AMG9810:

pre-control: 46.2 [32.8, 64.5] pmol.g\textsuperscript{-1}, n=8.

42°C t=15: 151.1 [62.2, 253.7] pmol.g\textsuperscript{-1}, p<0.01, n=8.

with AMG9810:

pre-control: 46.2 [32.8, 64.5] pmol.g\textsuperscript{-1}, n=8.

42°C t=15: 98.3 [75.9, 159.8] pmol.g\textsuperscript{-1}, p<0.05 vs. previous control, n=8.

Figure 3.58. The effect of the TRPV\textsubscript{1} antagonist AMG9810 on ATP release during heating. Data points are normalised as pmol.g\textsuperscript{-1} dry tissue weight and expressed as medians [25, 75] pmol.g\textsuperscript{-1}, n=8. Data sets were compared using repeated measures one-way Friedman ANOVA with Dunns post-hoc tests. Symbol, * vs. pre-control, # vs. previous control.
3.3.5.5 Protocol 25: Heating and ATP release from isolated urothelial cells.

The effect of heating (42°C) on urothelial cell ATP release was studied in the presence and absence of extracellular Ca$^{2+}$. In the absence of Ca$^{2+}$, a significant decrease in ATP release was seen during heating compared to control (37°C) in eight out of eleven bladders (figure 3.59A). Heating the superfusate of urothelial cells in the presence of Ca$^{2+}$ had the opposite effect (figure 3.59B).

![Figure 3.59](image.png)

**Figure 3.59.** The effect of heating (42°C) on urothelial ATP release from isolated urothelial cells in the absence (A) and presence (B) of extracellular Ca$^{2+}$.

Data points are individual paired comparisons of 37°C vs. 42°C normalised as nmol.cell$^{-1}$. Data sets were compared using paired Wilcoxon ranks test. Symbol, * vs. previous control.
3.3.6 Heating and tissue integrity.

3.3.6.1 The effect of varied heating (42, 46, 50°C) on bladder wall integrity heated with a heating coil.

When the bladder wall was exposed to heating (42-50°C) there was a temperature-dependent change of tissue integrity. The urothelial surface remained mainly intact when heated to 42°C, compared to 37°C. Heating to 46°C showed clear loss of urothelial integrity, which is even greater at 50°C (figure 3.60-3.65). Images of DAPI stained sections was only shown and analysed, as WGA staining produced inconsistent or minimal fluorescence.
Figure 3.60. H&E sections of bladder wall, with and without heating.
Bladder wall segments were exposed to heating; A) 37°C, B) 42°C, C) 46°C, D) 50°C, and stained with H&E. U – urothelium; SU – suburothelium; M – mucosa; D – detrusor. x20 magnification.
Figure 3.61. The mucosal region of the bladder wall stained with H&E, with and without heating.
A) 37°C, B) 42°C, C) 46°C, D) 50°C, and stained with H&E. U – urothelium; SU – suburothelium. x63 magnification.
Figure 3.6. van Gieson-stained bladder wall sections, with and without heating. A) 37°C, B) 42°C, C) 46°C, D) 50°C, and stained with van Gieson. U – urothelium; SU – suburothelium; M – mucosa; D – detrusor. x20 magnification.
Figure 3.63. van Gieson-stained bladder wall sections, with and without heating. Bladder wall segments are exposed to heating: A) 37°C, B) 42°C, C) 46°C, D) 50°C, and stained with van Gieson. U – urothelium; SU – suburothelium. x63 magnification.
Figure 3.64. DAPI-stained mucosal segments of the bladder wall, with and without heating. The urothelial lining of the bladder wall is analysed and compared between temperatures A) 37°C, B) 42°C, C) 46°C, D) 50°C, stained with DAPI. x40 magnification.
Intact preparations had an intact urothelial lining at 37°C, however, when preparations were heated there was a temperature-dependent reduction in the width and the percentage of the urothelial layer that was intact, as well as a decrease of nuclear diameter. N=6.

Percentage intact:

- 42°C: 88.8% [82.3, 97.9%], n=6;
- 46°C: 86.2% [72.3, 99.9%], n=6;
- 50°C: 66.3% [55.6, 78.0%], n=6 (figure 3.65A).

Width of urothelium:

- 42°C: 76.7% [67.7, 107.5%], n=6;
- 46°C: 67.4% [51.6, 69.6%], p<0.05, n=6;
- 50°C: 57.9% [36.2, 76.0%], p<0.05, n=6 (figure 3.65B).

Nuclear diameter:

- 42°C: 85.2% [76.0, 92.6%], n=6;
- 46°C: 80.7% [63.3, 88.3%], p<0.05, n=6;
- 50°C: 71.2% [47.4, 92.6%], p<0.05, n=6 (figure 3.65C).
Figure 3.6. The effects of heating (42, 46, 50°C) on urothelial integrity.
Data sets are expressed as medians [25%, 75% interquartiles], n=6. Comparisons are; A) % of intact urothelium, B) width of urothelium, C) nuclear size. Data sets were compared using one-way ANOVA with Wilcoxon post-hoc tests.
Exposing intact preparations to heating caused a significant reduction in the number of nuclei, proximal to the urothelium, when heated to 42°C compared to control at 37°C. This effect was larger at 46 and 50°C (figure 3.66-3.67). N=6.

37°C: 10.9 [8.1, 11.1] nuclei per unit pixel x10^{-5}, n=6.
42°C: 9.0 [8.4, 9.7] nuclei per unit pixel x10^{-5}, n=6.
46°C: 7.5 [7.2, 8.2] nuclei per unit pixel x10^{-5}, n=6.
50°C: 6.5 [6.2, 7.4] nuclei per unit pixel x10^{-5}, n=6 (figure 3.67).

Figure 3.66. DAPI-stained sub-urothelial segment of the mucosa adjacent to the urothelium, with and without heating.
A) 37°C, B) 42°C, C) 46°C, D) 50°C, stained with DAPI. x40 magnification.
Figure 3.67. The effect of heating (42, 46, 50°C) on nuclear count of sub-urothelial tissue integrity adjacent to the urothelium.

Data sets are nuclear counts per unit pixel and expressed as medians [25%, 75% interquartiles], n=6. Data sets were compared using one-way ANOVA with Wilcoxon post-hoc tests.

By contrast heating to 42, 46, and 50°C had no significant effects on nuclear counts in the region of the sub-urothelium nearer to the detrusor muscle layer (figure 3.68-3.69).

Note however, that the counts were smaller in this region compared to those in the layer adjacent to the urothelium.

37°C: 3.8 [3.3, 4.9] nuclei per unit pixel x10^{-5}, n=6.

42°C: 4.8 [4.2, 5.1] nuclei per unit pixel x10^{-5}, n=6.

46°C: 3.2 [2.9, 4.9] nuclei per unit pixel x10^{-5}, n=6.

50°C: 5.0 [4.1, 6.1] nuclei per unit pixel x10^{-5}, n=6 (figure 3.69).
Figure 3.68. DAPI-stained sub-urothelial segment of the mucosa near to the detrusor muscle layer with and without heating.
A) 37°C, B) 42°C, C) 46°C, D) 50°C, stained with DAPI. x40 magnification.
Figure 3.69. The effect of heating (42, 46, 50°C) on nuclear count of sub-urothelial tissue integrity adjacent to the smooth muscle layer.
Data sets are nuclei counts per unit pixel and expressed as medians [25%, 75% interquartiles], n=6. Data sets were compared using one-way ANOVA with Wilcoxon post-hoc tests.

The count of nuclei in the detrusor muscle layer of intact preparations did not change when tissue was exposed to heating at any temperature compared to the control at 37°C. N=6.

37°C: 6.2 [5.0, 7.8] nuclei per unit pixel x10^-5, n=6.
42°C: 7.3 [7.0, 8.6] nuclei per unit pixel x10^-5, n=6.
46°C: 6.2 [5.7, 7.0] nuclei per unit pixel x10^-5, n=6.
50°C: 6.3 [4.6, 7.8] nuclei per unit pixel x10^-5, n=6 (figure 3.70).
Figure 3.70. The effect of heating (42, 46, 50°C) on nuclear count in the detrusor smooth muscle layer of an intact preparation. Data sets are nuclei counts per unit pixel and expressed as medians [25%, 75% interquartiles], n=6. Data sets were compared using one-way ANOVA with Wilcoxon post-hoc tests.

3.3.6.1 The effect of heating (42, 46, 50°C) on the detrusor muscle structure.

Figures 3.71 and 3.72 show the effects of heating on detrusor-only strips muscle structure. Figure 3.73 data are expressed as mean values ± SEM as ‘n’ was less than 4 bladders, so median comparisons could not be used. Heating to any temperature showed reduced the nuclei count when compared to control at 37°C.

37°C: 6.6 ± 1.2 nuclei per unit pixel x10^{-5}, n=3.
42°C: 4.0 ± 0.8 nuclei per unit pixel x10^{-5}, n=3.
46°C: 5.5 nuclei per unit pixel x10^{-5}, n=2.
50°C: 4.9 ± 0.5 nuclei per unit pixel x10^{-5}, n=3.
Figure 3.71. H&E-stained detrusor sections, with and without heating. Detrusor only strips are exposed to heating; A) 37°C, B) 42°C, C) 46°C, D) 50°C, and stained with H&E. D – detrusor. x20 magnification.
Figure 3.72. van Gieson-stained sections of detrusor-only strips, with and without heating. A) 37°C, B) 42°C, C) 46°C, D) 50°C, and stained with van Gieson. C – collagen; D – detrusor. x20 magnification.
Figure 3.73. The effect of heating (42, 46, 50°C) on the nuclear count of detrusor smooth muscle in detrusor-only preparations. Data sets are nuclei counts per unit pixel and expressed as mean ± SEM. Data sets were compared using one-way ANOVA with Wilcoxon post-hoc tests.
3.4 Discussion.

This chapter explored the effects of heating to 42, 46, and 50°C on changes in contractility, in particular, spontaneous contractions. The study has addressed that heating to any temperature has the ability to reduce bladder spontaneous contractions. This reduction was evident as a reduced amplitude and greater reduction in the duration of contractions. Further, heating also increased the frequency of contractions. Therefore, differences in the reduction of these contractile variables made it important to express outcomes as an overall AUC of contractions. This phenomenon was possible to manipulate in a whole perfused pig bladder.

This reduction in contractility is only achieved when exposed to bladders with a whole bladder wall intact. Separation between regions of the bladder wall has contrasting effects on changes in contractility at different temperatures. Therefore, communication between layers of the bladder plays a fundamental role in reducing contractility during heating.

3.4.1 Heating to any temperature reduces bladder spontaneous contractions.

Exposure to localised heating has the potential to reduce the AUC of spontaneous contractions which was a consequence of reduced amplitude, duration and elevated frequency of contractions. This reduction in contractility was confirmed when heating was randomised. It is notable that the effects of heating to any temperature were not significantly different between each other and therefore heating to only 42°C is most practical. The effects of mild heating (42°C) were examined on different mechanisms of heating. It is shown that reducing contractility during heating to 42°C has the ability to
not only being replicated but can withstand this reduction for at least 1 hour. These effects always caused a reduction in AUC of contractions due to the decreased amplitude, duration and increased frequency of contractions. Furthermore, spontaneous contractions did recover after being exposed to heating (42°C). This allows for the possibility of routine administration of heating without causing any post-heating effects on contractility.

The use of the agents, ADP (30 µM) and high KCl (8 mM), to enhance spontaneous contractions also had some profound differences in contractility during heating. Although high KCl showed similar findings when preparations were pre-treated with CCh, heating in the presence of ADP changed the degree of reduction in the amplitude of contractions. It is shown that heating to 42°C again showed similarities to other data sets, but higher temperatures (46 and 50°C) caused little change in the amplitude of contractions which significantly differed from 42°C. Other contractile parameters still caused an overall decrease in the AUC of contractions. It is feasible to say that changes in the amplitude of contractions is unlikely to be a mechanism from the smooth muscle itself. This is because changes in the amplitude of contractions was still observed at higher temperatures when preparations were superfused with high KCl (enhances spontaneous contractions by smooth muscle cell membrane depolarisation). Therefore, these changes in the amplitude of contractions during higher heating could possibly be an action within the mucosal layer of the bladder wall.

Finally, this reduction during heating can be manipulated into a whole organ system as shown in the whole pig bladder model. This effect of heating from \textit{in vitro} contractility
studies to the *ex vivo* whole perfused pig bladder model further confirms that heating can reduce bladder spontaneous contractions.

### 3.4.2 The reduction of spontaneous contractions during heating (42°C) is not a mechanism of activated TRPV₁ channels.

It has been known for several years that TRPV channels are sensitive to varied heating. Temperatures at around 40-43°C tend to cause activation of TRPV₁ channels (Caterina et al., 1997). Therefore, to determine whether the reduction in contractility during heating to 42°C could be reversed, TRPV₁ antagonists were used. This method showed no reversal for the reduction of contractions during heating in the presence of both CPZ and AMG9810. Therefore it is probable that heating acts via a different mechanism. Previously, we have shown in rabbit bladder, heating to 42°C can be reversed using the TRPV₁ antagonist CPZ. However, as it cannot be seen in the pig bladder it may be possible that TRPV₁ doesn’t play a large role in pig during heat exposure (Hague *et al.*, unpublished data). A possibility could be the activation of other TRPV channels, in particular TRPV₂. Bender and colleagues first demonstrated that TRPV₂ channels become active at higher temperatures (≥50°C) (Bender *et al.*, 2005). Although bladder preparations were exposed to lower temperatures, it is difficult to exclude the possibility of activation of other TRPV channels, in particular, TRPV₂.
3.4.3 Where does heating affect the bladder wall?

As previously mentioned, intact preparations reduce spontaneous contractions at all temperatures. However, separation of layers within the bladder wall causes different effects during heating with distinct differences between temperatures.

3.4.3.1 Is the reduction of contractility an action of smooth muscle, or nerve-mediated?

Heating to 42°C had little action on smooth muscle and nerve-mediated contractions. However, 50% relaxation was decreased for EFS-induced contractions during heating (42°C). Therefore, it is likely that reduction of contractility in intact preparations during mild heating (42°C) must be through another means for example a direct effect of the mucosa. This effect has contrasting results when compared to our previous findings in rabbit bladder. We had shown that heating to 42°C significantly reduced CCh-induced contractions which was reversible. This effect however was not mirrored in nerve-mediated responses highlighting that heating rabbit bladder is a smooth muscle mechanism. Again, these contrasting results emphasises species differences in physiological responses (Hague et al., 2012). Higher heating (46 and 50°C) showed different effects to 42°C in pig bladder.

Heating to 46°C reduces smooth muscle induced contractions but did not affect nerve-mediated responses. Therefore, it is possible that the reduction during heating to 46°C comes from a direct action on the smooth muscle rather than changes in nerve-mediated responses. Removal of heating however caused an immediate desensitising effect on
nerve-mediated responses that recovered. This emphasises the possibility of heating acting through a multi-mechanistic system.

Severe heating (50°C) completely inhibited contractility of smooth muscle and nerve-mediated responses. These contractions did not recover when heating was removed or exposed to lower temperatures (42 or 46°C). This highlights the possibility that high heating to 50°C induces tissue damage and the effects of reduced contractility could to be a factor of reduced muscular strength rather than changes in cellular mechanisms. Although this cannot be excluded, the fact that spontaneous contractions recovered in intact preparations when heating (50°C) is removed, implies the possibility that this reduction, especially at higher temperatures, is a multi-mechanistic system rather than solely tissue damage. Alternatively, the methods of heating were different where the urothelial surface of intact preparations were heated and sub-mucosal temperature measured (up to 50°C). Due to the temperature gradient across the bladder wall, it indicates that temperature on the urothelial surface is exposed too much higher temperatures than the detrusor muscle itself. This therefore implies the possibility that tissue damage in intact preparations occurs at the point of the urothelial surface but doesn’t extend to layers of the detrusor muscle.

3.4.3.2 Does this reduction of contractility in intact preparations occur in the mucosal layer?

Just like smooth muscle and nerve-mediated responses, mucosal spontaneous contractions also have differing effects during varied temperature. There is a lack of effect for the
overall AUC during heating to 42°C, however, changes in the duration and frequency are evident. Heating to 46°C, vastly increases contractility which is seen to be heavily dependent on the amplitude rather than changes in duration and frequency. Again, severe heating (50°C) strikingly reduces contractility which seem to be permanently suppressed even during recovery periods or exposed to lower temperatures (42 or 46°C).

3.4.3.3 Could the effects of heating be through a mechanism of cell-to-cell interaction, diffusion of chemical agents, or both?

As previous findings in this project have shown intact preparations reduce contractility which recovers at all temperatures and is unspecific to regions within the bladder wall. This emphasises the possibility of heating to act as a means of cell-to-cell interactions, chemical release to underlying tissue layers or a combination of multiple mechanisms.

It has been reported that heat shock of ~43°C transiently induces phosphorylation of Cx43 in cultured human malignant cells (Hamada et al., 2003). As phosphorylation of gap junctions can lead to a reduced opening probability of the channel; it may potentially decrease cell-to-cell communication across the bladder wall (Saez et al., 2005). It has been shown that PKC is sensitive to heat and may become activated during heating that can lead to phosphorylation of Cx43 (Cesare et al., 1999). Therefore, this could potentially reduce electrical signalling across the whole bladder wall via gap junctions channel closure. As the electrical signalling is important for the spread of spontaneous activity, it may be a possible mechanism that the decreased spontaneous contractions in intact preparations is via this closure and reduced electrical signalling across the bladder wall.
wall which is therefore not seen in preparations where the bladder wall layers are separated (Ikeda et al., 2007, van der et al., 2004). Furthermore, as it was shown that spontaneous contractions are exacerbated when the mucosa and detrusor are intact, the spread of electrical and/or chemical currents via these gap junctions may play an integral part for spontaneous activity across the bladder wall, and any interferences of this could diminish the activity.

It has been reported that heat shock (~43°C for 2 hours) induces hyperthermic cell death in the presence of the gap junction intracellular communication inhibitor, lindane. In addition, they also found that lindane, in the presence of heat shock, attenuated the translocation of HSP70 to the nuclei (Hamada et al., 2003). Due to the nature of HSPs playing a large role in cell protection, it could be possible that the translocation of HSP70 during thermal shock induces a protective mechanism against damage or random coiling of cell proteins (Hamada et al., 2003). Further research demonstrated that Cx43 competes with cyclin D1 for interaction with HSP70. Cyclin D1 is an essential regulator for cyclin-dependent kinases to allow transition between G1/S in the cell cycle. Prevention of the interaction between HSP70 and cyclin D1 may therefore act as a means to protect the cell of incorrect protein folding (associated with heat stress) during the cell cycle (Hatakeyama et al., 2013).

Other HSPs, in particular HSP20, have been shown to have relaxative effects on smooth muscle (Woodrum et al., 2003, Ba et al., 2009). Although expression of HSP20 is little in the urinary bladder, other organs including the GI smooth muscle cells, which have
similar contractile motility, have shown rapid relaxation effects after phosphorylation of HSP20 (Batts et al., 2006, Batts et al., 2005). This is through interactions with mechanisms that control actin polymerisation. Therefore promoting depolymerisation of actin filaments and subsequent relaxation.

This project has showed that heating to any temperature induces ATP release, with 46°C having the largest increase. Much literature showed that an increase in ATP release can enhance contractile function via gap junctions; it is likely that the enhancement of ATP during heating acts through a different mechanism. Previous findings have demonstrated the importance of ATP sensing for controlling normal bladder function (Sadananda et al., 2009). It is quite possible that ATP release can directly or indirectly alter afferent signalling. Stress-induced ATP release, such as stretch, has been shown to target P2X₃ on sensory afferent neurons (Cook and McCleskey, 2000, Cockayne et al., 2000). ATP could halt afferent firing by desensitising these receptors on afferent neurons thus reduce the sensation of urgency. Another possible mechanism for increased release could be an indirect action with TRPV channels (Moriyama et al., 2003). However, the use of the TRPV₁ antagonist, AMG9810, did not diminish the increase in ATP release. Although this increase in ATP release was reduced in the presence of AMG9810 when compared to the previous intervention without AMG9810; this was not seen to be significantly different. Therefore it cannot be excluded that TRPV₁ channel activation may cause a partial effect on the increased ATP release during heating.

Furthermore, heating may promote the hydrolysis of ATP into ADP and/or adenosine. The increased production of these purines have many effects on the bladder including
relaxation. For example, ADP may spread and activate P2Y receptors on DSM cells. P2Y receptors on the DSM have shown to cause relaxation of the bladder via a cAMP-dependent mechanism (McMurray et al., 1998).

There is a substantial rise in ATP during the initial 15 minutes of heating to 42°C. The rise however was not sustained over the prolonged period of up to one-hour exposure to heating. This is probably due to the depletion and lack of replenished ATP stores.

The urothelium releases various other chemical mediators to provide the bladder wall with sensory inputs and control normal contractile function. The release of different chemicals including, ATP, NO, ACh, and PGs can alter bladder contractile function under stress-induced circumstances. NO is multifunctional that plays a large role in physiological and pathophysiological functions in smooth muscle. One function is the ability to induce smooth muscle relaxation. Although most findings illustrate the relaxing effects on nerve-evoked and tonic contractions, only few publications examined the effects of NO on spontaneous contractions in the bladder (James et al., 1993, Moon, 2002, Persson and Andersson, 1992). Moro et al., showed that increased levels of NO depresses the frequency and amplitude of contractions in mucosal preparations (Moro et al., 2012). Although we found that during heating an increase in the frequency of contractions, there is a possibility that increased mucosal NO production from heating could reduce the amplitude of contractions. Thus indicating the reduction of contractions would likely act through a multi-mechanistic system.
It is known that noxious stimulation including rising temperature can cause acute inflammation of visceral organs. Heating of the bladder therefore may result in contractile changes via an acute inflammation or damaged urothelium by activated and increased mast cell degranulation releasing vasoactive, inflammatory, and/or nociceptive mediators. These mediators include histamine, interleukins, COX enzymes and PGs, NO and many more which may alter bladder contractility (Grover et al., 2011). Although many of these should not be excluded, the likely candidates for alterations of bladder contractility would be NO and PG production. COX enzymes, in particular COX2, have been previously shown to become active during pro-inflammatory states (Lecci et al., 2000). This activation will lead to an increase PG production including PGI₂ and PGE₂. PGI₂ has also been shown to be produced during cellular damage and distension, however, previous studies demonstrated that antagonists against the PGI₂ receptor alleviated symptoms of OAB (Downie and Karmazyn, 1984, Khera et al., 2007). Therefore, it is unlikely that production of such PG would reduce contractility. There is evidence that supports a relaxation of the bladder via the production of PGE₂ via binding to EP₂ receptor (Coleman et al., 1994). The binding and activation of EP₂ leads to an increased production of cAMP and thus the promotion of relaxation (Frazier et al., 2005). However, Shemi and colleagues previously showed that cultured rat brain glial cells showed a significant reduction in PGE₂ production during ambient temperature of 42°C when compared to cultured cells at 39°C (Shemi et al., 2003). Although this is contradictory to our explanation, differences between cellular functions of the brain and bladder therefore makes it difficult to exclude the notion that PGE₂ production could reduce contractility in the bladder.
3.4.3.4 Heating causes changes to tissue integrity.

Exposure of intact preparations to radiant heating with a heating coil caused changes to tissue integrity. The urothelial lining was most affected, with a reduction of the thickness of the urothelium and shrinkage of nuclei within cells. This was especially evident at 46 and 50°C, but less so at 42°C. Heating to 42 or 46°C had no effect on the structural integrity of the urothelium, whereas preparations exposed to 50°C left some with little urothelial lining. Furthermore, changes to nuclear size with heat exposures extended to the sub-urothelial layer but not as far as the detrusor muscle layer, or the sub-urothelium proximal to muscle. This difference between the urothelial lining to the detrusor layer is likely to be due to a temperature gradient across the bladder wall, as the temperature did not exceed 42°C in the detrusor layer even though urothelium temperature was as high as 50°C.

Changes in the detrusor-only preparations, when altering superfusate temperature, showed a different trend. Heating to 42°C showed the greatest reduction in nuclear count compared to 46 and 50°C. However, relatively few preparations were used for this observation.

These changes to tissue integrity at higher temperatures are probably not the sole reason for reduced spontaneous activity due to the ability for spontaneous activity to recover from heating. In addition, the fact that changes to tissue integrity are less in the detrusor raises the question that spontaneous activity may not originate from the urothelium itself. It has been emphasised that the release of chemicals and resultant electrical activity originating
in the urothelium and immediate sub-mucosal space is crucial in controlling or generating spontaneous contractions in the bladder wall which then spreads to the detrusor layer. However, a damaged urothelial lining does not permanently reduce spontaneous contractions (due to their recovery on removing heating) suggesting that the sub-urothelium plays a more important role in initiating and/or controlling spontaneous activity. As previously mentioned, sub-urothelial cells generate their own Ca\textsuperscript{2+} transients and emphasises the fact that these cells probably have a substantial role.

Other studies have shown that heating, especially to temperatures of 46°C and greater have significant effect on cell damage and even death. Increase of temperatures in rat bladders for up to 1-hour exposure to microwaves were well tolerated below 44°C. However, temperatures in excess of 44°C for an hour had a significant increase in mortality rate. Although these exposures were examined \textit{in vivo}, these findings support the possibility of cell damage/death during exposures to higher temperatures (>46°C) (Haveman et al., 2003). As previously described (section 3.1) DNA damage can result from high temperatures (50-60°C). Hyperthermia to >46°C induced DNA denaturation in Chinese hamster ovary cells (Dewey et al., 1971, Warters et al., 1985). Other studies showed that microwave hyperthermia treatment on skeletal muscle showed no tissue damage with temperatures up to 43°C (Ichinoseki-Sekine et al., 2007). As also described above, nerve-mediated contractions were completely and irreversibly inhibited at 50°C, possibly due to nerve damage. This was corroborated by the observation that heating cultured neurons to 43°C caused cell death after 1.5-2.0 hours, whereas only 30 minutes
when heated to 45°C (White et al., 2007, White et al., 2003, Vogel et al., 1997). Therefore shorter durations (15 minutes) used in this study may have caused nerve damage at 50°C.

Heat stress on cells can induce apoptotic pathways. Apoptotic cell death, induced by heat stress is likely to be due to a combination of both oxidative stress and Ca\(^{2+}\) overloading. Mitochondria play a large role in the production of reactive oxygen species (ROS) and pro-apoptotic factors, including caspase (White et al., 2007). Even mild heating (43°C) caused a significant increase in caspase-3 activity promoting apoptotic cell death (White et al., 2003). Furthermore, heat stress to 42°C for 30 minutes in Jurkat cells (a human T lymphocyte cell line) down-regulated cellular FLICE-like inhibitory protein (FLIP) to increase formation of caspase-8 from procaspase-8. In addition, these cells were highly sensitised to FAS-mediated apoptosis, a receptor that cleaves procapase-8 to caspase-8 (Tran et al., 2003)(figure 3.74).

Overall, even relatively mild heating to 42-43°C can have significant effects on cellular integrity that can have permanent or transient consequences. Further work is required to investigate these effects specifically on urothelial, sub-urothelial and detrusor muscle cells from the bladder wall.

ROS are produced during heat-induced stresses on cells (Zhao et al., 2006, Slimen et al., 2014) and can lead to damage of many macromolecules, including proteins and DNA. Hydroxyl radicals can react with DNA by the addition to double bonds to base pairs, thus causing DNA damage (Cooke et al., 2003). Furthermore, heat stress inactivates
topoisomerase-II enzyme which is used to repair DNA (White et al., 2007). ROS have also been linked to oxidation of polyunsaturated fatty acids, amino acids in proteins, and deactivation of enzymes. Therefore, heating has the ability for pro-apoptotic responses (figure 3.74) but whether these changes to tissue integrity and cell damage are through apoptotic or necrotic pathways remains unclear.

3.4.3.5 Does heating cause conformational changes in to cell proteins important for tension generation?

It is evident that heating causes damage or conformational changes to many proteins and other complex structures. Denaturation of many proteins is seen even at temperatures below 37°C, but can be reversible and does not cause permanent cell damage. Therefore, the balance between denaturation and renaturation may change during heating.

Direct heating of proteins may result in permanent or temporary breakage of bonds which can affect their function. Indirect changes could be through the accumulation of ROS (section 3.4.3.4; (White et al., 2007)). These processes are likely to be dependent on the magnitude of heating. For example, smaller increases of temperatures (42°C) could cause conformational changes in protein or DNA rather than denaturation, whereas higher temperatures will have more profound denaturing effects. This could explain the complete inhibition of carbachol-induced contractures at 50°C, through say denaturation of muscarinic receptors.
In addition, heating could cause conformational changes of structural proteins, such as collagen fibres. Collagen fibres are found throughout the bladder wall and readily alter their configuration. Thermal stimuli (up to 50°C) cause unfolding of collagen fibres in the sub-endothelium of the arterial wall (Post et al., 1996). An additional study showed that heating causes shrinkage of collagen fibres, which caused a decrease in overall resting length of human tendon tissue leading to a reduced load (Vangsness et al., 1997). Such changes to extracellular matrix structure and possibly mechanical stiffness will modulate tension generation in multicellular tissues, as tension generated by muscle cells has to be transmitted throughout the intact muscle through the extracellular material.

3.4.3.6 A potential mechanism for reduction of bladder contractile function during radiant heating to 42°C.

It is likely that the action of heating on bladder spontaneous contractions has several causes. In particular, the mode of heating used – radiant heating - will affect different tissues in the bladder wall to variable extents. This method of heating was chosen as devices that may be used to heat the bladder as a means of reducing spontaneous contractions may be conveniently used in this configuration. Urothelial cells are more likely to be affected and was corroborated by histological observations and the fact that the contractile activity of mucosal and detrusor preparations remained unchanged during heating to 42°C; whereas intact preparation activity was reduced and ATP release from urothelium (mucosa surface) was equally altered at 42°C compared to higher temperatures.
Contractions using preparations that were either detrusor muscle only or mucosa preparations were unaffected by heating to 42°C, whereas responses from intact preparations were attenuated at this temperature. This implies that the mode of communication between mucosa and detrusor, which generates larger spontaneous contractions, was reversibly affected by heating. The particular mode of communication, release of diffusible agent or cell-to-cell communication remains to be established. However, results measuring ATP release during heating would imply it is not such as diffusible mediator as ATP release was actually increased on heating to 42°C. Figure 3.76, summarises potential communication routes that may be heat-labile.
The spread and generation of chemical and electrical activity from interstitial cells to underlining DSM via gap junctions is important in controlling spontaneous activity (A). The addition of heat to the bladder wall may minimise the spread of chemical and electrical activity from interstitial cells to underlining DSM by reduced gap junctions opening through PKC induced phosphorylation. This effect in addition to recoiling of collagen fibres may reduce spontaneous activity (B).
Chapter 4. The effect of sub-mucosal injections of inert bulking agents on pig bladder contractility.
4.1 Introduction.

The importance of mucosal and DSM communication has been emphasised in chapter 1 with respect to the regulation of bladder wall contractile function. Furthermore, decreasing bladder contractile function, in particular spontaneous contractile activity, could be a method of alleviating the symptoms of OAB. DSM contributes to the bulk of the bladder wall. However, an intact DSM and mucosa layer is essential for the generation of spontaneous contractions, associated with propagating Ca$^{2+}$ waves (Fry et al., 2012). Removal of the mucosa from the detrusor muscle layer therefore severely diminishes spontaneous contractions. Several factors are released from urothelial and sub-urothelial cells that can either diffuse or initiate cell-to-cell communication, to the DSM and have been accredited for the generation of spontaneous contractions. In spinal cord transected (T8-9) animals, there is an enhancement in spontaneous activity in isolated bladders (Ikeda and Kanai, 2008). Several changes occur in the bladder wall, including an increased number of ICs in the sub-urothelium. It has also been shown that blocking c-kit receptors, abundant on ICs, reduce spontaneous contractions (Sui et al., 2008, Biers et al., 2006). Thus, it is likely that ICs play a significant role in generating or exacerbating spontaneous contractions.

In addition, the release of agents such as ATP and ACh during mechanical and chemical stimuli, such as physical stretch or muscarinic receptor agonists themselves, modulate and generate large depolarising effects on ICs and spontaneous bladder wall contractions. Enhancement of spontaneous activity following stretch is blocked by atropine (M receptor antagonist), consistent with a role for released ACh. In addition, Ca$^{2+}$ and membrane
potential waves also induced by mechanical stretch originate near the urothelial and sub-urothelial interface spreading to the DSM. Further accentuating the importance of mucosal and DSM communication in the maintenance and enhancements of spontaneous contractions (Kanai et al., 2007).

The use of inert bulking agents may therefore act as a barrier or blockade for the spread of excitatory depolarising or chemical waves from the mucosa to the DSM. Therefore, a reduction of communication offers a means to decrease bladder wall spontaneous contractions.

4.2 Methods.

A full description of the methods is presented in chapter 2. Here there is a brief description of the protocols and specific experimental setup.

4.2.1 In vitro contractility.

4.2.1.1 Tissue preparation.

Freshly isolated bladder wall strips were dissected and mounted onto a tissue bath (as described in section 2.2).

4.2.1.2 Experimental protocol.

Following preliminary calibration of the force transducer, the preparation was pre-stretched and allowed to equilibrate for 45 minutes. After the stabilisation period, the preparation was exposed to:
1. CCh (1 μM) for 10 minutes and then washed out.

2. Normal Tyrode’s solution for one hour to allow for a stable measurement of spontaneous contractions.

The preparation was removed and placed on a Sylgard dish containing recently-gassed Tyrode’s solution (37°C). The preparation was: A) left without injection (null-injectate); B) injected with 0.2 ml Tyrode’s (vehicle control); C) injected with 0.2 ml PEG; D) injected with 0.2 ml coaptite. The injection or null-injectate period was no longer than five minutes. After the injection or null-injection, the preparation was remounted, pre-stretched to the same tension, and stabilised for 45 minutes. The protocol of brief exposure to 1 μM carbachol and measurement of spontaneous contractions was then repeated (figure 4.1). The measurement of spontaneous contractions was the basis for our phenomenon, thus an hour after CCh wash out was necessary to ensure stabilisation of contractions and baseline.

Figure 4.1. Protocol design to examine the effect of injection vs null injection on phasic (spontaneous activity) and tonic (CCh) contractions. Protocol 1 was first achieved as a control where the tissue was removed and either injected or not injected. From this protocol 2 was commenced.
4.2.2 H&E staining.

4.2.2.1 Examination of the site of injection.

Visual comparison of null-injectate vs injectate preparations was examined using H&E staining. Intact preparations were injected with either Tyrode’s, PEG, or coaptite or given no injection as described in section 4.2.1.3. For injectates only, undiluted methylene blue was mixed with the injectate for visual localisation of the injection site. Images were taken of the whole bladder wall with or without injectate using a CCD attached wide-field microscope at 5x objective as described in Methods, section 2.7.6.
4.3 Results.

Contractile variables were measured as characterised in Chapter 3 after injection with inert agents in the mucosa-detrusor interface, and compared to control data (injection with Tyrode’s or no injection).

4.3.1 The effect of injectates on phasic spontaneous contractile variables.

The effects of injecting bulking agents or Tyrode’s into the sub-urothelium was compared to that of the null-injectate procedure (figure 4.2A-D). The process of removing and then re-mounting the preparation, during which injection occurred, may itself cause changes to contractile activity. However, figure 4.2A-D shows no significant changes to the values of AUC, amplitude, frequency or duration after re-mounting.

- **AUC**: 128.6% [65.2%, 228.4%].
- **Amplitude**: 172.2% [79.5%, 304.5%].
- **Duration**: 94.2% [59.8%, 109.6%].
- **Frequency**: 106.7% [83.6%, 134.0%], n=10.
Figure 4.2. The effect of removing and remounting the preparation on spontaneous contractile variables.
All data sets were normalised to control (before remounting procedure) value and expressed as % medians [25%, 75%]. Contractile variables are; A) AUC, B) amplitude, C) duration, D) frequency. Data sets, before vs. after the remounting procedure, were compared using Wilcoxon rank tests. ‘N’ = 10.
The effect of injecting Tyrode’s solution or bulking agents was then measured and values were compared to,

1) Preparations where no injection occurred after the re-mounting procedure (null-injection).

2) Preparations compared to their own control (i.e. before removing, injecting and remounting procedure).

Values were compared to control (=100%), i.e. to those prior to the injection or null-injection procedure. The AUC (figure 4.2A) and amplitude (figure 4.2B) of spontaneous contractions were increased with the null-injectate preparations. The duration (figure 4.2C) and frequency (figure 4.2D) were unchanged. Thus, these changes represented those occurring in the interval between control and the procedure to remove and then remount the preparation.

Injection with Tyrode’s solution did not demonstrate a significantly reduced AUC, amplitude or duration of spontaneous contractions (figure 4.3A, B, C), but frequency was significantly reduced (figure 4.3D), when normalised to their own control. These conclusion were the same if data were compared to null injectate preparations (figure 4.4A-D).

Tyrode’s injection: AUC: 61.3% [31.8%, 104.8%].
Amplitude: 70.2% [40.5%, 106.4%].
Duration: 100.1% [97.1%, 112.1%].
Frequency: 62.1% [33.4%, 89.1%], n=6.
Figure 4.3. The effect of Tyrode’s injectate on spontaneous contractile variables compared to its own control (before remounting procedure).

All data sets were normalised to control (before remounting procedure) value and expressed as % medians [25%, 75%]. Contractile variables are: A) AUC, B) amplitude, C) duration, D) frequency. Data sets, before vs. after the remounting procedure, were compared using Wilcoxon rank tests. ‘N’ = 6.
Figure 4.4. The effect of Tyrode’s on spontaneous contractile variables compared to null-injectate.
All data sets were normalised to control (before remounting procedure) value and expressed as % medians [25%, 75%]. Contractile variables are; A) AUC, B) amplitude, C) duration, D) frequency. Data sets, null-injectate vs. Tyrode’s, were compared using Mann-Whitney U tests.
Injection with PEG significantly reduced the duration (figure 4.5C) of spontaneous contractions but not AUC, amplitude or frequency (figure 4.5A, B, D), when compared to their own control. No significant changes were obtained when data were compared to null-injectate preparations (figure 4.6A-D).

PEG injection:
- AUC: 68.6% [41.8%, 127.6%].
- Amplitude: 109.3% [47.5%, 115.3%].
- Duration: 89.1% [86.2%, 92.4%].
- Frequency: 113.7% [92.3%, 150.2%], n=9.

Injection with coaptite also generated no significant changes to AUC, amplitude or duration of spontaneous contractions when compared to their own control (figure 4.7A, B, C), but in this instance frequency was significantly increased (figure 4.7D). However, when data were compared to the null-injectate preparations there were significant reductions of both AUC and amplitude (figure 4.8A, B).

Coaptite injection:
- AUC: 55.7% [17.6%, 92.9%].
- Amplitude: 76.9% [21.8%, 112.4%].
- Duration: 89.1% [59.3%, 94.1%].
- Frequency: 133.3% [123.8%, 161.5%], p<0.01, n=9.
Figure 4.5. The effect of PEG injectate on spontaneous contractile variables compared to its own control (before remounting procedure).

All data sets were normalised to control (before remounting procedure) value and expressed as % medians [25%, 75%]. Contractile variables are; A) AUC, B) amplitude, C) duration, D) frequency. Data sets, before vs. after the remounting procedure, were compared using Wilcoxon rank tests. ‘N’ = 9.
Figure 4.6. The effect of PEG on spontaneous contractile variables compared to null-injectate.
All data sets were normalised to control (before remounting procedure) value and expressed as % medians [25%, 75%]. Contractile variables are; A) AUC, B) amplitude, C) duration, D) frequency. Data sets, null-injectate vs. Tyrode’s, were compared using Mann-Whitney U tests.
Figure 4.7. The effect of coaptite injectate on spontaneous contractile variables compared to its own control (before remounting procedure).

All data sets were normalised to control (before remounting procedure) value and expressed as % medians [25%, 75%]. Contractile variables are; A) AUC, B) amplitude, C) duration, D) frequency. Data sets, before vs. after the remounting procedure, were compared using Wilcoxon rank tests. ‘N’ = 9.
Figure 4.8. The effect of coaptite on spontaneous contractile variables compared to null-injectate.

All data sets were normalised to control (before remounting procedure) value and expressed as % medians [25%, 75%]. Contractile variables are; A) AUC, B) amplitude, C) duration, D) frequency. Data sets, null-injectate vs. Tyrode’s, were compared using Mann-Whitney U tests.
4.3.2 The effect of injectate or null-injectate on tonic CCh contractures.

The CCh contracture of null-injectate preparations significantly increased when normalised to its own control (before remounting procedure) (figure 4.9A). This trend was not seen for injection with Tyrode’s but was significantly increased for also PEG and coaptite injections (figure 4.9B-D). In contrast, this effect was different when comparisons were made vs. null injectate. Only a significant reduction was seen for PEG (figure 4.10A-C).

CCh contractures:  
Null-injectate: 156.2% [126.4%, 250.8%], n=11.
Tyrode’s: 115.1% [77.5%, 208.5%], n=6.
PEG: 119.5% [102.6%, 130.3%], n=11.
Coaptite: 133.2% [118.0%, 216.6%], n=10.
Figure 4.9. The effect of null-injectate and injectate on bladder CCh contracture compared to its own control (before remounting procedure).

All data sets were normalised to control (before remounting procedure) value and expressed as % medians [25%, 75%]. Data sets, before vs. after the remounting procedure, were compared using Wilcoxon rank tests. ‘N’ = 11 (A, null-injectate); 6 (B, Tyrode’s); 10 (C, PEG); 10 (D, coaptite).
Figure 4.10. The effect of null-injectate and injectate on bladder CCh contracture compared to null-injectate.

All data sets were normalised to control (before remounting procedure) value and expressed as % medians [25%, 75%]. Injectates are; A) Tyrode’s, B) PEG, C) coaptite. Data sets, null-injectate vs. Tyrode’s, were compared using Mann-Whitney U tests.
4.3.3 Histological examination of injection sites.

Tyrode’s solution produced a large separation between the mucosa and underlying DSM which is visible by the methylene blue stain when compared to control. PEG and coaptite also shows a visible separation which is observed (arrows on figure 4.11) by the clear region between the mucosa and DSM. Although this separation was smaller than that of the Tyrode’s injectate.

![Histological examination of injection sites](image)

**Figure 4.11. H&E staining of location of injectate or null-injectate.**
The bladder wall with A) null-injectate, or injectate B) Tyrode’s; C) PEG; D) Coaptite. U = urothelium, SU = sub-urothelium, M = mucosa, D = detrusor. Arrows represent injectate.
4.4 Discussion.

The use of an inert bulking agent reduces the AUC of spontaneous contractions, as well as the carbachol contracture. This effect was mainly dependent on reduced amplitude of contractions rather than duration. A time-control procedure (null-injectate) demonstrated an increase of both spontaneous contraction AUC and amplitude, but with no change for duration and frequency. It is against this trend that the effects of injections of inert agents, or Tyrode’s solution, on spontaneous contractions were tested. For Tyrode’s injection, the time-control (own control) showed a significant decline of frequency, and this was consistent when compared to the null-injectate control. PEG injectate preparations, when compared to their own control data only generated a significant decrease of duration, but this was not reflected in AUC. Even this effect was not evident when compared to null-injectate controls. The injection of coaptite did reduce the AUC and amplitude of spontaneous contractions when compared only to null-injectate controls. Therefore, the effect of injection of inert agents in the mucosa-detrusor interface produced small changes to spontaneous contractions, and less that those produced by heating.

With respect to CCh-induced contractions PEG, but not coaptite, injection reduced their amplitude when compared to null-injectate controls. However, these conclusions were derived from preparations that showed considerable enhancement of contractions when no injection was carried out which would make reliable conclusions from the injectate experiments more difficult to draw. However, these results suggest that coaptite may be the preferable agent to attenuate spontaneous contractions, without effecting tonic contractions mediated by the physiological contractile agonist, acetylcholine.
4.4.1 Do injectates block the diffusion of chemical agents across the bladder wall?

The use of a sub-urothelial injection increases the spacing between the mucosa and detrusor layers and may hinder of chemical and electrical communication between these layers. The urothelium releases many chemical mediators which act on underlying tissue layers. In particular there is a potential role for ATP release from urothelial cells to act on distant or neighbouring cells, including afferent nerves and sub-urothelial interstitial cells. Exogenous ATP enhances spontaneous contractions (Young et al., 2012, Wang et al., 2005, Ferguson et al., 1997). Thus by minimising the spread of ATP from urothelial cells to underlying tissue layers, it may act to reduce spontaneous activity. Purines, including ATP, facilitated the spread of Ca\textsuperscript{2+} transients within the sub-urothelium and to the DSM and urothelium. This spread of activity, once initiated, may propagate via gap junctions as they are blocked by agents such as glycyrrhetinic acid (Ikeda et al., 2007). By reducing the pathway for the spread of activity between mucosa and DSM, should in principle decrease spontaneous activity. Other P2Y receptor agonists such as UTP and ADP also promote signalling between mucosa and DSM suggesting this receptor subfamily is important in this inter-layer communication (Sui et al., 2008).

ACh is also important in sensory mechanisms for controlling normal bladder function as well as mediating mucosa-DSM communication (Kanai et al, 2008). Its non-neuronal release originates in the urothelium (Yoshida et al., 2006). ACh modulates the release of urothelial ATP (Kushida & Fry, 2015), possibly mediated by urothelial cell intracellular
Ca$^{2+}$ concentration (Kullmann et al., 2008). Thus, the use a bulking agent may prevent the spread of ACh from the urothelium to underlying tissue layers.

This possible transmitter influenced reduction of spontaneous contractions through blockage to sub-urothelial and DSM cells may not necessarily alter their sensitivity to afferent nerves. As these nerve fibres are abundant in the sub-urothelium which act as a sensor during filling. It could be speculated that the sensation of fullness remains intact. Thus keeping transitions between filling and voiding intact but reducing overall spontaneous contractions. Alternatively, this effect may lead to hypersensitivity due to an increase accumulation of diffusible agents. Previous studies have shown the use of P2X antagonists elevated the threshold and reduced peak activity of high threshold fibres in the bladder (Aizawa et al., 2011, Rong et al., 2002).

Transmitter release from urothelial cells changes during pathological conditions. Both ATP and ACh release substantially increase from tissue from patients with idiopathic detrusor overactivity or interstitial cystitis, and also from older compared to younger patients (Birder et al., 2003, Birder et al., 2004, Birder et al., 2012, Sugaya et al., 2009). Other chemical mediators also increase during pathological conditions including PGs and PGE$_2$ for example directly increases contractile function (Andersson et al., 1977, Park et al., 1999). Thus, the use of bulking agents to minimise the communication between mucosa and DSM via chemical mediators offers a treatment to manage pathological conditions such as OAB.
4.4.2 Accuracy of placement and spread of injectate.

The spread of injectate from the mucosa/DSM interface may have functional consequences for phasic and tonic contractions. Injection of Tyrode’s solution spreads readily through the bladder wall, whereas, PEG and especially coaptite remained localised to the sub-urothelium, proximal to the DSM. An increased spread of injectate may increase the spacing between DSM bundles, as well as the mucosa/DSM interface, transiently but any effect this may have on contractile function would be expected to dissipate as the solution is cleared. This would be less evident with PEG and coaptite injections and may have a longer-lasting action.

We have previously shown from gross observations of bladder wall preparations that PEG injections (37°C) showed a slight spread of compound within 24 hours (figure 4.12). However, this was probably due to leakage of injectate from around the injection site as histological observations showed no significant spreading from the mucosa/DSM interface region. Furthermore, the injectate did not subsequently spread for more than 96 hours at 4°C (Hague et al., unpublished data). However, as the melting point of PEG 600 is between 15-25°C, body temperature conditions would liquefy the compound and facilitate spreading in the longer term. In addition, the particle size of PEG is 4-100 μm which will also facilitate leakage. Coaptite may be preferable, especially when incorporated into a soft collagen mesh, as its particle size is greater (Vaizey and Kamm, 2005, Vaizey et al., 2007).
Figure 4.12. Spread of sub-urothelial PEG injection.
Different volumes (numbers in mls) of injectate with carbon particles were injected into the sub-urothelium; A) time = 0, B) time = 24 hours at 37°C (Hague et al., unpublished data).

4.4.3 Potential mechanism for reduced spontaneous contractions.

Figures 4.13A-B shows diagrammatically the communication between mucosa and the detrusor smooth muscle layer under normal conditions and in the presence of an inert injectable agent. Normally, release of urothelial mediators modulate detrusor activity either by their diffusion or by exciting intermediate cells such as ICs.
Figure 4.13. The communication between urothelial cells and underlying tissue layers in the presence and absence of injectate. The release of chemical agents from urothelial cells act on sub-urothelial cells to modulate Ca\(^{2+}\) transients. These have been shown to play a role in spontaneous activity. A) null-injectate, allows sufficient communication between the mucosa and DSM which is blocked by, B) injectate.
CHAPTER 5. DISCUSSION.
5.1 Summary and interpretation of principal findings.

Two novel approaches were used as potential therapeutic treatments to manage the overactive bladder: heating the bladder mucosa; injection of inert bulking agent in the suburothelial space. The hypothesis under test was that these approaches would reduce spontaneous contractions, as it has been observed previously that isolated preparations from patients with OAB have increased spontaneous contractions (Brading, 1997, Fry et al., 2010). With both applications, a reduction of spontaneous contraction was observed, using isolated preparations and ex vivo perfused bladders.

With respect to application of heat, the initial experiment was to determine whether heating to 42, 46, 50°C had different effects on spontaneous contractions. It was found that all temperatures reduced the tension integral (AUC) of spontaneous contractions. More detailed measurements were done to determine which component of spontaneous contractions were responsible for the reduction of the AUC where it was found that this was due to a reduced amplitude and shortening of contractions, offset by an increased frequency. The role of TRP channel activation by heating as a mechanism to reduce AUC was examined. The TRPV₁ channel subclass is activated during heating to 42°C (Venkatachalam and Montell, 2007, Avelino et al., 2013) and was considered as the primary target. However, reduction of spontaneous contractions on heating was unaffected by TRPV₁ receptor antagonism.

The bladder wall is a complex structure of urothelium, suburothelium (collectively the mucosa) and detrusor layers and further experiments were done to determine which of
these layers might contribute to the reduction of spontaneous contractions with heating. Preparations with the mucosa removed (detrusor-only) were used to measure the effect of heating on agonist-induced and nerve-mediated contractions to investigate the role of heating on detrusor muscle itself and the motor nerves to detrusor. Heating to 42°C had only minor effects on agonist-induced or nerve-mediated contractions; however, at higher temperatures (50°C) these contractions were completely abolished. It was therefore hypothesised that changes in spontaneous contractions during milder heating to 42°C were mediated either by changes to spontaneous contractions elicited by the mucosa or by interactions that may occur between mucosa and detrusor. Furthermore, at higher temperatures there may be additional tissue damage. The effect of heating on mucosa spontaneous contractions was similar to that on detrusor-only preparations, in particular at the smaller temperature increase to 42°C. Tissue integrity was examined at all temperatures. A smaller increase of temperature (42°C) had little effect on tissue integrity but higher temperatures (46 and 50°C) caused significant especially to the urothelium, in part due to larger temperatures at the urothelial face due to the method of heat delivery.

From these observations, it may therefore be hypothesised that reduction of spontaneous contraction magnitude with heating, in particular to a low temperature of 42°C, is due to alteration of sub-urothelial and detrusor smooth muscle interactions. Moreover, such interaction may have a protective effect as spontaneous contractions recovered at higher temperatures (50°C), even as nerve-mediated and agonist-induced detrusor responses were severely reduced at this temperature. Thus, sub-urothelial-detrusor interaction either via cell-to-cell interaction or diffusion of chemical mediators may be reduced. For
example, heat exposure does decrease gap-junction opening probability (Saez et al., 2005). In addition, PKC is activated to a greater extent during heating, and may decrease Cx43 gap junction opening (Cesare et al., 1999).

However, it was observed that heating actually increase urothelial ATP release and this will counter the hypothesis that decreased release of this purine would mediate the reduction of spontaneous contraction by reducing mucosa-detrusor interaction.

With respect to the use of injectables, the reduction of AUC was mirrored by a reduction of contraction amplitude. The use of different injectables (Tyrode’s, PEG, or coaptite) had similar effects on changes to the contractile variables, except that contraction frequency, which decreased, had no difference, and increased with respect to injecting Tyrode’s, PEG, and coaptite, respectively. All injectables also modulated the magnitude of the tonic CCh contraction: Tyrode’s injectate had the greatest effect and coaptite the least. Therefore, coaptite would be the most appropriate agent to reduce spontaneous contractions but least affect the normal cholinergic path of contractile activation. ATP and ACh release during bladder distension or stretch from urothelial cells increases spontaneous contractions (Kanai et al., 2007). Therefore, it is emphasised that these mediators play a large role in controlling spontaneous contractions in the bladder and their reduction by inert bulking agents could be through a reduced diffusion of these chemical mediators.
5.2 Novel approaches against OAB.

OAB is one of the most common and least understood pathologies in the bladder. There is a huge demand for therapeutic treatment due to the side-effects of current antimuscarinic therapeutics and the consequent lack of patient compliance. Therefore, these two novel approaches of heating and the sub-urothelial injection of inert agents make them highly attractive as paradigms to alleviate the symptoms of OAB. As previously discussed, OAB is significantly associated with an increased spontaneous activity in isolated preparations. Therefore, by introducing processes to reduce these contractions, they offer a means to prevent the sensations associated with OAB symptoms.

5.2.1 Heating as a novel target against OAB.

Heating is a potential therapy against OAB due to its ability to reduce spontaneous contractions. However, there is a need to strictly control the temperature to which the bladder is exposed too. For example, mild heating (42°C) has the ability to reduce spontaneous contractions with minimal changes to tissue integrity. However, whilst greater heating (50°C) also reduced activity, changes to tissue integrity were also evident. Furthermore, at this temperature a profound inhibition of agonist-mediated and nerve-mediated contractions was seen. Therefore, the use of higher temperatures may reduce OAB symptoms but could promote side-effects including sensation of pain, detrusor underactivity and urinary tract infections due to stasis of urine in the lower tract. Sensitisation to painful stimuli, such as heating, can result from tissue damage or intense nervous stimulation. Sensitisation to pain varies among internal organs. For example, the liver, kidneys, and lungs can suffer considerable damage before induction of pain. Hollow
viscera, including the bladder tend to have a lower threshold to pain (Julius and Basbaum, 2001, Wood and Perl, 1999).

The pain threshold in many peripheral nerves is around 42°C. After this, inflammatory mediators decrease the threshold considerably. Therefore it will be very important to measure the pain threshold if heating is to be used as a potential therapeutic. Furthermore, the increased ATP release during heating may activate P2X3 receptors on afferent nerves that could induce pain (Burnstock, 2001). Thus there are many factors that need to be considered before any therapeutic treatment based on bladder heating can be used clinically.

In clinical practice, the use of a permanent implanted heating coil would be most feasible and would reduce repeated invasive surgical techniques. One approach for implanting a heating coil, would be attached to an intravesical catheter with associated pressure monitor attached. This would monitor pressure changes in the bladder from OAB patients and to titrate appropriate amounts of heating. Thus this approach could minimise OAB contractions with a minimum of side-effects.

5.2.2 Inert bulking agents as a novel target against OAB.

The use of inert bulking agents may also be used potentially to treat the symptoms of OAB due to their ability also to reduce spontaneous contractions. Bulking agents are ideal candidates as a therapy due to their current use in clinical practice and demonstrated biocompatibility. They are currently being used against stress urinary incontinence (SUI)
as a sub-urothelial injection into the bladder trigone/proximal urethra. These agents form an inert bulk around the bladder neck to prevent leakage of urine during stresses on the bladder, i.e. coughing (Herschorn, 2005). However, injection of these compounds often needs to be repeated, often at six-month intervals, due to breakdown.

For clinical practice, the volume, number of injections, migration of injectate, and long-term effectiveness are highly important. All these points must be addressed to confirm their success as a long-term treatment against OAB. In principle, the use of coaptite seems to be the most ideal bulking agent due to its low migration, biocompatibility, and a consistent volume of a collagen mesh even when the solid particles are broken down (Vaizey et al., 2007). The number of injections will need to be determined as complete inhibition of communication within the bladder wall could lead to bladder retention. Partial blockage at different regions throughout bladder wall might be sufficient.

Overall, the use of heating seems to be a better candidate in terms of finely controlling spontaneous contractions and monitoring the ‘time to micturate’ to manipulate normal bladders. However, the ability to produce a clinical device remains uncertain due to potential side-effects - i.e. painful sensations. Therefore, for economic purposes and ease of use in clinical practice, bulking agents may be preferred. However, these also have concerns in terms of the need for repeated injections. It may be considered that incorporating coaptite into botulinum toxin (BoTox) injections may be a combined therapy with long-lasting effects.
5.3 Critique of methodology.

Although these studies provide detailed and concise findings about reduction of spontaneous contractions, they did not use human bladder wall samples, especially from patients with OAB. The pig bladder has been used as a useful model of the human bladder in terms of its size, physiological contractile function and perfusion. When heating the bladder, different effects on contractility have been shown with rabbit, guinea pig and pig tissue. For example, heating to 42°C did not decrease agonist-induced contractions in the pig bladder, whereas in the rabbit bladder the opposite was shown. Moreover heating reduced spontaneous contractions in rabbit bladder only on removal of the intervention (Hague et al., unpublished data). Therefore it is important to explore the effects of heating in the human bladder. Furthermore, although an ex vivo model was established to complement the in vitro phenomena (reduced AUC is a consequence of reduced amplitude and greater shortening of contractions), it is necessary to observe this effect in vivo. In vivo studies would give us a clearer understanding of the effects of heating on the micturition reflex.

The use of a heated coil to raise bladder wall temperature is a convenient construction for a clinical device. However, a limitation identified by this study was that changes to bladder wall thickness would influence the temperature gradient across the wall. In addition, although the area of exposed bladder mucosa remained consistent, the distance between the mucosa surface and heating coil may have varied. The sub-urothelium temperature increased to the same degree between preparations, however, the temperature gradient between the urothelium surface and DSM may have varied. Thus, the positioning
of a heating coil in the intact bladder would be important. Also, from a technical aspect slight differences in the position of the thermistor probe in the sub-mucosa-detrusor interface might have generated some error to the actual temperature recorded in this space. It could not be determined if there was a temperature gradient in this space and to determine this, very small thermistor probes would have to be used. However, because the base of the sub-mucosa has such a copious blood supply and would in effect ‘cool’ heated tissue, this would tend to diminish any gradients in this region.

Although we did see changes to pH during heating, these changes were seen to show no effect on spontaneous contractions. However, it is also possible that heating may have altered the pCO₂ composition of the Tyrode’s solution. Typically a decrease in pCO₂ would lead to an increase in pH. This has been widely reported including heat-induced changes to pCO₂ in blood (Gothgen, 1984). It could be possible that changes in contractility maybe a direct action of changes to pCO₂ of Tyrode’s rather than heat itself.

The reduction of spontaneous contractions was maintained for at least an hour during heating, but for a clinically-effective device the effect would have to be much longer. It is not known if prolonged heating, even if for intermittent periods, over months or years could cause adaptation of the effect. The next stage of development could use an implantable device in an in vivo model.
Changes to tissue integrity also remains a problem. Heating to higher temperature did cause changes to tissue integrity, but whether this was due to cell damage was not determined and this would require labelling with markers of apoptosis and/or necrosis.

This study has shown a clearer understanding of the mechanisms by which heating the bladder wall reduces spontaneous contractions but the precise pathways remain to be characterised. Heating is likely to disrupt interactions between the mucosa and detrusor layers rather than affecting the contractile properties of the individual layers themselves. Also intriguing is that although higher temperatures severely reduced contractions in individual layers, the effect was somewhat less with intact preparations. To further study a disruption of mucosa-detrusor interaction optical imaging (Ikeda et al., 2007, Kanai et al., 2007), may be used to explore the spread of Ca$^{2+}$ and membrane potential waves between these layers during heating.

The use of injectables also showed some limitations. Although their use clearly reduced spontaneous contractions, it remains unclear to whether these changes are due to the injectate itself or physical damage from the injection needle. A useful control experiment would be to determine the effect, if any, of merely piercing the mucosa-detrusor interface with a needle.

Furthermore, there was a large variability between different preparations with some interventions that may be due to changes in the tissue area during the experiment. In particular with experiments using injectables, this could have arisen from re-clamping of
the tissue preparation after injection. When the tissue preparation was re-clamped, any slight differences in the position of clamping could increase or decrease the contractile force of the tissue preparation. In addition, the area of tissue infiltrated with injectable would disrupt communication to different extents.

5.4 Conclusion and further experiments.

This research has shown some novel findings for the effects of heating and inert bulking agents on spontaneous contractions in the bladder. These approaches were successful in significantly reducing spontaneous contractions. The large subset of this thesis (heating) showed that it could be through a means of sub-urothelial and DSM interactions. It has further explored and contradicted previous theories to show that the urothelium may not be as important in mediating or controlling spontaneous contractions as we initially thought. Thus our findings suggest that both approaches may be recognised and used in clinical practice to treat OAB patients. However, further detailed studies into the mechanisms of action must be elucidated.

Heating. To better understand the effects of heating on reducing spontaneous contractions, further experiments may be proposed. Firstly, it is important to determine whether the reduction of spontaneous contractions is by a mechanism of altered diffusion or changed cell-to-cell communication between the mucosa and the underlying smooth muscle. To examine this the following may be carried out:
1. Remove the mucosa and determine changes to spontaneous contractions when the mucosa is merely placed on top of the detrusor in the presence and absence of heating.
   a. Preliminary data shows that placing the mucosa layer on top of the detrusor enhances spontaneous contractions but heating does not reduce this enhancement.

2. Examine the role of gap junctions during heating to test the hypothesis that the reduction of spontaneous contractions could be due to reduced opening of gap junctions between adjacent cells.
   a. Heating in the presence or absence of gap junction blockers (e.g. carbenoxolone) can be carried out.
   b. The phosphorylation states of Cx43 and PKC should be quantified using biochemical techniques for example western blots. This will determine whether heating is an effect of reduced gap junction conductance via PKC phosphorylation.

3. Measure the transfer or spread of electrical activity across the bladder wall using optical imaging techniques.

4. To quantify the effects of enhanced ATP release on bladder spontaneous contractions.
   a. The use of adenosine or P2Y specific blockers may reverse the effects of heat-induced reduction of spontaneous contractions.

5. Measure the transfer or spread of electrical activity across the bladder wall using optical imaging techniques.
An additional experiment to describe the effects of heating can be to determine whether the changes to tissue integrity is due to cell death. To do this:

6. Quantify the extent of cell damage or death with heating using apoptotic and/or necrotic markers.

Further studies could explore the possible role of other TRP channel subtypes:

7. Use TRPV$_2$ channel antagonists during heating and measure spontaneous contractions. This channel sub-type is activated at higher temperatures than TRPV$_1$ channels.

These experiments must be translated to an *ex vivo* preparation if the concept of mild heating to reduced overactive bladder activity may be extended to clinical use. This may with the further evaluation of isolated perfused pig bladder preparation described in Chapter 3.

**Injectables.** To understand better the effects of injectables on reducing spontaneous contractions, further experiments are required.

Firstly, a confirmation of location of the injection is necessary. This may be best done by including black carbon particles in the injectate to quantify their location.
Secondly, it needs to determined if the method of injection (metal needle or a plastic insert) itself causes tissue damage. This would be done by following the same protocol and piercing the sub-urothelium with a needle.

Thirdly, the influence of injectate on stress-induced urothelial transmitter release (ATP and ACh) needs to be determined.
References.


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