Pharmacological investigation of porcine bladder function and sensory activity

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DEDICATION

For Mum and Dad

For all the sacrifices you have made over the years for me.

You taught me to believe that I can achieve anything I set my mind to!

To my brothers and sisters - Caroline, Roy, Lilian, Anastasia, Larry, Miriam

My nephews and niece - Rhoda, John-Roy, Charles and BJ

For your encouragement throughout my thesis, here's to the first Dr. in the family!

My partner

Ryan Hall - For all your love, support and encouragement especially this past year. I know it has been challenging for you, but I hope that now this is done, we can embrace the coming change. Here's to the future.

My adopted family - Kathie, Ian, Gavin and Mandi

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ABSTRACT

Overactive bladder (OAB) is a common condition of the lower urinary tract (LUT) that is characterised by urinary urgency, with or without incontinence, accompanied by frequency and nocturia as a result of involuntary urinary bladder contractions. OAB affects millions of people worldwide and has a significant impact on quality of life. A significant amount of research has been undertaken, and is still on-going, investigating the underlying mechanisms of bladder dysfunction and the development of new therapeutic agents for the treatment of LUT conditions. It is now clear that the simplistic ideology of the bladder acting as a passive reservoir is far more complicated, and involves complex mechanisms between the different components of the bladder wall.

The recently emerging role of the bladder urothelium and sub-urothelium in regulating bladder functions has laid the foundation for a hypothesis supporting a mechanosensory basis for OAB. The urothelium expresses several different types of receptors and ion channels, and upon stimulation releases mediators such as ATP, nitric oxide and acetylcholine. These mediators act on receptors in the urothelium and in the underlying nerves and muscle to mediate bladder activity, and although these mechanisms are still not completely understood there is evidence to suggest that in pathological conditions, these pathways may be altered. The aim of this project was to investigate the mechanisms, structures and transmitters involved in these mechano-sensory pathways in the bladder wall using *in vitro* pharmacology, histology, immunohistochemistry and mediator assays

Spontaneous phasic contractions (PCs) developed in tissue strips isolated from the dome, body and trigone regions of the pig bladder, and the amplitude and frequency of these PCs was measured. Functional and structural differences were observed between the different bladder regions, particularly in the trigone versus the dome and body. Removal of the mucosa significantly increased the development time of PCs in the dome and body but not in the trigone. Mucosa removal also significantly decreased the frequency of contractions in the dome and body but not in the trigone. Since increased PCs of the bladder are associated with OAB, the mechanisms regulating these contractions were explored. Phasic contractions amplitude could be increased by low concentrations of the muscarinic receptor agonist carbamylcholine (carbachol - CCh), and functional differences were observed in the trigone compared to the dome and body regions. The muscarinic receptor subtype involved in mediating the effect of CCh was also investigated, but from the results obtained it was not possible to determine which subtype was responsible.

Research into the role of interstitial cells (ICC) in the bladder has grown in the past decade since this specialised class of cells was discovered in the urinary bladder. It is postulated that these cells may be involved as mediators of mechanosensory function in the bladder. The expression of c-Kit, a tyrosine kinase receptor, on ICC allowed for the functional role of these cells in PCs of the bladder to be investigated, and to attempt to determine their localization in the different bladder layers using immunohistochemistry. Imatinib, a tyrosine kinase receptor inhibitor, had no effect on the amplitude or frequency of PCs in tissue strips from the different bladder regions, suggesting that c-Kit positive ICC may not be involved in PCs in the pig bladder. A population of cells staining positive for vimentin, but lacking specific c-Kit staining, was identified but cannot be conclusively identified as ICC.

Investigation and measurement of ATP release using a luciferase assay kit in response to stretch was also performed in the dome and body regions of the bladder. ATP release was found to increase with stretch. The presence of a heterogeneous population of cells in the bladder including detrusor smooth muscle cells, urothelial cells and ICC makes it difficult to determine the exact source of ATP, although literature suggests the urothelium to be the dominant source.

In summary, the data presented in this thesis support a role for the urothelium and suburothelium the complex physiological processes of the pig urinary bladder. Further research is required into the integrated physiology of the bladder wall, and this may contribute to the discovery of new targets for pharmacological agents to treat lower urinary tract conditions.

PUBLICATIONS

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ABBREVIATIONS

ATP-γ-S	adenosine 5'-O-(3-thiotriphosphate)
2-MeSATP	2-(methylthio)ATP
4-DAMP	4-Diphenylacetoxy-N-methylpiperidine methiodide
abl	Abelson proto-oncogene
AC	adenylate cyclase
ACh	acetylcholine
ADP	adenosine diphosphate
ANOVA	analysis of variance
ATP	adenosine triphosphate
BK_{Ca}	calcium activated large conductance potassium channel
BOO	bladder outlet obstruction
BPH	benign prostatic hyperplasia
BSA	bovine serum albumin
BTX	botulinum toxin A
BzATP -	3'-O-(4-benzoyl)benzoyl ATP
cAMP	cyclic adenosine monophosphate
CCh	carbamylcholine chloride (carbachol)
cGMP	cyclic guanosine monophopshate
c-Kit	tyrosine protein kinase ***Kit***
CML	chronic myeloid leukaemia
CSLM	confocal scanning laser microscopy
DAG	diacylglycerol
dH_2O	distilled water
DO	detrusor overactivity
DRG	dorsal root ganglia
DS	donkey serum
EFS	electrical field stimulation
FITC	fluorescein isothiocyanate
GDP	guanosine diphosphate
GFP	glomerular filtration pressure
GI	gastrointestinal
GISTs	gastrointestinal stromal tumours
GPCR(s)	G-protein coupled receptors
GTP	guanosine triphosphate
H&E	hematoxylin and eosin
HGN	hypogastric nerve
IC	interstitial cells
ICC(s)	interstitial cells of Cajal
ICC-DMP	interstitial cells of the deep muscular plexus
ICC-DT	detrusor interstitial cells
ICC-IM	intramuscular interstitial cells
ICC-LM	longitudinal muscle interstitial cells
ICC-LP	lamina propria interstitial cells

ICC-MP	interstitial cells of the myenteric plexus
ICC-SM	interstitial cells of the sub-mucosa
ICC-SS	interstitial cells of the sub-serosal
ICC-SU	sub-urothelial interstitial cells
ICS	international continence society
I_gG	immunoglobulin G
IHC	immunohistochemistry
IMP	inferior mesenteric plexus
IMS	industrial methylated spirit
IP_3	inositol triphosphate
K_{ATP}	potassium sensitive ATP channel
Kd	dissociation constant
LUTS	lower urinary tract symptoms
MB	muscle bundle
MTS	Masson's trichrome stain
NA	noradrenaline
NANC	non-adrenergic non-cholinergic
NGF	nerve growth factor
NK1	neurokinin receptor 1
NK2	neurokinin receptor 2
NO	nitric oxide
OAB	overactive bladder
Oxo-M	oxotremorine methiodide
PA	phasic activity
PBS	phosphate buffered saline
PC (s)	phasic contraction
PDGFR	platelet derived growth factor
PEL	pelvic nerve
PNS	parasympathetic nervous system
PNTR	presynaptic neurotransmitter release
PPADS	pyridoxal-5'-phosphate-6-azo-phenyl-2,4-disulfonate
Rho A	rho kinase A
RNA	ribonucleic acid
ROCK	rho kinase
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
SBB	sudan black B
SCF	stem cell factor
SCI	spinal cord injury
SEM	standard error of the mean
SHP	superior hypogastric plexus
SMA	smooth muscle actin
SMC(s)	smooth muscle cell
SN	sciatic nerve
SNS	sympathetic nervous system
SU	suburothelium

TK-I	tyrosine kinase region I
TK-II	tyrosine kinase region II
TM I	transmembrane region 1
TM II	transmembrane region 2
TNP-ATP	2',3'-O-(2,4,6-trinitrophenyl) ATP
TRPV1	transient receptor potential V1 vanilloid receptor
TTX	tetrodotoxin
TXRED	texas red
U	urothelium
UDIF	urothelium derived inhibitory factor
UDP	uridine diphosphate
UTP	uridine triphosphate
VUJ	vesico-ureteric junction
WHO	world health organization
α,β-MeATP	alpha,beta-methylene ATP

CHAPTER 1

GENERAL INTRODUCTION

INTRODUCTION

The urinary system is composed of a pair of kidneys which form urine, ureters that carry the urine from the kidneys to the bladder, the urinary bladder which stores the urine and periodically eliminates it, and the urethra through which the urine is excreted. The main functions of the urinary system are elimination of waste products from the blood, elimination of these waste products into the environment and homeostatic regulation of blood plasma volume and solute concentration (Martini and Nath 2009). The kidneys filter on average 125ml of plasma per min of which 124ml/min is reabsorbed and 1ml/min is excreted as urine. Thus of the 180L of plasma filtered in day, 1.5L of urine is formed (Sherwood 2008) with the urinary bladder vital to the urinary system as the major player in the storage and elimination of the toxic wastes excreted into the urine.

1.1 URINARY TRACT ANATOMY

1.1.1 THE URETERS

The ureters are a pair of muscular tubes that extend inferiorly and medially from the renal pelvis to the bladder base and are firmly attached to the posterior abdominal wall (Martini and Nath 2009). The walls of the ureters are composed of 3 layers - the inner mucosa made up of transitional epithelium, a middle layer of both circular and longitudinal smooth muscle and an outer connective tissue layer. The ureters function under low pressure to transport urine from the kidneys to the bladder and this occurs via intrinsic peristaltic contractions. The urine drains into the bladder at the vesico-ureteric junction which, if correctly functioning, allows for unidirectional flow of urine and contains a mechanism to prevent retrograde flow of urine back into the ureters from the bladder. This protects the ureters from bladder infections and the high pressure encountered in the bladder during voiding (Chapple 2011).

1.1.2 THE URINARY BLADDER

The urinary bladder is a hollow, muscular organ located in the pelvic cavity and is primarily involved in the collection, storage and emptying of urine (Andersson and Arner 2004). The bladder is anchored by the middle and lateral umbilical ligaments towards the umbilicus, while the anterior, posterior and inferior surfaces of the bladder are anchored to the pelvic and pubic bones by tough ligamentous bands (Martini and Nath 2009). Depending on its state of distension, its size varies and it can hold anywhere between 400ml-1000ml of urine (Chu and Dmochowski 2006). The bladder can be anatomically divided into three sections: - the bladder dome which forms the top

of the bladder, the bladder body which is located above the ureteral orifices and the bladder base comprised of the trigone, anterior bladder wall, urethral-vesical junction and deep detrusor (Andersson and Arner 2004). (Figure 1.1) The bladder wall, is composed of several layers of cells namely the urothelium, sub-urothelium, detrusor muscle and adventitia or serosa.

1.1.3 THE BLADDER WALL LAYERS

The urothelium is the transitional epithelial lining of the urinary tract and extends from the renal pelvis to the urethra. It is composed of 3 layers of cells - a basal layer comprised of small cells attached to the basement membrane, an intermediate layer of moderately sized cells and an apical superficial layer of hexagonal "umbrella" cells (Apodaca *et al.,* 2003). The umbrella cells are characterised by rigid membrane plaques composed of uroplakin membrane proteins that have an important role in the maintenance of urothelial permeability and physical integrity (Riedel *et al.,* 2005). These umbrella cells maintain the urothelial barrier against small molecules such as water, urea and protons via tight junction complexes composed of specialised membrane lipids. They are organised into heterodimers interconnected with extensive junctional complexes (Birder 2005). The basal cells are involved in the continuous regeneration of worn out or damaged urothelial cells.

The bladder urothelium was traditionally seen as passive protective barrier between the urinary tract and its contents. This simplistic view has changed over the years with emerging evidence of the highly specialized functions of the urothelium. Besides its protective function, the urothelium has been shown to be a responsive structure capable of detecting chemical, mechanical and thermal stimuli, and expresses a number of receptors such as purine receptors (Ferguson *et al.,* 1997; Burnstock 2001; Birder *et al.,* 2003; Chopra *et al.,* 2008), acetylcholine receptors (Chess-Williams 2002; Beckel *et al.,* 2006; Kullmann *et al.,* 2008), adrenoceptors (Birder *et al.,* 2002) and adenosine receptors (Yu *et al.,* 2006). The urothelium also expresses several different ion channels including amiloride and mechanosensitive sodium channels (Carattino *et al.,* 2008) and TRP channels (TRPV1, TRPV2, TRPV4, TRPM8) (Birder *et al.,* 2002; Birder and de Groat 2007; Kullmann *et al.,* 2009). Urothelium cells have also been shown to release chemical mediators including ATP, acetylcholine, prostaglandins, nitric oxide (NO), peptides, neurotrophins and cytokines which are all capable of activating, modulating or inhibiting sensory nerves, ICC and the detrusor smooth muscle (Apodaca *et al.,* 2007; Birder and de Groat 2007; Birder 2010) Figure 1.2. Evidence has shown that the urothelium also releases an unidentified urothelium

derived inhibitory factor (UDIF) that exerts an inhibitory effect on the underlying detrusor muscle inhibiting its contractility (Hawthorn *et al.,* 2000). The UDIF is mainly released in the bladder dome and the bladder neck and its release is mediated by muscarinic receptors and not α-adrenoceptor as it had originally been proposed (Templeman *et al.,* 2002).

The sub-urothelium is located between the basement membrane of the urothelium and the luminal surface of the detrusor. It is made up of a rich network of sensory and effector nerve fibres (Birder 2001; Dickson *et al.,* 2006), a network of blood vessels and capillaries embedded in a connective tissue matrix (Miodonski *et al.,* 2001; Hossler and Kao 2007). Within this layer is also a network of interstitial cells whose physiological role is unclear but are thought to contribute to sensory processing and mediating communication between the urothelium and detrusor (McCloskey 2010; McCloskey 2011). Evidence of the urothelium's role in detection of stimuli has emerged from ultrastructural and immunohistochemical studies that have shown the presence of both afferent and efferent nerves in close proximity to and within the urothelium (Jen *et al.,* 1995; Kunze *et al.,* 2006; Birder *et al.,* 2007). Additionally, the sub-urothelial population of interstitial cells may play a role in bladder sensory pathways (Sui *et al.,* 2004; Brading and McCloskey 2005; Ikeda *et al.,* 2007; Grol *et al.,* 2008). These cells may act to amplify bladder sensory responses and mediate signals between the urothelium and the detrusor directly or via afferent nerve fibres (Fry *et al.,* 2007). The extensive linking of interstitial cells via numerous gap junctions and their close contact to nerves, in addition to their ability to respond to neurotransmitter release, makes them an ideal communication network between the urothelium and underlying bladder layers and nerves (Sui *et al.,* 2004; Brading and McCloskey 2005; Ikeda *et al.,* 2007; McCloskey 2010). A type of smooth muscle labelled the muscularis mucosae has also been identified within this layer (Paner *et al.,* 2007; Heppner *et al.,* 2011). The presence of a similar layer in the GI tract has been shown to develop spontaneous phasic contractions (Uchida and Kamikawa 2007) and although its role is well documented in the GI tract, its functional significance in the bladder is relatively unknown.

Within the literature there is a great deal of controversy regarding the nomenclature of the urothelium and its underlying layers - from the urothelium, sub-urothelium, sub-mucosa, lamina propria etc. This may be due to the comparison of these layers to those of the gastrointestinal tract. In this thesis, the term mucosa will be used, and includes the urothelium and the underlying tissues down to the detrusor muscle bundles.

The detrusor muscle, also known as the muscularis layer, is composed of 3 layers of smooth muscle (SM) fibres interspersed with connective tissue, rendering the bladder elastic. The inner and outer layers of the detrusor consist of longitudinal smooth muscle, with a circular layer in between allowing the detrusor to expand and contract during bladder filling and emptying (Martini and Nath 2009). The detrusor muscle bundles are large, often a few mm in diameter, and composed of smaller sub bundles (Brading 1987). The individual smooth muscle cells of the detrusor exhibit common features identical to other smooth muscle cells in the body and are generally long spindle shaped cells with a central nucleus. They have a diameter of 5-6μm in the region where the nucleus is located, and measure a few hundred microns in length (Andersson and Arner 2004). Among the smooth muscle cells can be found cells with long dendritic processes parallel to the muscle fibres and containing vimentin, an intermediate filament expressed by cells of mesenchymal origin (Smet *et al.,* 1996). In the trigone region, SM bundles are smaller and clearly differentiated into two layers, unlike in the dome and body where they are not arranged in distinct layers and run in all directions (Speakman *et al.,* 1987). The orientation and interaction between bladder SMCs is important as this determines the behaviour of the bladder wall and affects its shape and intraluminal pressure (DeLancey *et al.,* 2002).

Normal kidney function has an important role in the behaviour of the normal detrusor, especially during bladder filling. Urine production is dependent on low glomerular filtration pressure (GFP) and any increase in bladder pressure above the GFP ultimately prevents filtration and subsequently urine production ceases (Brading 1997b). It is therefore imperative that during bladder filling, intravesical pressure be maintained at a low threshold. The detrusor muscle layer is very compliant, which allows it to accommodate urine storage and then rapidly expel large volumes of urine in response to sudden increases in intravesical pressure following nervous stimulation, allowing for a synchronous contraction and hence micturition.

1.1.4 THE URETHRA

The urethra extends from the neck of the bladder to the exterior of the body and its length varies between males (20cm) and females (3 – 4cm). The male urethra is divided into 3 sections – proximal, membranous and spongy urethra (DeLancey *et al.,* 2002). The urethra in both sexes passes through the urogenital diaphragm, and is composed of two distinct muscle layers and an inner epithelial membrane which lines the urethral lumen and is continuous with the bladder urothelium. The outer circular muscle layer is made up of relatively small striated muscles with the inner muscle layer

comprised of slender smooth muscle bundles (DeLancey *et al.,* 2002). The urethral muscle bundles are capable of exerting tone on the urethral lumen over prolonged times effectively preventing urine leaking until bladder pressure exceeds urethral pressure and subsequently contraction of the detrusor enables micturition (Oelrich 2005).

1.2 INNERVATION OF THE BLADDER

Bladder filling and voiding is under the control of the nervous system in conjunction with the detrusor muscle, urethral smooth muscles and the urethral sphincters. As the nervous system matures, voluntary control of micturition develops and is dependent on learned behaviour thereby making the micturition reflex very complex. There are 3 sets of peripheral nerves, belonging to the parasympathetic nervous system (PNS), sympathetic nervous system (SNS) and the somatic nervous system, which are involved in the innervation of the lower urinary tract. The pelvic parasympathetic nerves excite (contract) the bladder and relax the urethra, the lumbar sympathetic nerves inhibit the bladder body and contracts the bladder base and urethra, whilst the pudendal nerves contract the urethral sphincter (Yoshimura and Chancellor 2003) (Figure 1.3).

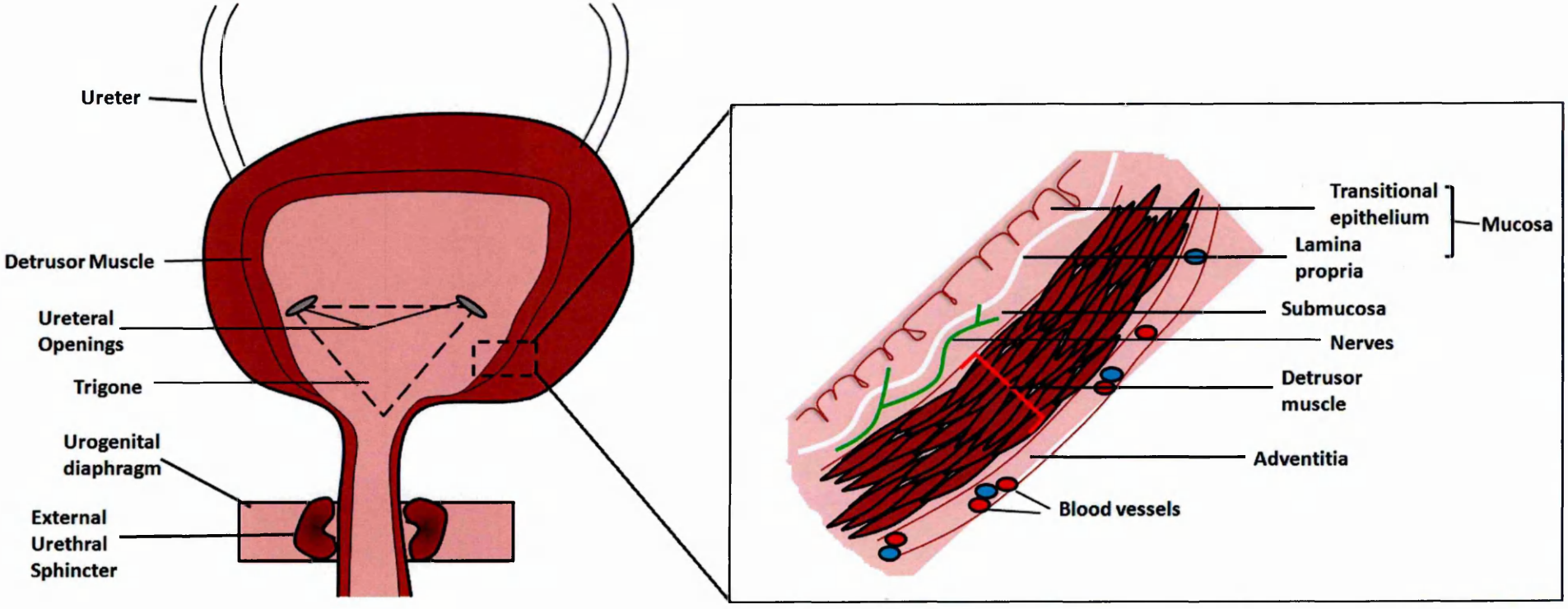


Figure 1.1- Basic anatomy of the urinary bladder. Adapted from (Herbrandson 2005).

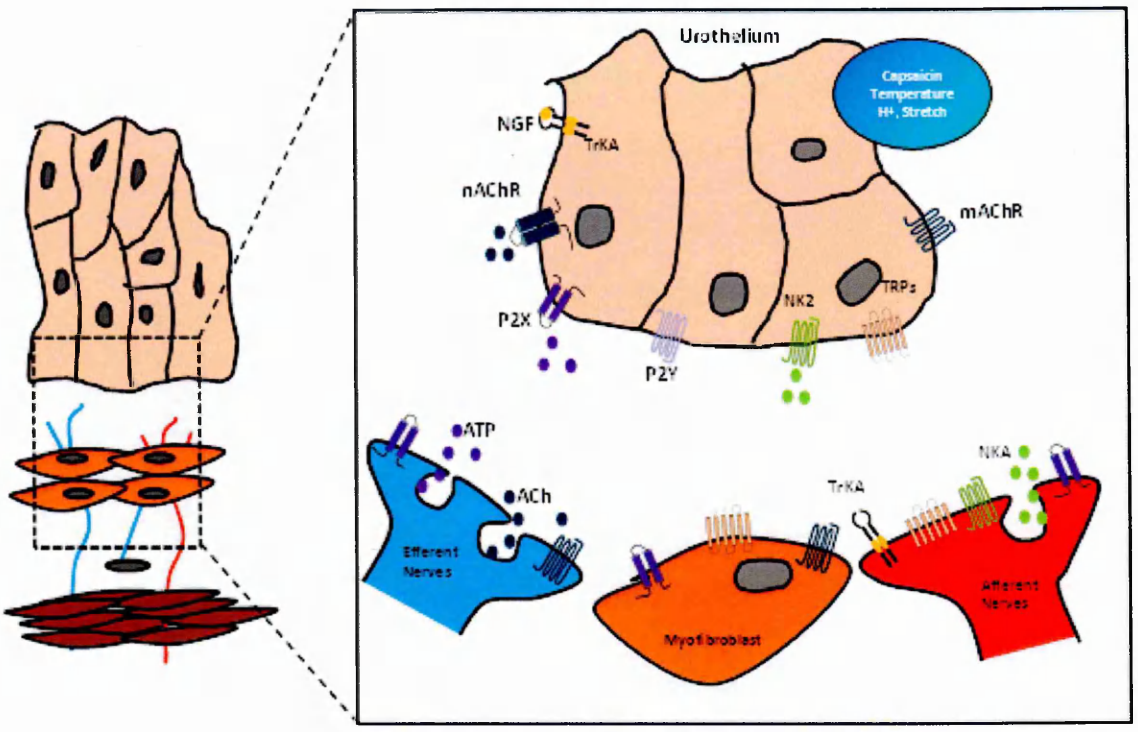


Figure 1.2 – Urothelium model highlighting various interactions between urothelium cells, bladder nerves, detrusor smooth muscle and ICCs. Mediators released due to urothelial receptor stimulation target bladder afferent and efferent nerves and other cell types within the bladder wall. Urothelial cells are also targets of neurotransmitters released from nerves; ACh, acetylcholine; Adr, adrenoceptor; BR, bradykinin receptor; H^+, proton, MR, muscarinic receptor; NA, noradrenaline; NGF, nerve growth factor; NR, neurokinin receptor; NicR, nicotinic receptor; NO, nitric oxide; P2R, purinergic 2 receptor unidentified subtype; P2X and P2Y; purinergic receptors; PG, prostaglandin; SP, substance P; trkA, receptor tyrosine kinase A, high affinity receptor for nerve growth factor; TRPs, transient potential channels. Adapted from (Birder 2010).

1.2.1 Parasympathetic Pathway

Parasympathetic pre-ganglionic neurons innervating the detrusor muscle and the vascular supply to pelvic genital organs, via the pelvic nerves and the vesicle plexus (Williamson-Kirkland and Berni 1980), originate in the S2-S4 sacral region of the spinal cord and converge in the sacral parasympathetic nucleus region (Yoshimura and Chancellor 2003) (Figure 1.3a). The parasympathetic post-ganglionic nerves release both acetylcholine (ACh) and non-adrenergic non-cholinergic transmitters (NANC) (ATP and NO) (Fowler *et al.,* 2008). The major excitatory mechanism of the bladder is via cholinergic transmission which releases ACh and results in detrusor contraction via M_3 muscarinic receptors and subsequent micturition (Fowler *et al.,* 2008). ATP released via the NANC excitatory transmission acts on P2X purinergic receptors of the detrusor further enhancing contraction, while NO exerts inhibitory input to the urethral smooth muscles allowing for relaxation and urine outflow (Andersson and Arner 2004) (Figure 1.3b)

1.2.2 Sympathetic Pathway

The inferior mesenteric ganglion connects the sympathetic preganglionic nerves to the post ganglionic neurons in the bladder, as well as to the postganglionic paravertebral ganglia and the pelvic ganglia (Yoshimura and Chancellor 2003). These sympathetic neurons innervate the bladder neck, vas deferens, seminal vesicles and the prostate via the sympathetic chain and the hypogastric plexus (Williamson-Kirkland and Berni 1980). ACh release mediates ganglionic transmission in the sympathetic pathway, acting on ganglionic nicotinic receptors, causing release of noradrenaline (NA) and activation of β-adrenoceptors, which inhibit the detrusor allowing the bladder body to relax. In addition, activation of α-adrenoceptors in the urethra and bladder neck, causes contraction of the bladder base and the urethral sphincter (Yoshimura and Chancellor 2003) (Figure 1.3b) closing the outlet and allowing the bladder to fill and store urine.

1.2.3 Somatic Pathway

The S2-S4 sacral spinal cord region contains the efferent somatic neurons responsible for innervation of the external urethral sphincter and the external anal sphincter via the pudendal nerve (Williamson-Kirkland and Berni 1980) (Figure 1.3a). These efferent somatic neurons release ACh that acts on skeletal muscle-type nicotinic receptors resulting in contraction of the urethra (Yoshimura and Chancellor 2003). (Figure 1.3b)

1.2.4 Afferent Pathways

The sacral dorsal root ganglia contain the pelvic and pudendal afferent nerves, while the rostral lumbar dorsal root ganglia contain the hypogastric afferent nerve. These afferent nerves contain axons that relay information from the lower urinary tract to the lumbosacral spinal cord and higher brain bladder control centres (Yoshimura and Chancellor 2003) and are composed of myelinated (Aδ) and unmyelinated (C) axons (Fowler *et al.,* 2008). The Aδ fibres are activated at low bladder pressures (5-15 mmHg) and respond to stretch as the bladder fills and to bladder contraction during voiding (Jänig and Morrison 1986). Afferent C fibres on the other hand are insensitive to bladder filling and respond to noxious stimuli such as chemical irritation (Janig and Morrison 1986) and cooling (Jiang *et al.,* 2002). Aδ fibres transmit information on bladder filling to the CNS and higher brain centres while C fibres, in the case of pathological conditions, convey nociceptive information leading to feelings of bladder pain (Beckel and Holstege 2011).

A dense network of sensory nerves has been identified in the sub-urothelial layer of the bladder in both animals and humans (Gosling and Dixon 1974; Gabella 1995), with some fibres showing projections into the urothelium and this may be crucial in the relaying of sensory information from the urothelium (Birder 2001; Wiseman *et al.,* 2002). ATP, ACh, prostaglandins and various other inflammatory mediators have been shown to either modulate afferent nerve firing or produce action potentials (England *et al.,* 1996; Genzen *et al.,* 2001; Dang *et al.,* 2005; Dang *et al.,* 2008). The release of these substances from the urothelium and smooth muscle cells within the bladder wall has led to the inclusion of them as part of the sensory limb of the LUT (Apodaca *et al.,* 2007).

1.3 Micturition Reflex

The micturition cycle (Figure 1.4) is a coordinated process between the neural pathways in the brain and the spinal cord to control the activities of the urinary bladder and urethra (Chu and Dmochowski 2006). These neural pathways help maintain the reciprocal relationship between the bladder and the urethra and are organized as 'on and off' switching circuits (Fowler *et al.,* 2008). Bladder filling occurs at low luminal pressure when the bladder detrusor is relaxed and the urethral sphincter is closed. Bladder emptying on the other hand is facilitated by the rapid contraction of the detrusor muscle and relaxation of the urethral sphincter (Andersson and Arner 2004).

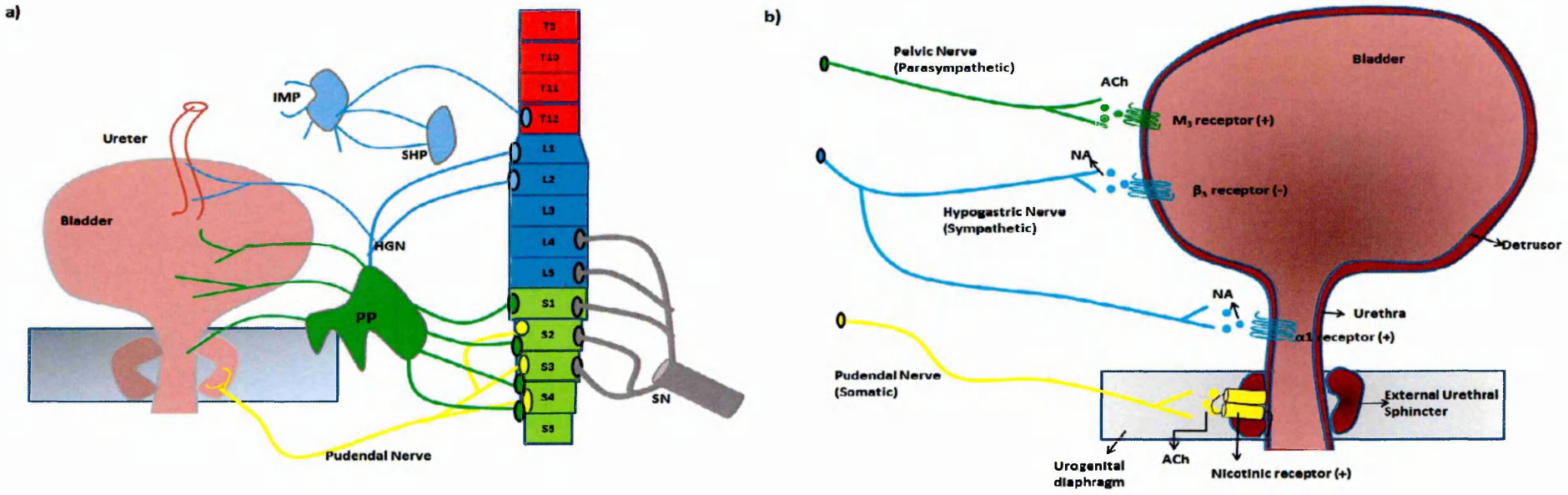


Figure 1.3 – Innervation of the urinary bladder - a) sympathetic fibres (blue) exit the spinal cord at the thoracolumbar region (T_{11} - L_2) and pass along the paravertebral sympathetic chain via the inferior mesenteric plexus (IMP) and hypogastric nerve (HGN) to enter the bladder base and urethra. Parasympathetic preganglionic neurons exit the spinal cord (S2 - S4) and send fibres along the pelvic nerves (PEL) via the pelvic plexus (PP) and onto the bladder body and the internal urethral sphincter. Somatic neurons arising from the S2 - S4 motor neurons innervate the external urethral sphincter via the pudendal nerve; b) neurotransmitter mechanisms that regulate the lower urinary tract and micturition; SHP - superior hypogastric plexus, SN - sciatic nerve, ACh - acetylcholine, NA - noradrenaline. Adapted from (Fowler *et al.*, 2008).

1.3.1 BLADDER FILLING

During the bladder filling phase of the micturition cycle the somatic and the sympathetic nervous systems work together to enhance bladder compliance and to generate sufficient outlet resistance to maintain continence (Michel and Peters 2004). Urine storage is reliant on the ability of the urinary bladder to increase in size with increasing urine volume without an appreciable rise in bladder intravesical pressure. During bladder filling afferent activity increases, conveying the state of bladder distension, and subsequent desire to void (Chapple 2011). The increase in bladder size is thought to trigger the urothelium to generate transmitter release leading to increased afferent signalling mediated by the A-δ sensory fibres (Abrams 2003; de Groat 2004). The increase in bladder compliance during filling is mediated by noradrenaline which is released from sympathetic hypogastric nerves. This stimulates β-adrenoceptors in the detrusor, which in humans mainly involves the β_3-subtype, and thus detrusor relaxation. Sympathetic nerves also inhibit the parasympathetic stimulatory pathway, restraining detrusor contractility whilst allowing contraction of the internal and external urethral sphincters to allow bladder filling (Chu and Dmochowski 2006) without any considerable changes in bladder intravesical pressure.

Detrusor relaxation is accompanied by enhanced tone in the bladder neck, urethra and pelvic floor. Resistance in the bladder neck and proximal urethra is mainly due to smooth muscle tone, whereby NA stimulates α_1-adrenoceptors to elicit contraction (Testa *et al.,* 1993; Taniguchi *et al.,* 1997; Nasu *et al.,* 2009). The distal urethra is primarily composed of striated muscle forming the external urethral sphincter (Bannowsky and Juenemann 2003). Its action is supported by the pelvic floor muscles and contraction of these muscles is mediated by nicotinic receptors activated by ACh released from somatic pudendal nerve (urethra) and sacral nerve fibres (pelvic floor) (Michel and Peters 2004; Michel and Hegde 2006).

1.3.2 BLADDER EMPTYING

Bladder volume during the filling phase is monitored by the pelvic afferent nerves and they relay information on the bladder state to the pontine micturition centre in the brain via the periaqueductal gray matter (Fowler 2002). The micturition reflex is initiated once the bladder volume has reached a critical threshold, via afferent nerves signalling to the micturition centre in the brain, thereby causing sensation of bladder fullness (Chancellor and Yoshimura 2004). The voluntary supraspinal pathways that initiate voiding are triggered once the urine volume in the bladder reaches 75% of bladder capacity causing the desire to void (Chu and Dmochowski 2006). Once this occurs,

the micturition centre sends inhibitory input to the sympathetic and somatic centres in the spinal cord - to relax the internal and external sphincters - and stimulatory input to the parasympathetic centres leading to bladder activation via ACh release and M3 receptor activation causing detrusor contraction and bladder emptying.

1.3.3 BLADDER PHASIC ACTIVITY

Bladder rhythmic activity has been described since the studies of Mosso and Pellacani in 1882. Since then, *in vitro* and *in vivo* experiments on various animal species have confirmed that the bladder is rhythmically active during the bladder filling phase and this activity has been referred as autonomous activity (Drake *et al.,* 2003; Gillespie 2004) and sometimes as phasic activity, spontaneous activity and non-micturition contractions. This activity involves waves of contraction and localised stretches of the bladder wall as it fills, and is different to micturition contractions (Gillespie 2004). The mechanisms generating these two types of bladder contractions, how they are regulated and their physiological significance has been investigated.

Using guinea pig bladder, Drake *et al.* (2003) showed that isolated resting bladder displayed small spontaneous increases in intravesical pressure resulting in waves that propagated throughout the bladder wall (Drake *et al.,* 2003). This activity was unaffected by atropine and tetrodotoxin thereby proving that it was not mediated by the CNS but rather an inherent property of the bladder itself. Muscarinic agonists increased the amplitude and frequency of this autonomous activity and this was inhibited by atropine, with TTX having no effect. The lack of inhibition of nicotinic responses by atropine and TTX implied that the postganglionic parasympathetic nerves responsible for micturition contractions did not generate this autonomous activity (Gillespie 2004) and therefore a different underlying mechanism.

Two hypotheses were suggested to explain the origins of bladder autonomous activity. The neurogenic theory suggested that the unstable contractions were caused by neural plasticity within the CNS giving rise to generalised, nerve-mediated excitation of the detrusor. This may result from inappropriate expression of spinal bladder reflexes, sensitisation of afferent terminals or as a result from damage to pathways arising from higher brain centres to the bladder ultimately causing inappropriate activation of the micturition reflex (de Groat 1997), whereas the myogenic theory suggested bladder phasic contractions are due to changes from within the bladder causing increased excitability and likelihood of spontaneous contractions due to enhanced electrical coupling between muscle cells (Brading 1997a). Since autonomous activity, phasic sensory discharge and non-micturition contractions are all features of the normal

bladder filling, any modification to the mechanisms underlying them might be responsible for changes in pathological conditions (Gillespie 2004).

Bladder strips from various LUT conditions exhibit abnormal spontaneous mechanical activity (Sibley 1984; German *et al.,* 1995; Mills *et al.,* 2000). The abnormal activity observed contains elements of fused titanic tension which is not present in normal bladder. Evidence of altered bladder PCs has been described in numerous studies. Examination of human spinal cord injury bladders compared to normal bladder showed that there SCI bladders highly expressed connexion-43 gap junctions which in turn increased intracellular communication and subsequent increased bladder PCs (Haferkamp *et al.,* 2004). Similarly, cell culture experiment performed on BOO cells also showed increased electrical coupling between muscle bundles via increased gap junctions, thereby contributing to increased PCs present in BOO (Li *et al.,* 2007). Vahabi *et al.* (2011) using a streptozotocin-induced diabetic rat model which is commonly used to study LUT conditions showed pathological bladder had increased PCs compared to control bladders (Vahabi *et al.,* 2011a)

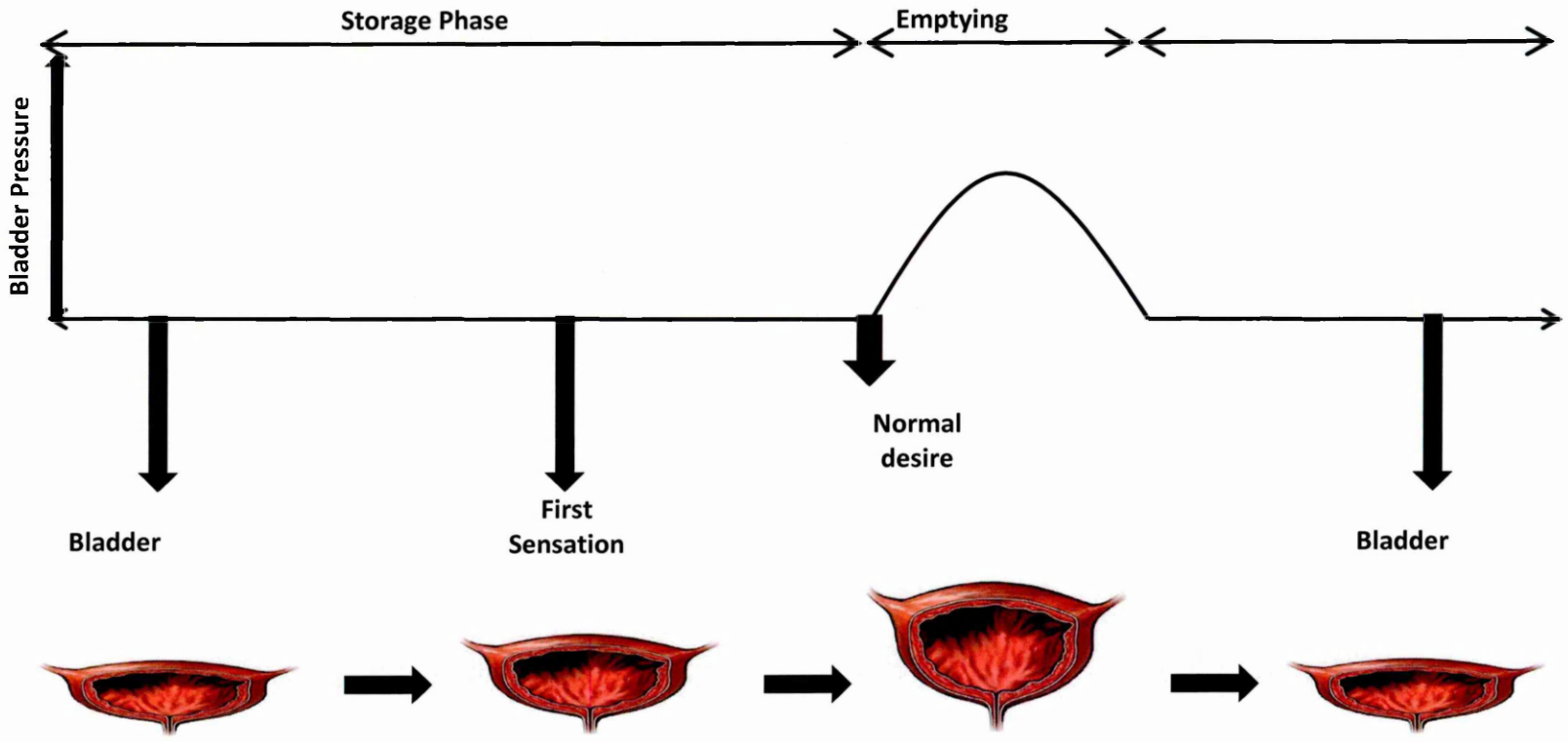


Figure 1.4 - Micturition cycle showing bladder filling with no increase in bladder pressure. This is maintained until impulses relayed from the sacral region of spinal cord to the brain signal a conscious desire to void at which point the parasympathetic nerves initiate detrusor contraction due to an increase in bladder pressure and thus emptying of the bladder. Adapted from http://www.hvurology.com/services/bladder.php (Hudson Valley Urology P.C. 2007)

1.4 Bladder Pharmacology

Bladder pharmacology is complex, with numerous types of receptors and ion channels found throughout the bladder, and involved in the various functions. These include muscarinic receptors, adrenoceptors, nicotinic receptors and purinergic receptors

1.4.1 Muscarinic Receptor Subtypes and Classification

Currently, there are five muscarinic receptor subtypes that have been identified and are designated M_1-M_5 (Caulfield and Birdsall 1998). The M1-M5 genes encode for muscarinic receptor glycoproteins which have been shown to mirror structural features, of the seven transmembrane helix, G-protein coupled receptor family (Caulfield and Birdsall 1998). A comparison of the different muscarinic receptor subtypes in various tissues is shown in Table 3.1 below. Bladder smooth muscle contains a mixed population of the different subtypes with the M_2 and M_3 muscarinic receptors being found on the detrusor smooth muscle. In addition, muscarinic receptors can be found on the urothelium and on presynaptic sympathetic and parasympathetic nerve endings (Caulfield and Birdsall 1998).

Muscarinic receptors are classified as being part of a family that when activated, are able to exert their effect by coupling to a G-protein. G-protein coupled receptors (GPCRs) are composed of seven transmembrane helices which contain an extracellular N-terminus and an intracellular C-terminus. In their inactive conformation, the Gα subunits are bound to guanine diphosphate (GDP). Upon activation by a ligand (agonist), the receptor undergoes a conformational change and substitutes the GDP for guanine triphosphate (GTP) thus activating it (Rang and Dale 2007). Once activated, there are two principal signal transduction pathways involving GPCRs: the phophatidylinositol pathway and the cAMP pathway, shown in Figure 1.5.

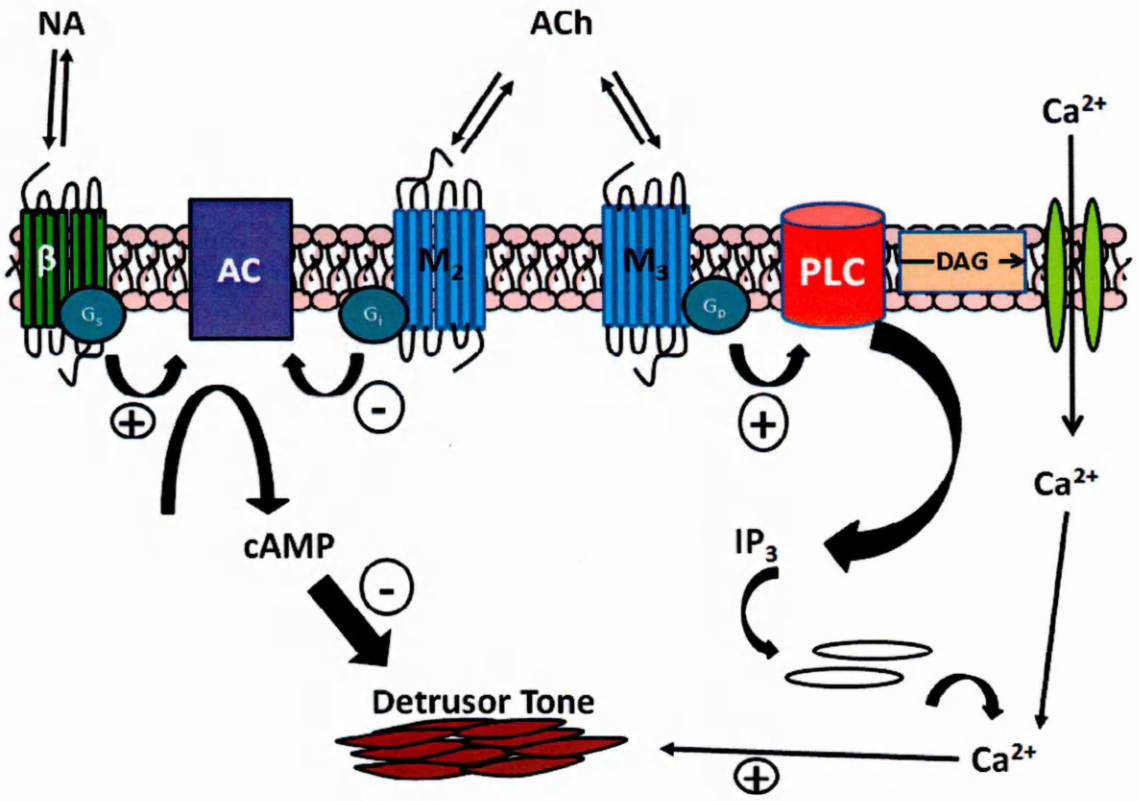


Figure 1.5 - M_2 and M_3 muscarinic receptor second messenger systems in the detrusor. Direct detrusor contraction occurs via second messenger diacylglycerol (DAG) and inositol triphosphate (IP_3) upon receptor activation by acetylcholine (ACh). On binding to M_2 receptors, ACh causes indirect detrusor contraction by inhibition of cAMP levels thereby reversing the relaxation effect due to β-adrenoceptors following stimulation by noradrenaline (NA). Adapted from (Chess-Williams 2002).

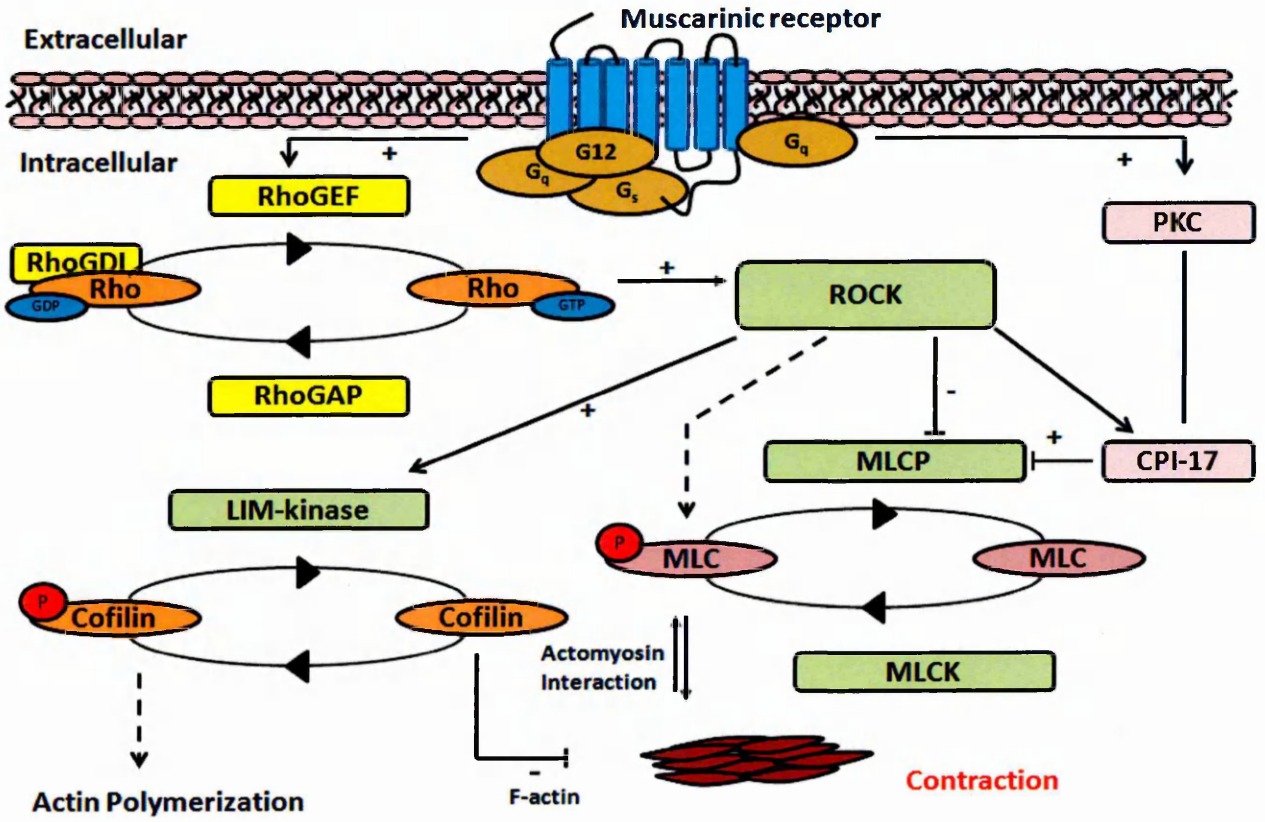


Figure 1.6 - Summary of Rho kinase coupling to muscarinic acetylcholine receptors and its downstream effector mechanisms. Rho-GEF stimulation by muscarinic acetylcholine receptors results in catalysis of Rho-GDP to Rho-GTP which subsequently interacts with its main effector ROCK which modulates smooth muscle contraction via several mechanisms. Adapted from (Peters *et al.*, 2006).

Muscarinic Receptor	G Protein Coupling	Location	2nd Messenger	Cellular Response
M1	$G_q/11$	Brain Sympathetic ganglia **Parasympathetic nerves**	IP_3/ DAG	Neuronal Depolarization
M2	G_i/o	Heart, SMCs **K^+ Channels** **Detrusor and urothelium** **Parasympathetic nerves**	AC	Decreasing PNTR Indirect Muscle Contraction
M3	$G_q/11$	**Smooth Muscle - Detrusor** Glands, Brain **ICCs**	IP_3/DAG	Muscle contraction, Gland secretion Decreasing PNTR
M4	G_i/o	Forebrain Striatum **Parasympathetic nerves**	AC	Ca^{2+} Channel Inhibition
M5	$G_q/11$	Substantia nigra	IP_3/ DAG	unknown

Table 1.1 - Muscarinic receptor subtype expression in human tissue and their respective second messenger pathways with particular emphasis on those present in the bladder shown in bold. AC – adenylate cyclase; DAG – diacylglycerol; ICCs -interstitial cells of Cajal; IP3 – inositol triphosphate; PNTR – presynaptic neurotransmitter release; SMCs - smooth muscle cells. Adapted from (Caulfield and Birdsall 1998; Scarpero and Dmochowski 2003).

1.4.2 MUSCARINIC RECEPTORS ON THE DETRUSOR

The distribution of muscarinic receptors in the bladder is not uniform. The bladder dome has the highest density of muscarinic receptors whereas the bladder neck contains the lowest (Saito *et al.,* 1997). Even though the M_1-M_5 muscarinic receptor subtypes have been seen in bladders of various species, the M_2 and M_3 receptors are the most abundant, occurring at a ratio of 3:1 respectively in several species including humans and pigs, and 9:1 in rats (Chess-Williams 2002). In spite of the M_3 receptor being the minor population, in all species to date it is responsible for direct detrusor contraction. It is thus the main mediator of detrusor contractions in the bladder (Chess-Williams 2002; Stevens *et al.,* 2007). The functional role of the majority M_2 receptor subtype is unclear but it has been proposed that it may have a role in indirectly mediating bladder contractions by enhancing M_3 receptor activated contractile responses. This has been shown using M_2, M_3 and M_2/M_3 double knockout mice (Ehlert *et al.,* 2005). Another study using rats proposed that M_2 receptor stimulation inhibits sympathetically mediated relaxation via β-adrenoceptors, which in turn leads to more efficient bladder emptying (Hegde and Eglen 1999). In certain diseased states and experimental models, M_2 receptor function has emerged in studies of neurogenic human bladder (Pontari *et al.,* 2004) and following outflow obstruction in rats (Braverman *et al.,* 1998; Braverman and Ruggieri MR 2003). Bladders from denervated rats show increased M_2 receptor density, exhibiting functional affinity more similar to M_2 than M_3 receptors (Braverman *et al.,* 1998).

M_2 and M_4 muscarinic receptors coupled via the G_i and G_o subunit inhibit adenylate cyclase subsequently reducing cAMP formation, while M_1, M_3 and M_5 receptors are coupled to $G_{q/11}$ and stimulate phospholipase C causing the release of intracellular calcium from the sarcoplasmic reticulum initiating detrusor contraction (Scarpero and Dmochowski 2003; Rang and Dale 2007).

Bladder phasic contractions and tone can also be activated and enhanced via the Rho kinase signalling pathway shown in Figure 1.6. Rho kinases (ROCKs) are serine/threonine kinases that are involved in various cellular functions of which there are two known isoforms namely ROCK-I and ROCK-II (Fukata *et al.,* 2001; Riento and Ridley 2003). Rho A, a small GTPase regulated by guanine-nucleotide-exchange factors (GEFs) is the main activator of ROCK (Peters *et al.,* 2006). A role for ROCK in bladder function via muscarinic acetylcholine receptors has been reported and extends to not only these but also to purinoceptors, neurokinin and bradykinin receptors (Wibberley *et al.,* 2003; Quinn *et al.,* 2004; Schneider *et al.,* 2004; Takahashi *et al.,*

2004; Braverman *et al.,* 2006). Stimulation of GEFs by muscarinic receptors results in the activation of Rho A which in turn results in GTP binding to Rho. This subsequently interacts with ROCK leading to increased myosin light chain phosphorylation and contractility via myosin light chain phosphatase inactivation. ROCK also promotes actin fibre formation by activating LIM-kinase which effectively inactivates cofilin - an actin depolymerisation factor - eventually enhancing contraction. ROCK inhibition by compounds such as fasudil and Y27632 results in SM relaxation (Peters *et al.,* 2006).

1.4.3 Muscarinic Receptors on the Urothelium

Muscarinic receptors have been demonstrated on the urothelium and suburothelium of the bladder. Porcine urothelium was found to express a higher density of muscarinic receptors compared to the detrusor layer (Hawthorn *et al.,* 2000). Studies on the rat and human urothelium layer confirmed the presence of mRNA and receptor proteins for all the receptor subtypes (M_1-M_5) (Tyagi *et al.,* 2006). The expression pattern of the muscarinic receptor subtypes in humans shows the presence of M_1 subtype in basal cells, M2 on umbrella cells, homogenous distribution of M_3 and M_4, with the M_5 occurring in a decreasing gradient from the luminal surface to the basal cells (Bschleipfer *et al.,* 2007). In human bladder mucosa, the M_2 subtype is the most abundant as confirmed by RT-PCR results (Mansfield *et al.,* 2005).

1.4.4 Presynaptic muscarinic receptors in the bladder

Muscarinic receptors are also found on both the parasympathetic and sympathetic nerve endings regulating acetylcholine (ACh) and noradrenaline release respectively (Chess-Williams 2002). These receptors can either enhance or inhibit transmitter release. The facilitatory mechanism seems to involve activation of pre-junctional M_1 receptors that in turn increase ACh release, while the inhibitory mechanism does the opposite by decreasing ACh and is mediated by either M_2 or M_4 receptor subtypes (Somogyi and de Groat 1999).

1.4.5 Adrenoceptor receptors in the bladder

The effects of noradrenaline from the sympathetic system and adrenaline from the adrenal glands are mediated by α- and β-adrenoceptors receptors. Functional, molecular biology and radioligand binding techniques have identified the various subtypes of adrenoceptors present in the bladder. Initially, β-adrenoceptors were classified into β_1- and β_2-subtypes, but in the last 20 years, a third subtype β_3-AR has been identified (Michel and Hegde 2006). Although all three subtypes have been shown in bladders from various species, in the human bladder, greater than 95% of all

β-AR mRNA belongs to the β_3-subtype (Nomiya and Yamaguchi 2003). In the rat and pig bladders $\beta_3 \geq \beta_2$ subtype, while in the rabbit and mouse bladder, the β_2 subtype is predominant. The guinea pig bladder expresses all three subtypes (Michel and Vrydag 2009). β-adrenoceptor distribution in the bladder is varied and is species dependent, with β_3-adrenoceptors being predominant in pig and humans (Yamanishi *et al.,* 2002). Bladder smooth muscle relaxation upon β-adrenoceptor stimulation has been shown in many species including rat, guinea pig, cats, dogs, pigs, monkeys and humans, with the maximum relaxation effect being similar across species (Michel and Vrydag 2009). Bladder relaxation occurs mainly in the detrusor and not necessarily in the bladder neck, and involves to a minor extent cAMP formation, whereas BKCa potassium channel and L-type calcium channels appear to play an important role (Brown *et al.,* 2008; Frazier *et al.,* 2008; Hristov *et al.,* 2008). The urothelium also contains β-adrenoceptors, which upon stimulation activate the adenylate cyclase pathway in bladder urothelial cells and initiates an increase in intracellular calcium triggering NO production (Birder *et al.,* 2002).

The α_1-adrenoceptors are classified into three subtypes namely α_{1A}-, α_{1B}- and α_{1D}- (Schwinn and Price 1999; Schwinn and Roehrborn 2008). In the bladder, they are located in the urothelium, smooth muscle and afferent nerves. The different subtypes can be distinguished pharmacologically based on their affinities for α_1-adrenoceptor antagonists (Michel and Vrydag 2009). Walden *et al.* (1997) have extensively researched the localization of this class of receptors and they found that in the prostate and urinary bladder of monkeys, rats and humans, the α_{1A}-subtype is the major subtype present on the smooth muscle cells (Walden *et al.,* 1997). The distribution of all the subtypes is species specific (Schwinn and Roehrborn 2008). The α_{1A}-subtype regulates smooth muscle tone in the bladder neck whereas the α_{1D}-subtype is involved in bladder function at the level of the spinal cord. The α_{1B}-subtype primarily functions to regulate blood pressure via contraction of small resistance vessels (Yamada and Ito 2011).

Beside muscarinic receptors, the bladder possesses a whole range of other receptors that have different roles in bladder function. ATP acts on purinergic receptors and play a role in modulation of bladder afferent signalling. Within this family of receptors are the metabotropic P2Y receptors and the ionotropic P2X receptors discussed in more detail in chapter 5. ATP is primarily released from the urothelium of various species (Ferguson *et al.,* 1997; Sadananda *et al.,* 2009; Kumar *et al.,* 2010). The bladder also contains tachykinin receptors consisting of substance P, neurokinin A and neurokinin B which act on NK1, NK2 and NK3 receptors respectively (Andersson 2002; Canda *et al.,*

2006). NK1 and NK2 receptors are present in the detrusor, whereas NK2 is also present on the bladder urothelium (Ishizuka *et al.,* 2000; Birder 2010). These receptors have both sensory and efferent functions including perception of pain in bladder, regulation of micturition threshold and nerve excitability (Maggi 1995; Gu *et al.,* 2000; Andersson 2002).

Bradykinin receptors are expressed in the bladder detrusor smooth muscle and on basal and apical cell of the urothelium and help in detrusor contractility. The effects of bradykinin are mediated by B1 and B2 receptors and they have a role in normal and pathological conditions (Lecci *et al.,* 1999; Chopra *et al.,* 2005; Fabiyi and Brading 2006). In addition, endothelin-1 receptors stimulate proliferation of urinary bladder detrusor contractions (Ukai *et al.,* 2006; Ukai *et al.,* 2008).

Our understanding of normal bladder function, though much increased in recent years, is still limited, as is our understanding of the pharmacology of pathological conditions of the bladder, thus further research in this area is needed.

1.5 Overactive Bladder

1.5.1 Definition

The International Continence Society (ICS) has defined Overactive Bladder (OAB) as urinary urgency with or without urge incontinence usually accompanied by frequency and nocturia (Abrams *et al.,* 2003). The key symptom of OAB is urgency, the sudden compelling desire to void that is impossible to defer (Abrams 2003). Frequency is defined as a patient complaint of voiding too often, whereas nocturia is the complaint of waking up more than 2-3 times a night to void. Urinary incontinence is the complaint of involuntary leakage of urine, while urge incontinence is the involuntary loss of urine immediately preceded by or accompanied by urgency (Abrams *et al.,* 2003). Other types of urinary incontinence include stress incontinence, whereby one has involuntary leaking of urine upon exertion of force such as coughing or sneezing, and mixed incontinence where urgency accompanies the involuntary leakage upon exertion of force (Chu and Dmochowski 2006)

1.5.2 Prevalence of OAB

According to the World Health Organisation (WHO), bladder problems affect an estimated 200 million people worldwide and have a great impact on the quality of life of affected individuals (Hunskaar *et al.,* 2000). OAB is a condition that affects people of all walks of life regardless of age, sex or race. In a population based survey in 6

European countries by Milsom *et al.* in 2001, 16,776 individuals were interviewed directly or by phone. They found the overall prevalence in individuals ≥40 years to be 16.6% (Milsom *et al.,* 2001). Frequency was the most reported OAB symptom at 85%, followed by urgency at 54% and urge incontinence at 36%. 60% of affected individuals had sought medical assistance but only 27% were undergoing medical treatment. (Milsom *et al.,* 2001). A longitudinal population survey of LUTS in women ≥20 years of age between 1991 and 2007 by Wennberg *et al.* showed cumulative incidences of UI, urgency and OAB were 21%, 20% and 20% respectively (Wennberg *et al.,* 2009). There was an increase in overall prevalence of OAB going from 17% in 1991 to 26% in 2007 (Wennberg *et al.,* 2009). With regards to age and OAB, the incidence of OAB symptoms increases with advance in age and this was equally apparent in both men and women (Milsom *et al.,* 2001; Irwin *et al.,* 2006).

1.5.3 Causes of OAB

The symptoms of OAB are attributed to the involuntary contraction of the bladder detrusor muscle during the filling phase and the causes are widely varied. There are the transient reversible causes such as an excess intake of alcohol or caffeine, stool impaction, certain pharmaceutical drugs and restricted mobility (Chu and Dmochowski 2006). Non-transient causes of OAB can be grouped as follows: neurogenic due to some kind of spinal cord injury, myogenic due to obstruction or hyperplasia, inflammatory caused by interstitial cystitis and lastly idiopathic due to no known defined cause (Semins and Chancellor 2004). The International Continence Society (ICS) has grouped detrusor overactivity (DO), a urodynamically measurable phenomenon, into two types - phasic and terminal. Phasic DO is commonly seen in idiopathic cases and has characteristic waveform and may or may not lead to urinary incontinence. Terminal DO is characterised by a single involuntary contraction at the point when the controllable desire to void is normally experienced but cannot be suppressed, leading to bladder emptying (Abrams 2003).

1.5.4 Pathophysiology of OAB

The exact mechanisms underlying OAB are still largely unknown but studies in both humans and animal models have shown the following changes in affected individuals:- increased spontaneous myogenic activity, altered responsiveness to stimuli, fused tetanic contractions and changes in smooth muscle ultrastructure (Steers and Klausner 2008) Patchy denervation, enlarged sensory neurons, hypertronic ganglion cells and enhanced spinal micturition reflex are some of the findings observed upon examination of the micturition reflex and innervation in models of OAB (Steers and Klausner 2008).

The normal detrusor is not well coupled electrically and relies on the densely innervated muscle bundles to respond to stimuli from the SNS or the PNS. Biopsies performed on severe OAB patients have revealed patchy denervation leading to an increase of connective tissue between the muscle bundles leading to complete denervation and detrusor hypertrophy (German *et al.,* 1995). Enhanced contractile activity of the bladder can result from increased electrical coupling of the muscles via close abutments between myocytes and the presence of protrusion junctions. This then allows electrical stimuli to spread across the muscle generating uninhibited contractions in response to even the slightest low grade stimuli (Steers and Klausner 2008).

Nerve growth factor (NGF) is a naturally occurring molecule that is actively involved in the stimulation and differentiation of nerves and neuronal growth after injury. The patchy denervation observed in OAB bladders suggests certain death of bladder neurons (Chu and Dmochowski 2006). Data from animal models associated with increased nerve growth factor have shown altered membrane conductance, and thus excitability of dorsal root Ganglia (DRG) neurons and this is further supported by evidence of increased NGF in patients with OAB, benign prostatic hyperplasia and interstitial cystitis (Chu and Dmochowski 2006).

The urothelium was traditionally seen as just a passive barrier that protected the underlying bladder layers from the toxic effects of urine. Ongoing research has changed this perception as the urothelium's role is emerging as an important one in the normal functioning of the bladder. The urothelium and suburothelium have been shown to have a rich supply of sensory (afferent) nerves and during bladder filling, several neurotransmitters are released and act on these afferent nerves to convey impulses to the CNS causing bladder emptying (Kumar and Chapple 2005). The mediators released by the urothelium and suburothelium can initiate or depress bladder activity. ATP released after urothelial stretch acts on $P2X_{2/3}$ purinergic receptors on afferent nerves to initiate voiding (Kumar and Chapple 2005). This is supported by a study which showed that mice, which were deficient of P2X receptors, exhibited a decrease in urinary frequency and an increase in bladder volume and capacity (Chu and Dmochowski 2006). Other urothelial mediators contributing to the mechanosensory theory are nitric oxide (NO) - an afferent nerve inhibitor - which has been shown to relax the bladder, and an unidentified urothelium derived inhibitory factor (UDIF) that depresses bladder contractions (Kumar and Chapple 2005).

1.5.5 MANAGEMENT OF OAB

Due to the varied causes of the symptoms of OAB, afflicted patients must undergo a basic evaluation that covers complete history, urinalysis and a physical examination (Chu and Dmochowski 2006). The lower urinary tract is innervated by nerves within the S2 to S4 region of the spinal cord. A neurologic assessment focusing on this region is also recommended to evaluate bladder function (Dwyer and Rosamilia 2002). Presence of bacteria, haematuria and pyuria can be analysed by cultures and urinalysis (Dwyer and Rosamilia 2002). Effective management and treatment of OAB requires several approaches working in conjunction - both pharmacological and non-pharmacological (Ouslander 2004). Pharmacological intervention often involves using anticholinergic drugs that block bladder muscarinic receptor mediated pathways. Extreme cases of OAB may require surgical denervation of the bladder to reduce the overactivity. Non-pharmacological interventions involve alteration of lifestyle, changing diet, performing pelvic exercises, restricting caffeine and alcohol intake and, last but not least bladder training (Lavelle *et al.,* 2006).

1.6 PHARMACOLOGICAL TREATMENT OF OAB

Pharmacotherapy directed at the afferent and efferent mechanisms that control the bladder is the 1st line treatments of OAB in the U.K. The majority of drugs used are directed peripherally, to act on muscarinic receptors of the bladder (Kumar and Chapple 2005). They target the symptoms rather than the cause, since the aetiology is not understood, and are therefore limited in efficacy. The following are some of the major treatment options available for OAB.

1.6.1 ANTICHOLINERGICS

Anticholinergics are the most commonly used agents and include oxybutynin, tolterodine, darifenacin, solifenacin, propiverine and trospium (Sellers and Chess-Williams 2012). Of the more established anticholinergics available for use, oxybutynin, tolterodine and trospium are available worldwide, with propiverine availability being restricted to only the U.K. The most recently introduced agents are darifenacin and solifenacin (Abrams and Andersson 2007), fesoterodine (Khullar *et al.,* 2008) and imidafenacin, which is currently only approved and marketed in Japan (Michel and Hegde 2006). Although this class of drugs is the first line pharmacotherapy used for OAB treatment, their use is significantly hindered due to their significant side effects such as dry mouth, constipation, blurred vision and urinary retention, thereby causing major non-compliance issues (Sellers and McKay 2007). The action of these agents at other muscarinic receptors in other body systems is the main cause of the reported

adverse effects. In the bladder, anticholinergics target the muscarinic receptors on the detrusor with relative selectivity for M_2 or M_3 receptors, in an attempt to limit adverse reactions. They work mainly by decreasing the amplitude of normal and involuntary contraction of the bladder while at the same time increasing the bladder volume at first contraction and thus increasing bladder functional capacity (Kumar and Chapple 2005). There is increasing evidence suggesting that these agents may also be acting on receptors present in the urothelium/sub-urothelium, ICC and afferent nerves. To help ease the side effects associated with anticholinergics, novel delivery systems have been developed e.g. extended transdermal release mechanisms, which have considerably decreased side effects and are well tolerated (Hill *et al.,* 2006; Kaplan *et al.,* 2006). Due to the side effects of anticholinergics, there has been a drive to develop novel more effective drugs.

1.6.2 α_1-ADRENOCEPTOR ANTAGONISTS

Previous pharmacological and molecular biology studies indicated that selective α_{1A}-adrenoceptor antagonists could be effective in the treatment of urinary obstruction in patients with benign prostatic hyperplasia (BPH) with fewer cardiovascular side effects than conventional anti-muscarinics (Walden *et al.,* 1997; Andersson and Hedlund 2002; Michel 2010). Terazosin, doxazosin, alfuzosin, tamsulosin and silodosin are the currently prescribed α_1-adrenoceptor antagonists for the treatment of BPH and LUTS (Schwinn and Roehrborn 2008). The selectivity of these antagonists is varied with alfuzosin, doxazosin and terazosin being relatively long acting and non-subtype selective showing equal selectivity for the 3 subtypes. Tamsulosin on the other hand is relatively selective for α_{1A}- and α_{1D} adrenoceptors both *in vivo* and *in vitro* with less affinity for α_{1B}-subtype (Noble *et al.,* 2009). These agents have been shown to alleviate the irritative symptoms associated with BPH such as frequency, nocturia and urgency in addition to relieving the dynamic component of bladder outlet obstruction (Sellers and McKay 2007) via an unknown mechanism. The proposed putative mechanism involved is thought to be at the level of the CNS and the spinal cord.

1.6.3 β-ADRENOCEPTOR AGONISTS

Drugs targeting β-adrenoceptors in the LUT are agonists, specifically β_3-selective agonists. The majority of this class of drugs are under various states of preclinical or clinical development. Mirabegron (YM 178) is the most advanced candidate for this class of agents, having shown positive clinical proof of concept results as well as dose ranging studies (Chapple *et al.,* 2008; Chapple *et al.,* 2010). The mechanism of action is to mediate relaxation of the detrusor, and it has been shown that the newly

developed β_3 adrenoceptor agonists work in this way on the human detrusor (Yamanishi *et al.,* 2006). There is a population of β_3-receptors that have been identified in different cell types in the bladder including afferent nerve terminals (Limberg *et al.,* 2010). Studies performed on bladder sheets from SCT mice have shown that β_3-receptor agonist can reduce afferent firing by direct action on these nerves (Kanai *et al.,* 2011).

1.6.4 Botulinum Toxin A (BTX-A)

Botulinum toxin (BTX) is a neurotoxic protein produced by Gram positive *Clostridium botulinum (Apostolidis 2011).* Of the seven serotypes of the botulinum toxin (BTX), BTX-A and BTX-B are the most commonly used in the treatment of LUT disorders (Charrua *et al.,* 2011). The use of BTX in the treatment of bladder dysfunction symptoms has been very effective, although it is not fully understood how it works in the bladder. The benefits are long lasting over a period of several months and can be repeated (Aoki and Guyer 2001). The mechanism of action of BTX involves connection of the toxin to the neuronal membrane, subsequent internalization and finally it is able to exert its intracellular effects. Its mechanism of action in the bladder was originally thought to involve prevention of vesicular release of ACh therefore decreasing involuntary contractions of the detrusor, but ongoing research has challenged this idea. It has been proposed that its mechanism of action may be on afferent neurotransmission, as postulated by Apostolidis *et al.* (2006). These authors proposed that BTX-A efficacy is dependent on inhibition of several neurotransmitters including ACh, ATP and substance P, and downregulation of purinergic $P2X_3$ and TRPV1 (Apostolidis *et al.,* 2005) thus causing decreased activation, bladder contractility and electrical coupling (Apostolidis *et al.,* 2006). BTX-A is currently being used clinically although it has not yet been licensed due to insufficient information on its mechanism of action.

1.6.5 Alternative treatments for OAB

Substance P, neurokinin A and neurokinin B represent the tachykinin family of peptides. They can be found in both the CNS and PNS and are involved in the sensory arm of micturition (Sellers and McKay 2007). Locally at the bladder, they are released from capsaicin sensitive afferent nerves (Holzer 1988) and facilitate neurotransmitter release, smooth muscle contraction and neurogenic inflammation (Maggi *et al.,* 1993). Tachykinin released at the bladder can act via neurokinin receptor 2 (NK2) found on the detrusor muscle to induce OAB (Sellers and McKay 2007). NK2 receptor antagonists have been shown to decrease hyperactivity in rats but not yet in humans

(Lecci *et al.,* 1998). The most recent development pertaining to the use of tachykinins in control of bladder activity is its use at the spinal cord level.

Bladders from several species including humans have been shown to contain the following potassium channels - ATP sensitive (K_{ATP}) channels, calcium activated large conductance channels (BK_{Ca}) and small conductance channels (SK) (Sellers and McKay 2007). *In vitro* studies and animal models have demonstrated that K channel openers can inhibit bladder contractility (Darblade *et al.,* 2006). The use of these drugs *in vivo* in humans has been plagued with obstacles such as vascular effects that warrant further investigation into OAB treatment.

The bladder urothelium and sensory nerves contain the transient receptor potential V1 vanilloid receptors (TRPV1) which have recently been linked to OAB pathogenesis. Women with sensory urgency had increased expression of TRPV1 in the trigone mucosa (Liu *et al.,* 2007). TRPV1 agonists such as resiniferatoxin works by desensitizing C-fibre afferents by binding to the receptor and this subsequently increases bladder capacity - for a limited period of time - in OAB patients with burning urinary sensation (Cruz *et al.,* 1997). A novel vanilloid receptor TRPA1 has recently been identified in the bladder neurons (Andrade *et al.,* 2006) and is a new drug target for treatment of bladder urgency.

Neuromodulation is used in patients that are unable to tolerate anticholinergics, have refractory OAB or other co-existing neurogenic conditions. This technique uses mild electrical stimulation of pelvic nerves (S3 nerve root) via centrally implanted electrodes in the sacral foramen (Thon *et al.,* 1991). A consensus has not been reached as to the exact mechanism of action. Suitable candidates have a permanent surgical unilateral implant that stimulates the S3 nerve root and is attached to a small pacemaker placed within the sub-dermal pocket in the buttock region (Kohli and Patterson 2009).

In summary the current treatment options are limited and there is a need to develop novel or more effective therapies for this distressing disorder. In order to achieve this, a greater understanding of both normal and pathological functioning of the bladder is required.

1.7 THESIS AIMS AND OBJECTIVES

The aims of the thesis are:-

- Investigate the phasic contractions that develop naturally in isolated pig bladder strips and determine any functional and structural heterogeneity between the different regions of the pig bladder using an *in vitro* tissue organ bath technique.
- Explore the effect of the mucosa (urothelium/sub-urothelium) on the development of phasic contractions and determine whether these PCs can be modulated cholinergically.
- Functionally investigate the role of interstitial cells in the development of PCs using imatinib (Glivec); a tyrosine kinase inhibitor, and determine the expression and localisation of bladder ICCs using immunohistochemistry and confocal microscopy.
- Investigate transmitter release (ATP) due to mechanical stretch on pig bladder strips obtained from the different bladder - dome, body and trigone.

CHAPTER 2

FUNCTIONAL CHARACTERISATION OF PHASIC CONTRACTIONS IN THE PIG URINARY BLADDER.

2.1 INTRODUCTION

It is widely accepted that during the filling phase involuntary phasic contractions (PCs) occur in the urinary bladder of several species, including man. This was first discovered in 1882 by Mosso and Pellacani from their studies on healthy female volunteers and from experimental studies on dogs. In their experimental studies the PCs were eliminated following spinal cord transection, suggesting the contractions were controlled via central neural mechanisms (Mosso and Pellacani 1882). The presence of PCs in the bladder was corroborated ten years later, in 1892 by Sherrington, who observed PCs in bladders of monkeys, cats and dogs. His experiments went on to show the sustained presence of PCs even after spinal transection and complete peripheral denervation (Sherrington 1892), and following bladder removal and maintenance *in vitro* the contractions still persisted, leading to the conclusion that the phasic activity was an inherent property of the bladder (Sherrington 1892). These two major findings set the stage for the on-going discussion to this day as to the origin and role of PCs in the bladder.

2.1.1 ORIGIN AND ROLE OF BLADDER PHASIC CONTRACTIONS

Since the work of Mosso & Pellacani and Sherrington, several studies have been performed to investigate bladder phasic activity and its origins, and several hypotheses have been proposed. Using a cat model, Gjone established that bladder PCs are influenced by a dual sympathetic and parasympathetic innervation (Gjone 1965). Plum and Colfelt (1960) showed that following denervation, if no damage occurred to the bladder; phasic activity persisted and subsequently coined the phrase "autonomous bladder" to explain the inherent PCs that occur (Plum and Colfelt 1960). Further experiments using spinal anaesthesia and parasympathetic ganglion blockage did not inhibit the PCs and this solidified the notion that the bladder phasic activity was an intrinsic property of the bladder wall.

Isolated bladders from many species including man are rhythmically active *in vitro* and this 'autonomous' activity has been shown to involve localised stretches of the bladder wall and waves of contraction (Gillespie 2004). Although the exact mechanism involved in this autonomous activity is unknown, various theories have been put forward speculating the origin of these bladder PC waves. Initially, it was thought that the PCs were myogenic in origin but this may not be the case. *In vitro* studies using isolated rabbit bladders exhibited continued phasic contractions even in the presence of atropine and tetrodotoxin which had no effect on both the electrical and mechanical activity of bladder smooth muscle thus suggesting a myogenic origin (Liu *et al.,* 1998).

It has also been postulated that PCs may involve a network of pacemaker cells in the bladder wall in conjunction with interstitial cells driven by both excitatory and neural inputs (Drake *et al.,* 2003; Gillespie *et al.,* 2003). The fact that the autonomous phasic activity is different to that generated by the typical detrusor micturition contractions caused by post-ganglionic parasympathetic nerves leaves open the possibility that within the bladder is a complex, coordinated mechanism capable of eliciting PCs and rises in intra-vesical pressure (Gillespie *et al.,* 2003). Several studies have identified a distinct sub-urothelial layer of cells with similar morphological characteristics as myofibrobrasts (Sui *et al.,* 2002; Wiseman *et al.,* 2003; Wu *et al.,* 2004). These cells form a dense electrical network between the urothelium and the afferent nerves underneath, which is capable of responding to neurotransmitter release. In addition to this, their close connection to sub-urothelial afferent nerves makes them ideally placed to exert a modulatory effect on bladder function (Wu *et al.,* 2004). Interstitial cells (ICC) and myofibroblasts cells in this region expressing muscarinic M3 receptors are capable of eliciting PCs that may be propagated via the network of large myelinated and small unmyelinated fibres (Wiseman *et al.,* 2002).

The urothelium may also play a role in eliciting or modulating bladder PCs, due to the fact that it is capable of releasing various mediators such as ACh, ATP and NO that may act on the underlying afferent nerves, ICC and detrusor muscle present in the bladder. (Birder 2006). A recent study reported that removal of the mucosa from bladder strips from the pig bladder dome region resulted in delayed development of PCs compared to strips with the mucosa present (Akino *et al.,* 2008). This finding supports a role for the urothelium/suburothelium in PC development or regulation, and points to the possibility of the sub-urothelium being the ideal site of integration and coordination of PCs.

Bladder phasic contractions from normal detrusor strips have been described as being distinctly irregular and arise from baseline. This is in contrast to contractions observed in detrusor strips from bladders in different pathological conditions, which appear as fused tetanic contractions (Brading 1997b). The role of these bladder phasic contractions is still not clearly understood. It is possible that they may be involved in the pathways that relay information on bladder fullness although their origin and exact physiological role is unknown. The relationship of these PCs to the non-voiding contractions reported in urodynamic studies needs to be investigated further, but it is clear that in pathological conditions, these PCs are increased.

2.1.2 SMOOTH MUSCLE PHASIC CONTRACTIONS IN PATHOLOGICAL CONDITIONS

Functional differences have been reported in the phasic activity of isolated bladder strips from normal human bladders and those from patients with pathological conditions (Sibley 1984; Harrison *et al.,* 1987; Kinder and Mundy 1987) and animal models (Sibley 1984; Sibley 1987; Sethia *et al.,* 1990; Bramich and Brading 1996). Functionally, bladders from pathological conditions exhibit increased electrical coupling between muscle bundles. This results in enhanced cell to cell coupling, thought to be responsible for the fused tetanic contractions observed in the unstable bladder, which is absent in normal detrusor. This enhanced abnormal phasic activity has been reported in various pathological conditions. Cultured SMCs from rat bladders with bladder outlet obstruction showed enhanced cell-to-cell communication compared to those obtained from normal rats (Li *et al.,* 2007). Inhibition of this enhanced communication using a gap junction inhibitor led to the conclusion that increased cell-to-cell communication may contribute to the increased PCs observed in BOO. Isolated bladder strips from streptozotocin-induced diabetic rats - a model of bladder dysfunction - showed increased PCs compared to strips from control rats (Nakahara *et al.,* 2004; Stevens *et al.,* 2006; Vahabi *et al.,* 2011b). In human studies, bladders obtained from patients with detrusor overactivity due to spinal cord injury (SCI) exhibited increased expression of connexin-43 gap junctions between SMCs (Haferkamp *et al.,* 2004). This shows enhanced intracellular communication between SMCs and subsequently increased PCs.

Although there is a paucity of information on the PCs in individual regions of the bladder, a few differences have been noted. Experiments investigating the effect of removal of the urothelium on spontaneous activity of the bladder have shown the trigone to be different to the dome, in that the trigone exhibits no delay in the development of PCs in comparison to the dome. This finding suggests that the urothelium has no effect in the development of PCs in the trigone and may therefore lack the ability to release mediators that may enhance PCs (Akino *et al.,* 2008). Immunohistological studies on guinea pig bladder have shown mucosal heterogeneity between the dome, lateral wall and trigone. This study reported that there were differences in the distribution of cells that stained positive for neuronal NO synthase and cGMP between the urothelium and sub-urothelium in the trigone, lateral wall and dome (Gillespie *et al.,* 2004; Gillespie *et al.,* 2005). A recent study looking at structural differences between the bladder dome and trigone examined mRNA expression of cold cut biopsies and revealed that the dome contained a relatively higher SM content in

comparison to the trigone, reflecting a well-developed network of sub-urothelial myofibroblasts and muscularis mucosae in the dome (Sánchez *et al.,* 2011).

In pathological conditions bladders show marked structural differences compared to normal bladders. The use of light and electron microscopy has shown detrusor from unstable bladder to contain increased elastin and collagen between the muscle bundles, subsequently causing patchy structure and loss of intrinsic nerve connections (Gosling *et al.,* 1986; Harrison *et al.,* 1987). Using electron microscopy Elbadawi *et al* (1993) described widened intercellular spaces, scarce intermediate cell junctions which are common in normal tissue and finally increased protrusion junctions and cell-cell support. The cell-cell abutments and protrusion junctions were proposed to be involved in the generation of OAB myogenic contractions and the increased electrical coupling between muscle cells (Elbadawi *et al.,* 1993a; Elbadawi *et al.,* 1993b).

2.2 CHAPTER AIMS

The aims of this chapter were:-

- To characterize the phasic contractions that develop *in vitro* in isolated pig urinary bladder strips in the different regions of the bladder dome, body and trigone,
- To determine the role of the mucosa in the development of these PCs and any functional heterogeneity between the different regions.
- To relate this to differences in the histology of the bladder layers.

2.3 MATERIALS AND METHODS

2.3.1 EXPERIMENTAL ANIMALS

Urinary bladders from White Landrace and Duroc female pigs were acquired fresh from the local abattoirs. The pigs were aged between 4-6 months and body weights were between 70-80kgs. The bladders were separated from the abdominal viscera and cut at the proximal urethra/bladder neck. The bladders were immediately placed in cold (4°C) modified Krebs' bicarbonate buffer containing 118.4mM NaCl, 11.7mM D-Glucose, 24.9mM $NHCO_3$, 4.7mM KCl, 1.15mM $MgSO_4$, 1.15mM KH_2PO_4 and 1.9mM $CaCl_2$ for transport to the laboratory.

2.3.2 TISSUE PREPARATION

Upon arrival at the laboratory, harvested bladders were processed immediately. The bladders were stripped of any excess fat and ligaments and these were disposed of according to the university guidelines regarding animal tissue handling. The ventral side of the bladder was identified and the bladder was then cut into the 3 major regions - dome, body and trigone as shown in figure 2.1. Firstly, the dome was removed by dissecting across the top 1/3 of the bladder. The bladder was then cut open longitudinally through the ventral side to expose the urothelium lining and the trigone region of the bladder. The trigone was separated from the bladder body and all three sections were then placed in cold Krebs' (4°C) and stored in the refrigerator. The tissue was used within 48 hours.

2.3.3 BLADDER STRIP PREPARATION FOR FUNCTIONAL STUDIES

Bladder strips measuring approximately 1-1.5cm length x 2-4mm width x 4-6mm depth were dissected from the three regions - dome, body and trigone. One strip per pair was left intact whilst the second strip was carefully denuded of the mucosa by dissecting away the urothelium and the underlying loose sub-urothelial layer, down to the detrusor along the natural plane of dissection as previously described (Sellers *et al.*, 2000; Templeman *et al.*, 2002; Templeman *et al.*, 2003; Akino *et al.*, 2008).

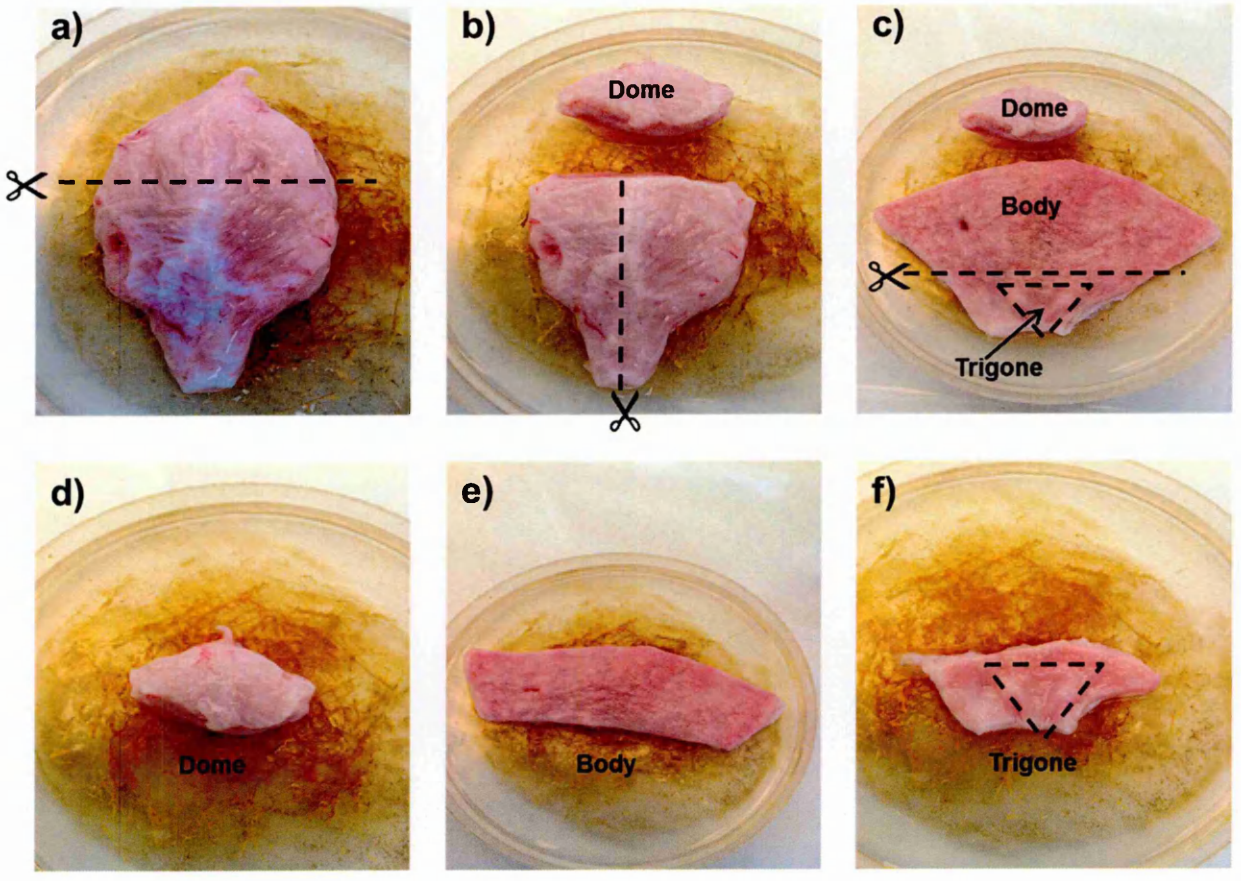


Figure 2.1- Dissection of the pig urinary bladder - a) Plane of dissection to separate the dome from the body and trigone, b) Longitudinal cut through the ventral side to expose the trigone shown in c). Dome, body and trigone regions respectively following separation (d-f).

2.3.4 *In Vitro* Tissue Bath Preparation

Bladder strips were mounted in tissue baths, attached at one end to tissue holders and at the other end by Dynamometer UF1 force transducers (Pioden Controls Ltd. UK) using surgical thread Figure 2.2. The tissue strips were then maintained in warmed Krebs' solution at 37°C and continuously gassed with 95% O_2 and 5% CO_2. A pH of 7.4 was maintained under these conditions due to the buffering established by the CO_2 and $NaHCO_3$. Tissue strips were allowed to equilibrate under a load of 1g for 60 minutes with the Krebs' buffer replaced every 15 minutes. The tension developed by the tissues was measured via the isometric force transducers connected to a Powerlab data acquisition system (ADInstruments, UK) which converted the analogue signal to digital and using 'Chart5' software (ADInstruments, UK) for analysis of the acquired data.

2.3.5 Investigation of Basal Phasic Activity

Bladder strips were observed for the development of phasic contractions and measurements of amplitude and frequency were taken at one and two hours following tissue set up. Development of phasic activity in the different bladder regions was also recorded

Amplitude and frequency measurements of the phasic activity were taken over a five minute period at the 1 hour and 2 hour time points. Amplitude was the peak amplitude in the selected 5 minute period (Figure 2.3). For frequency, 30% of the peak amplitude was calculated and used as a threshold to define a single contractile event as used previously in the laboratory (Vahabi *et al.,* 2011a) and proposed by Imai *et al.* (2001). Any contractions above the threshold line were counted as single contractile events as shown in Figure 2.3. Wet tissue weight was recorded at the end of each experiment in order to normalize data to account for differences in tissue weight and size.

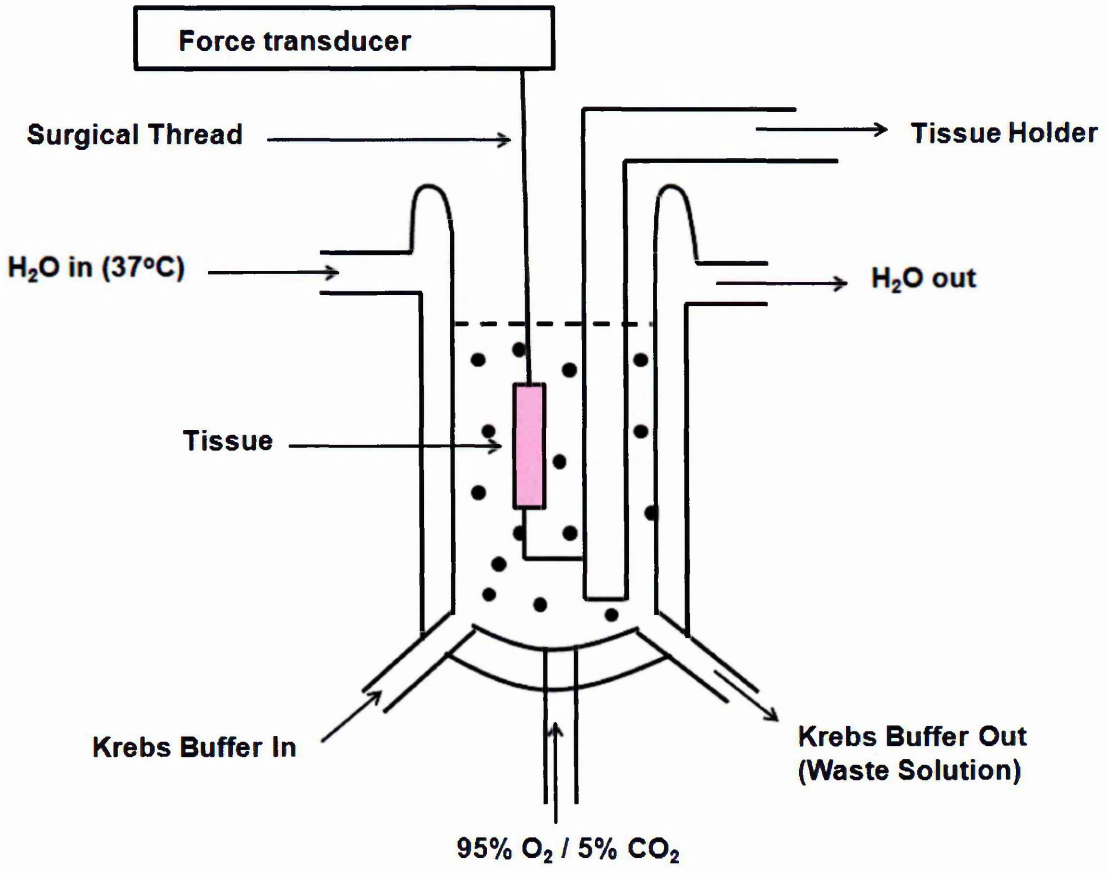


Figure 2.2 - Double-walled glass tissue bath (Adapted from (Longhurst and Uvelius 2001).

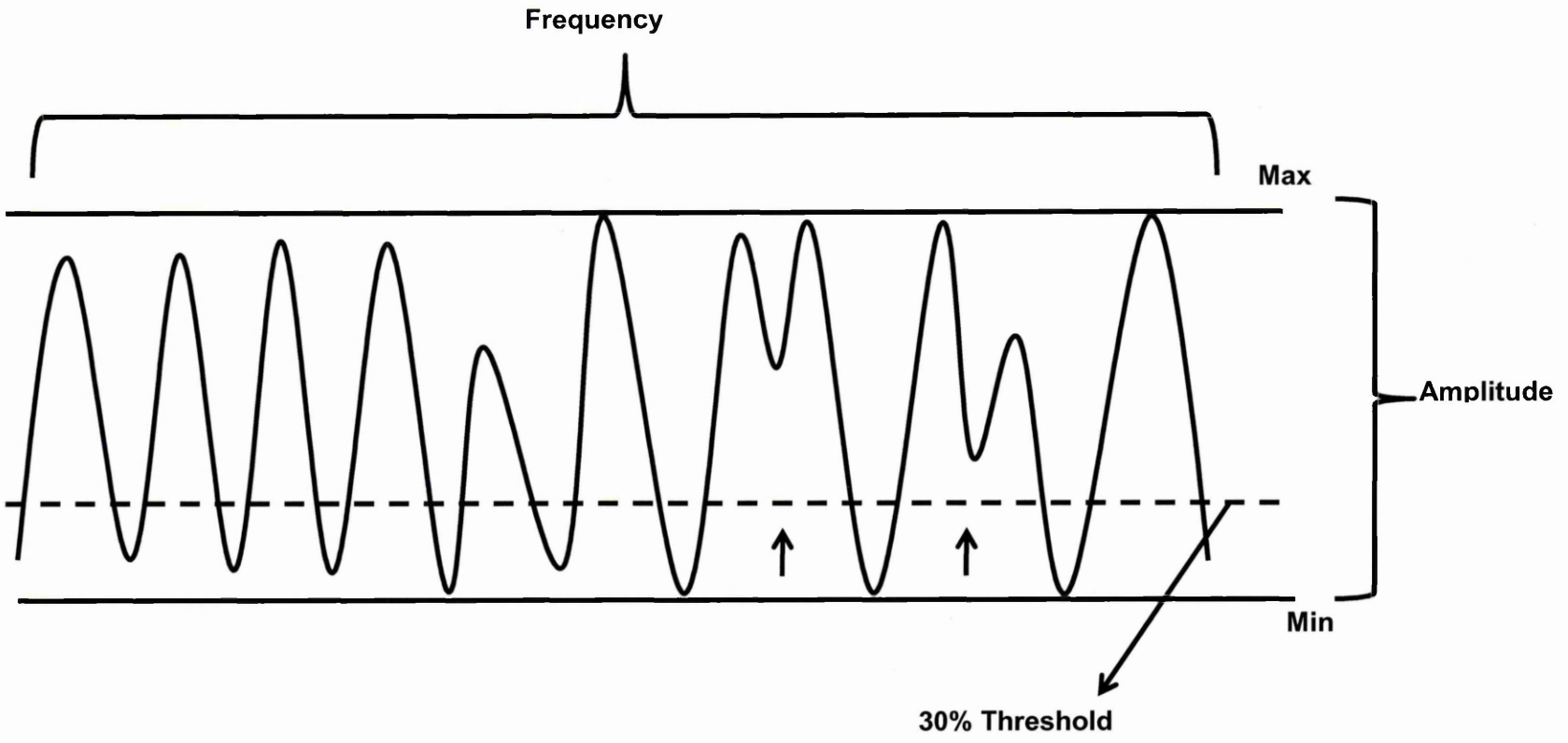


Figure 2.3 - Calculation of amplitude and frequency of phasic contractions in pig bladders over a selected 5 minute period. Amplitude was the peak amplitude during the selected period. For frequency measurements, 30% of the peak amplitude was calculated and any contractions that exceeded this threshold (shown by dotted line) were counted as a contractile event. Any superimposing contractions that did not cross the threshold line (shown by arrows) were counted as single contractions. Adapted from (Imai *et al.*, 2001).

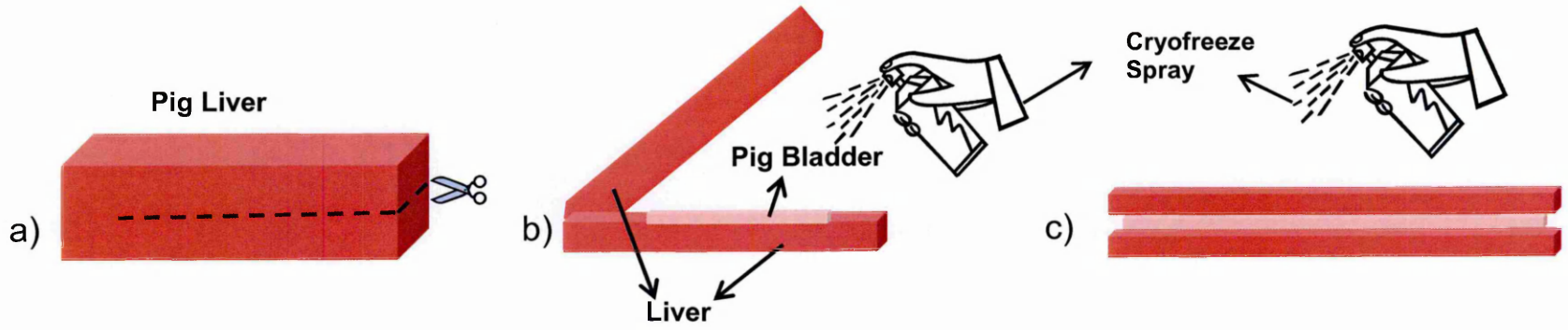


Figure 2.4 - Preparation of bladder tissues for sectioning. Liver-bladder-liver sandwiches were prepared. This technique ensured that the bladder urothelium remained intact during sectioning.

2.3.6 HISTOLOGICAL ANALYSIS OF THE PIG BLADDER

Two methods of tissue preparation were investigated to determine which provided better structural integrity of the tissue for subsequent histological staining and measurement of the different bladder layers thickness. For each tissue preparation method, six pieces of tissue from each bladder region were prepared accordingly for sectioning and staining.

2.3.6.1 Liver-bladder-liver sandwich preparation

The first tissue preparation method involved using bladder sections (1.5x1.5x0.5cm) from the three bladder regions sandwiched between pieces of pig liver to provide support for the urothelium as shown in Figure 2.4. The liver-bladder-liver sandwich was immediately sprayed with cryofreeze spray and placed at -80°C before sectioning at 10micron thickness using a cryostat and mounting on polylysine coated slides. Sections were subsequently fixed in cold (4°C) paraformaldehyde for 15 minutes, washed in 1X phosphate buffered saline (PBS) then immersed in cold methanol (-20°C) for 4 minutes, cold acetone (-20°C) for 2 minutes and finally washed again with 1X PBS ready for histology staining.

2.3.6.2 Paraffin embedded tissue preparation

In the second method, fresh pig bladder tissues were fixed using neutral buffered formaldehyde 10% v/v (Leica Microsystems, Milton Keynes, U.K.) for at least 2 hours. These were then placed onto IP III histology cassettes (Leica microsystems, Milton Keynes, U.K.) and placed in a Shandon Elliott duplex tissue wax automatic processor (Shandon Elliott Inc., PA, U.S.A.) whereby the tissue was initially dehydrated in 50% industrial methylated spirit (IMS) for 90 minutes, 70% IMS for 60 minutes, three times in 99% IMS for 60 minutes and twice in 99% IMS for 90 minutes. The tissue was then cleared three times using Sub-X for 90 minutes and finally immersed in molten wax for 90 minutes and the last step in the processor in molten wax for 120 minutes. The tissue was then transferred into a glass container of molten wax and placed in a vacuum oven at a temperature of 60° - 70°C at 60cmHg pressure for 30 minutes to eliminate any air pockets remaining in the tissue. Tissue mould bases (Leica microsystems, Milton Keynes, U.K.) were filled with molten wax and the tissue was removed from the histology cassettes and aligned to the correct orientation in the base. The tissue cassette was then placed over the mould and pressed into place ensuring that wax filled the cassette and this was then placed on a cold plate to cool to allow the wax to set ready for sectioning at 3 microns on a Leica SM 2400 microtome ((Leica microsystems, Milton Keynes, U.K.).

2.3.7 *HISTOLOGICAL STAINING*

Two staining techniques were used and compared in order to measure the bladder layers in the three bladder regions, haematoxylin and eosin stain (H&E) and Masson's trichrome stain (MTS), which stain different components of the tissue sections thus enabling distinguishing of the different structures present.

2.3.7.1 Haematoxylin and Eosin staining

Prior to staining, the paraffin wax embedded tissues underwent a de-waxing stage. This was performed by immersing the slides in Sub-X three times for five minutes followed by a rehydration step whereby the slides were placed in IMS three times for 5 minutes. Frozen and fixed liver-bladder-liver sandwich sections and de-waxed paraffin embedded sections were immersed in filtered Harris haematoxylin solution for 1 minute and rinsed in tap water until clear. The slides were then immersed in 1% eosin for 1 minute and again rinsed in tap water until clear. Subsequent dehydration in ascending alcohol solution (50% - 1 minute, 70% - 1 minute, 80% - 1 minute, 95% - 2 minutes and 100% - 2 minutes) was performed and then the sections were cleared using xylene for 2 minutes in the fume hood. DPX (Sigma Aldrich, Dorset, U.K.) was used to mount coverslips and the slides were left to air dry ready for microscopic analysis.

2.3.7.2 Masson's trichrome stain

A MTS kit (Sigma Aldrich, Dorset, U.K.) was used. Fixed sections were allowed to mordant in pre-heated Bouin's solution at 56°C for 15 minutes and allowed to cool in tap water. The yellow colour that developed on the slides was removed by several rinses in tap water. The slides were then immersed in Weigert's iron haematoxylin solution for 5 minutes, rinsed with tap water for 5 minutes and then placed in Biebrich scarlet-acid fuchsin stain for 5 minutes followed by a rinse with tap water. The slides were then immersed in phophotungstic/phosphomolybdic acid solution for 5 minutes, aniline blue solution for 5 minutes, 1% acetic acid for 2 minutes and the slides were finally rinsed and dehydrated through increasing alcohol concentrations, cleared in xylene and allowed to air dry for cover slip mounting with DPX.

2.3.7.3 Bladder layer measurements

Each tissue section from each of the three bladder regions had 6 measurements taken for each layer - urothelium, suburothelium and detrusor. The measurements obtained were combined and averaged to provide the results presented in this chapter. Figure 2.5 shows an example of how measurements were taken.

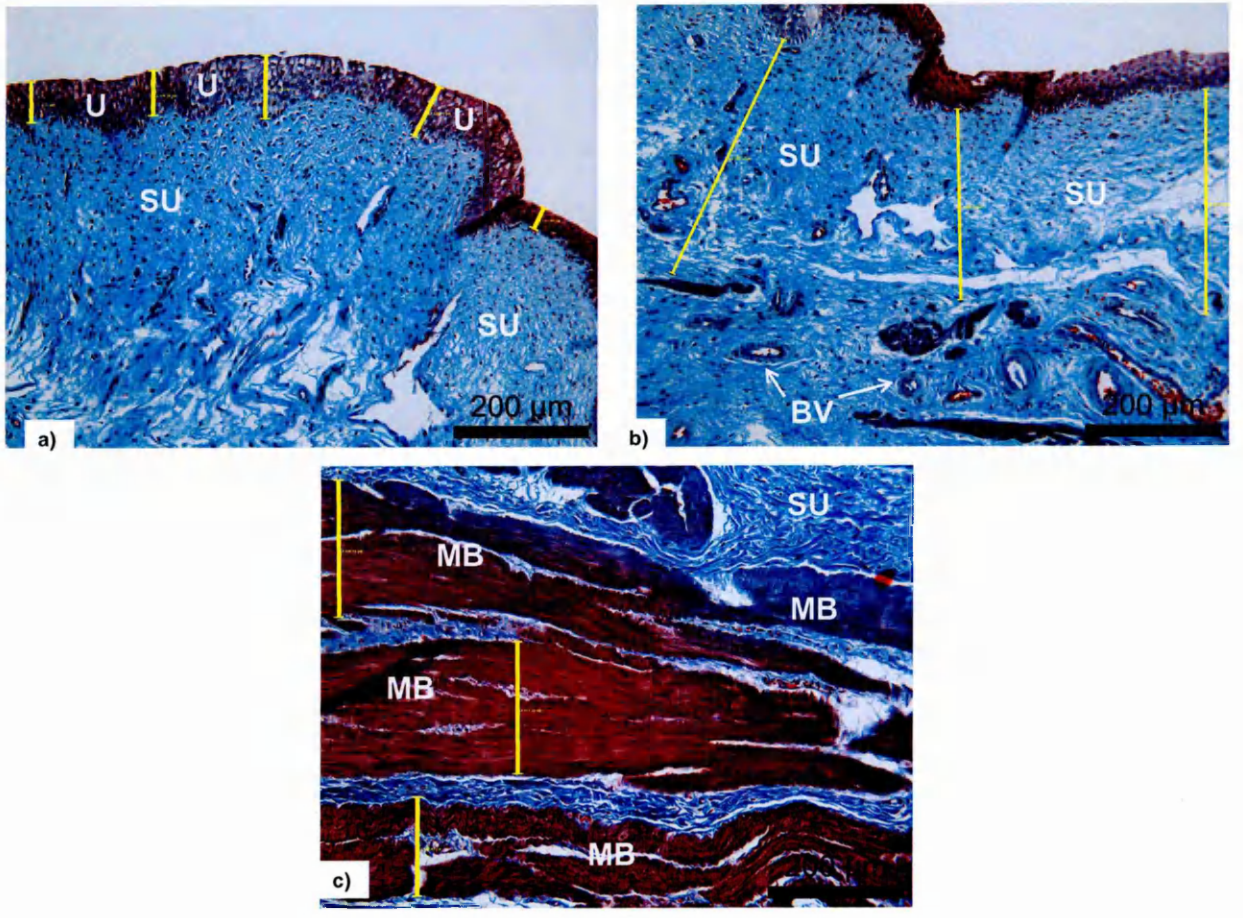


Figure 2.5 - Example of measurements taken using Q capture pro 8.0 imaging software for bladder layer thickness. A) urothelium, B) suburothelium and C) detrusor smooth bundle fibres of the body region; U - urothelium, SU - sub-urothelium, MB - muscle bundles, BV - blood vessels; Images taken at 20X magnification.

2.3.6 *DATA AND STATISTICAL ANALYSIS*

All data are expressed as mean ± standard error of the mean (SEM). Amplitude measurements are shown as absolute grams of tension per mg of tissue weight to account for differences in tissue weight. Frequency is the number of contractions per 5 minute period. Comparison of data from intact vs. denuded bladder strips was performed using an unpaired Student's t test. A comparison of data from the 3 bladder regions was performed using a one-way ANOVA with a Bonferroni post-hoc test. $p<0.05$ was considered significant.

Urothelium, sub-urothelium and detrusor layer thickness was compared across the three bladder regions. Comparison of thickness dimensions within each layer of the bladder was performed using a Student's t test whereas inter-region analysis was performed using a one-way ANOVA with a Bonferroni post-test.

2.4 RESULTS

Representative PCs that developed in bladder strips from the dome, body and trigone are shown in Figures 2.6 - 2.8 respectively. The PCs that developed in intact strips from the three regions appear similar in shape with all contraction peaks appearing regular with a constant baseline (Figure 2.6a, Figure 2.7a & Figure 2.8a). Removal of the mucosa did not alter the baseline but did affect the waveform with the shape of contractions becoming fused and irregular (Figure 2.6b, Figure 2.7b & Figure 2.8b).

2.4.1 TIME TO DEVELOPMENT OF PCS AND EFFECT OF MUCOSA REMOVAL IN PIG BLADDER DOME, BODY AND TRIGONE

Phasic contractions were observed in 96.7% of all bladder strips examined from the three bladder regions. For each bladder region, 10 intact and 10 denuded strips were examined (n=10 animals). 90% of the strips from the bladder dome developed PCs in comparison to 100% of strips from the body and trigone. One strip of intact and one strip of denuded dome did not develop PCs. The time taken to develop PCs in intact strips from the dome, body and trigone regions was 17.2 ± 4.0 minutes, 17.7 ± 7.4 minutes and 10.7 ± 3.9 minutes respectively (Figure 2.9a). In contrast, removal of the mucosa significantly increased the time to develop PCs in denuded dome strips to 60.3 ± 6.7 minutes (Figure 2.10a, ***$p<0.0001$). In the body region, mucosa removal also increased the time for PC development to 44.4 ± 11.8 minutes, although this was not significantly increased versus intact tissues (Figure 2.10b). For the trigone, mucosa removal did not affect the time taken to develop PCs, being 7.3 ± 2.1 minutes in denuded strips and not significantly different to the intact strips (Figure 2.10c).

Comparison of the time to development of PCs in intact strips in the three bladder regions showed no significant differences (Figure 2.9a). In contrast, in the denuded bladder strips, comparison between the dome, body and trigone showed the time to development of PCs to be significantly less in trigone vs. the dome and body regions (Figure 2.9b).

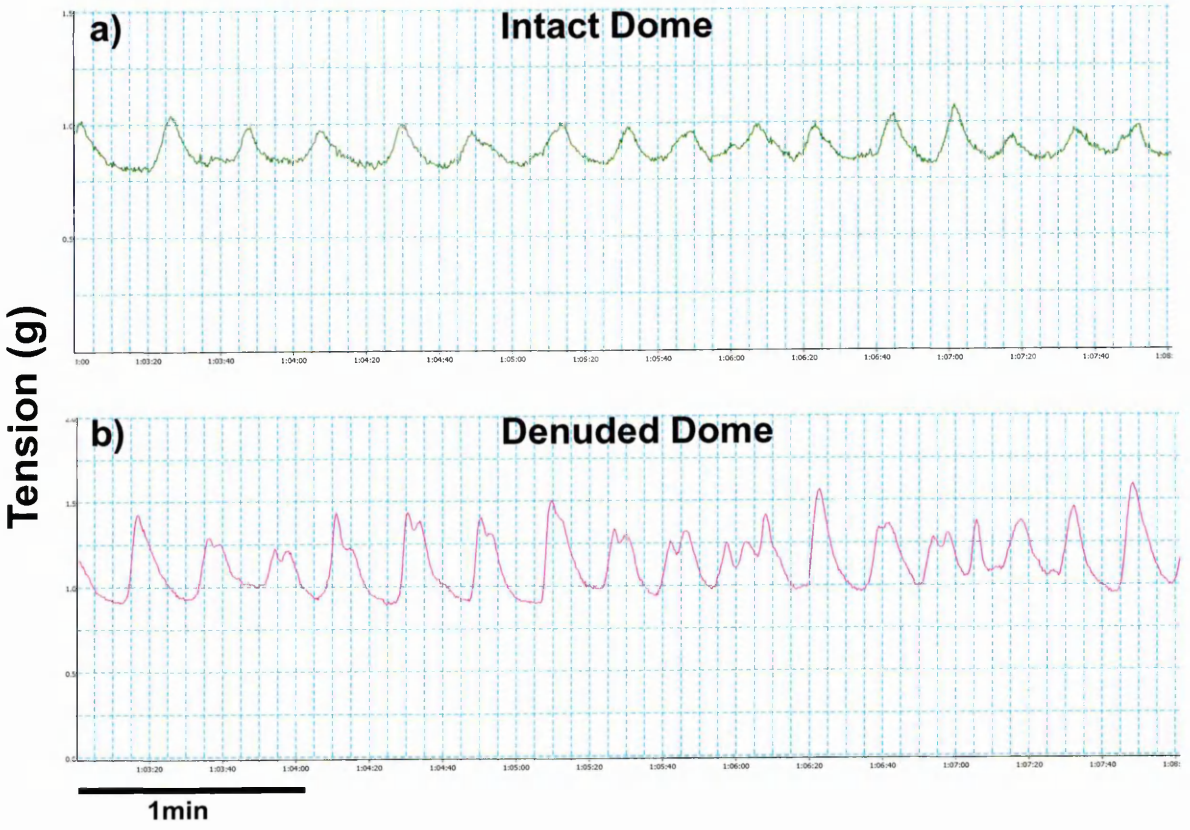


Figure 2.6 - Typical chart recordings of phasic contractions in bladder strips from a) intact and b) denuded dome.

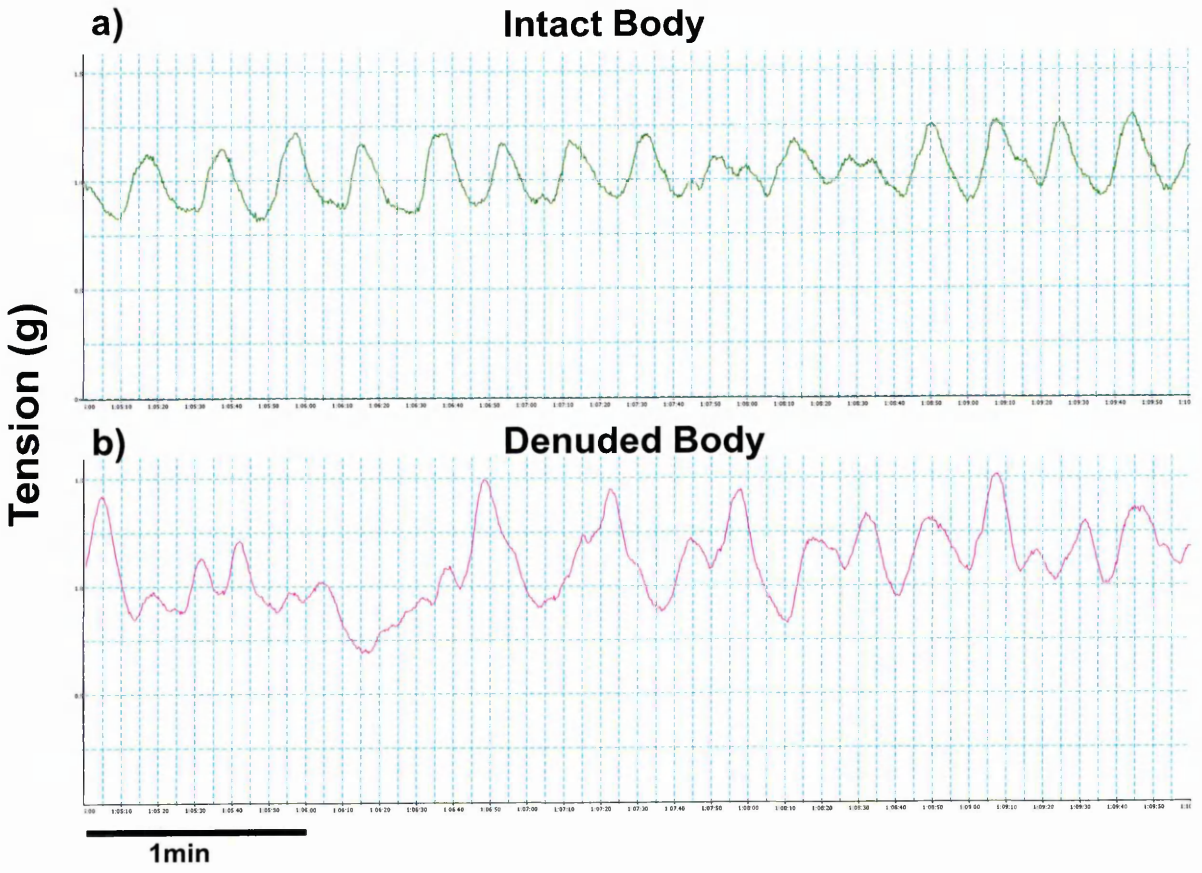


Figure 2.7 - Typical chart recordings of phasic contractions in bladder strips from a) intact and b) denuded body.

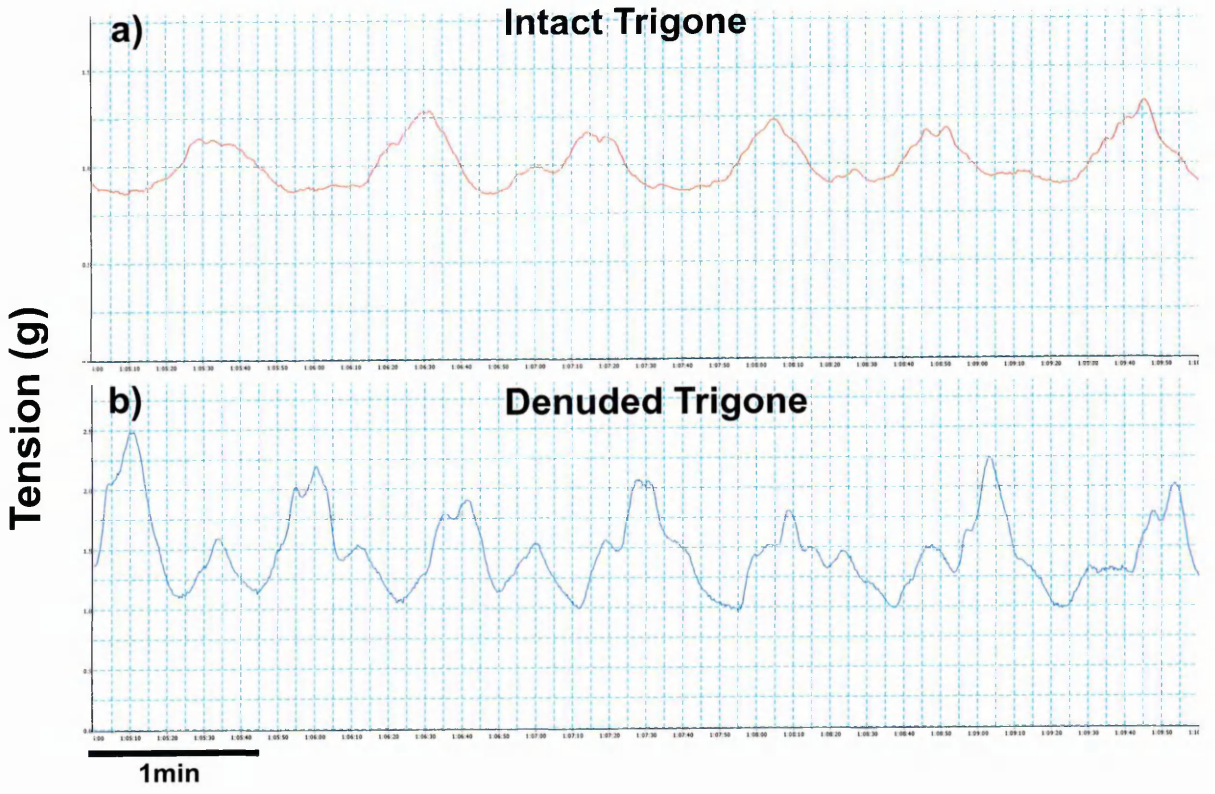


Figure 2.8 - Typical chart recordings of phasic contractions in bladder strips from a) intact and b) denuded trigone.

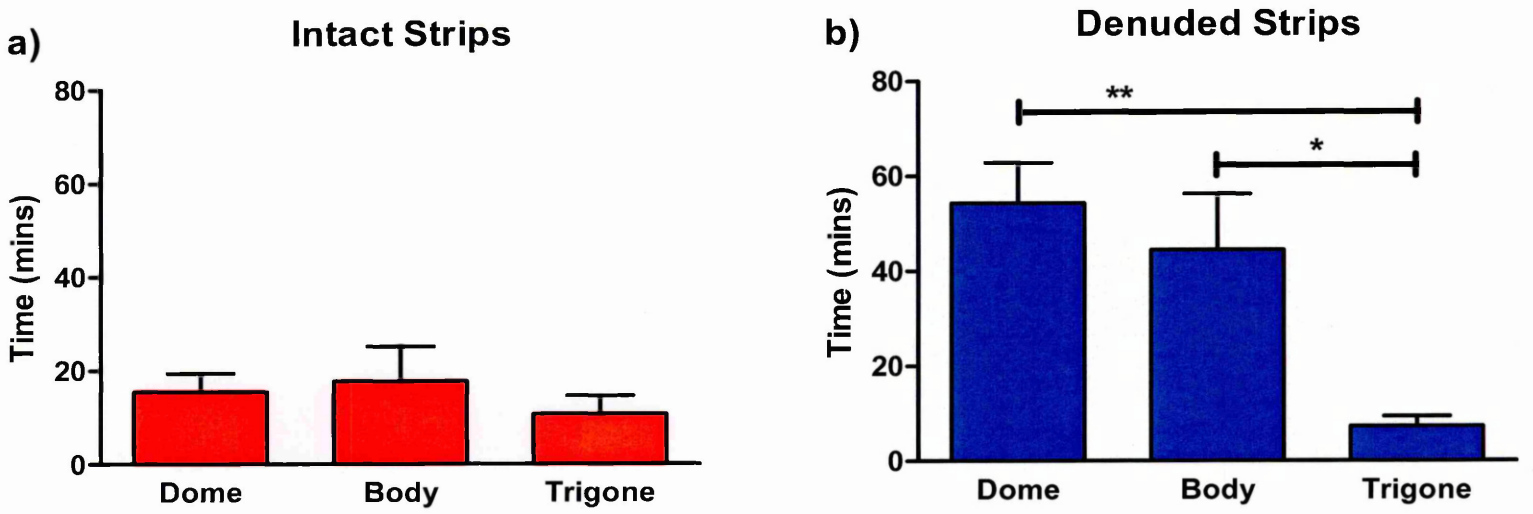


*Figure 2.9 - Time to development of PCs in a) intact and b) denuded pig bladder strips from the dome, body and trigone. Data are mean ± SEM; *p<0.05 vs. body, **p<0.01 vs. dome, n=9 (dome) and n=10 (body and trigone).*

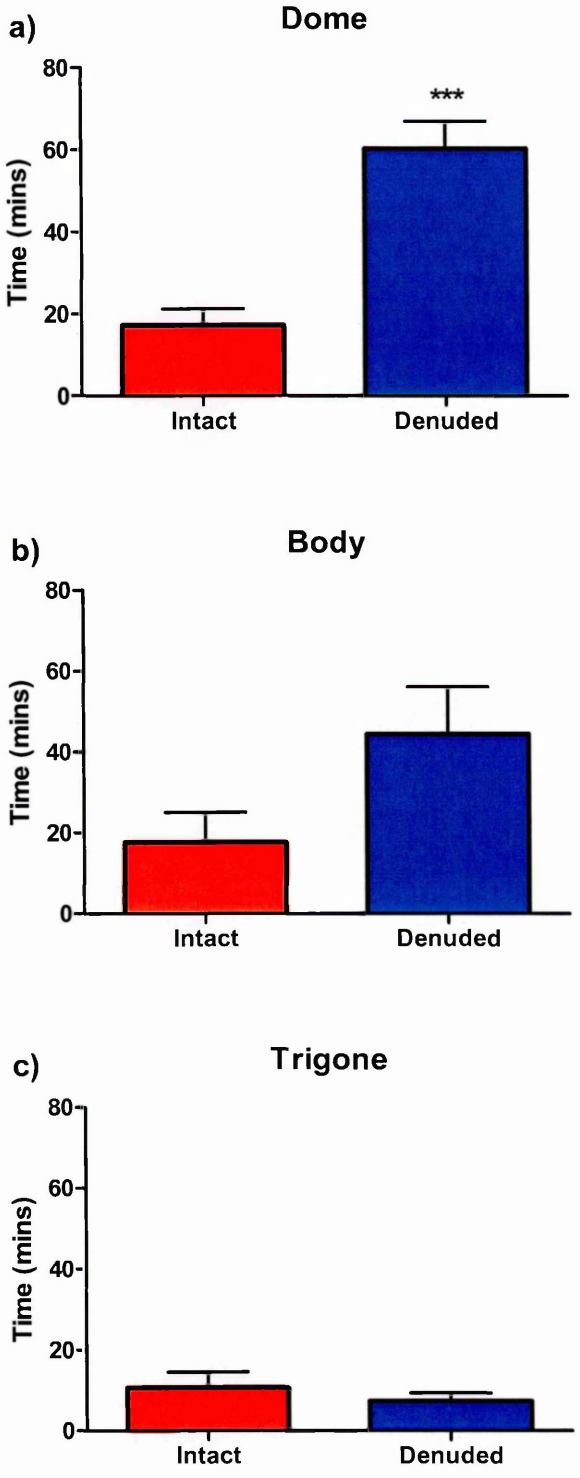


*Figure 2.10 - Time to development of PCs in intact vs. denuded strips of a) dome, b) body and c) trigone. Data are mean ± SEM; n=9 (dome), n=10 (body and trigone). ***p<0.0001 vs. intact.*

2.4.2 *AMPLITUDE AND FREQUENCY OF PHASIC CONTRACTIONS IN PIG BLADDER STRIPS AND THE EFFECT OF MUCOSA REMOVAL*

The amplitude of PCs in intact bladder strips was significantly greater in strips from the trigone region versus the dome and body (Figure 2.11a). Following removal of the mucosa, the amplitude of PCs remained significantly greater in denuded strips versus strips from the dome and body regions Figure 2.11b). Removal of the mucosa resulted in only a slight increase in amplitude of PCs in the body and trigone regions although not significantly so compared to intact strips (Figure 2.12).

Frequency of PCs was greater in intact strips of the dome and body versus the trigone although not significantly so (Figure 2.13 & 2.14). Removal of the mucosa significantly decreased the frequency of PCs in the dome and body whereas in the trigone, removal of the mucosa only slightly and non-significantly increased frequency (Figure 2.13 and 2.14).

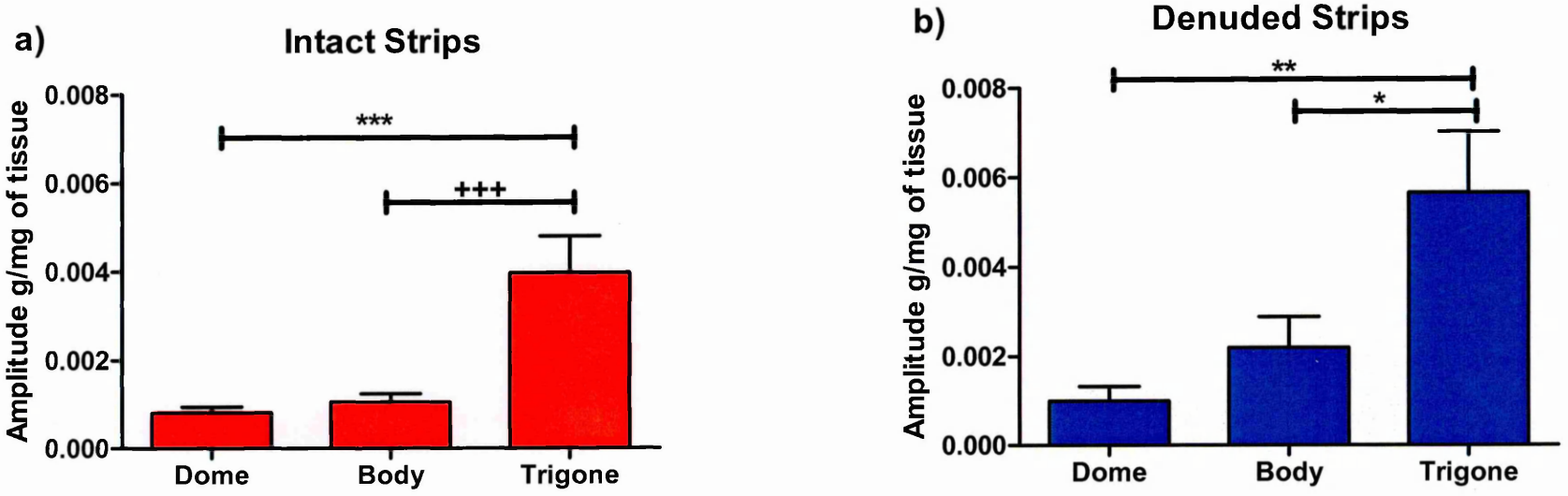


*Figure 2.11 - Comparison of amplitude of PCs between the dome, body and trigone in a) intact and b) denuded strips. Data are normalized for tissue weight and presented as mean ± SEM. n=10; *p<0.05 vs. denuded body, **p<0.01 vs. denuded dome, ***p<0.0001 vs. Intact dome, +++p<0.0001 vs. intact body.*

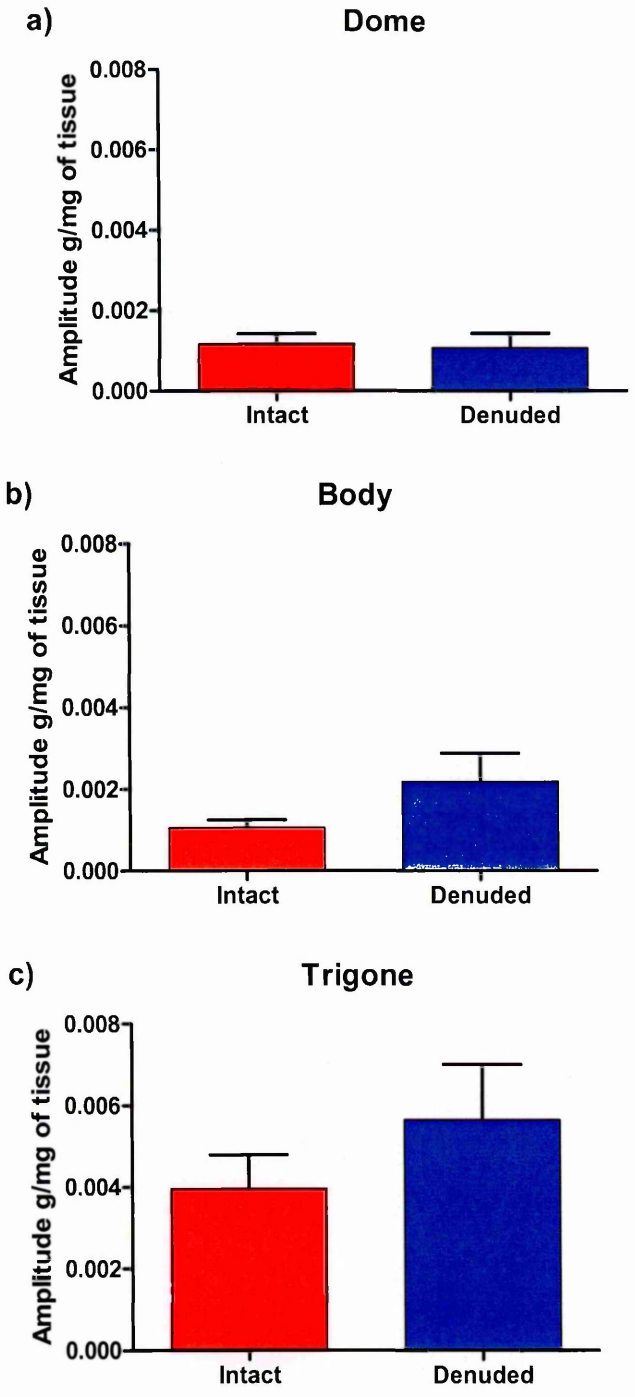


Figure 2.12 - Amplitude of PCs in intact vs. denuded strips of pig bladder a) dome, b) body and c) trigone. Data are normalised for bladder strip weight and presented as mean ± SEM, n=9 (dome) n=10 (body and trigone).

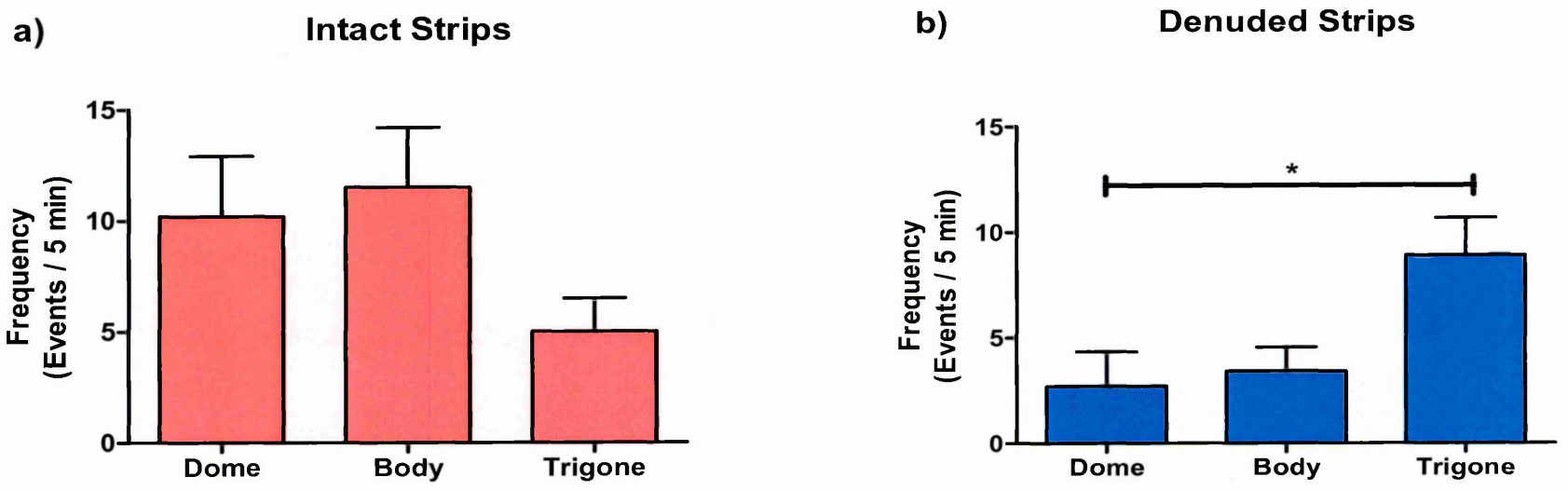


*Figure 2.13- Comparison of frequency of PCs between the dome, body and trigone in a) intact and b) denuded strips. Data are presented as mean ± SEM. n=10; *p<0.05 vs. denuded dome.*

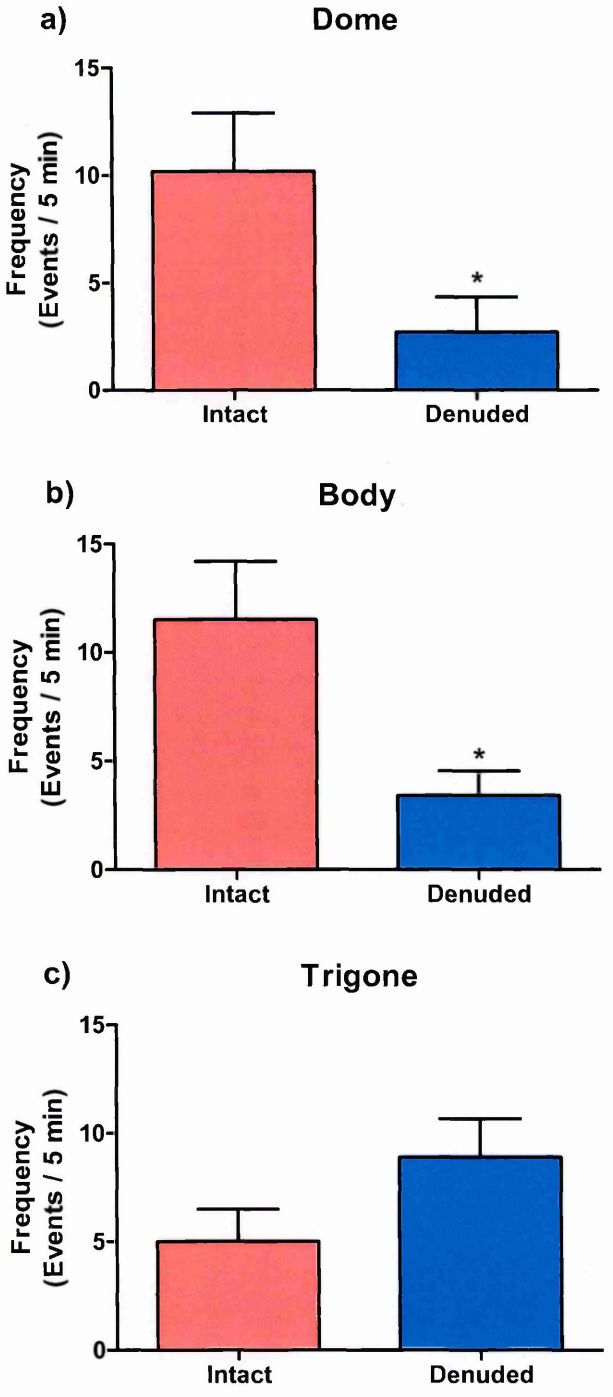


Figure 2.14- Frequency of PCs in intact and denuded strips of pig bladder a) dome, b) body and c) trigone. Data are normalised for bladder strip weight and presented as mean ± SEM, n=9 (dome) n=10 (body and trigone); *p<0.05 vs. intact

2.4.3 *Effect of time on the amplitude and frequency of PCs*

Comparison of the amplitude of PCs at 1hr vs. 2hrs in intact strips of the dome, body and trigone bladder regions showed no significant differences at the two time points (Figure 2.15a). Denuded strips on the other hand showed significant increase in the amplitude of PCs at 2hrs vs. 1hr time point in all three bladder regions (Figure 2.15b). With regards to frequency, intact strips from the three regions were not significantly different in the number of contractions over time (Figure 2.16a), whereas in the denuded strips, a significant increase in the frequency of PCs at 2hrs compared to 1hr was observed in the dome and body. In contrast the denuded strips of trigone showed similar frequency at the two time points (Figure 2.16b).

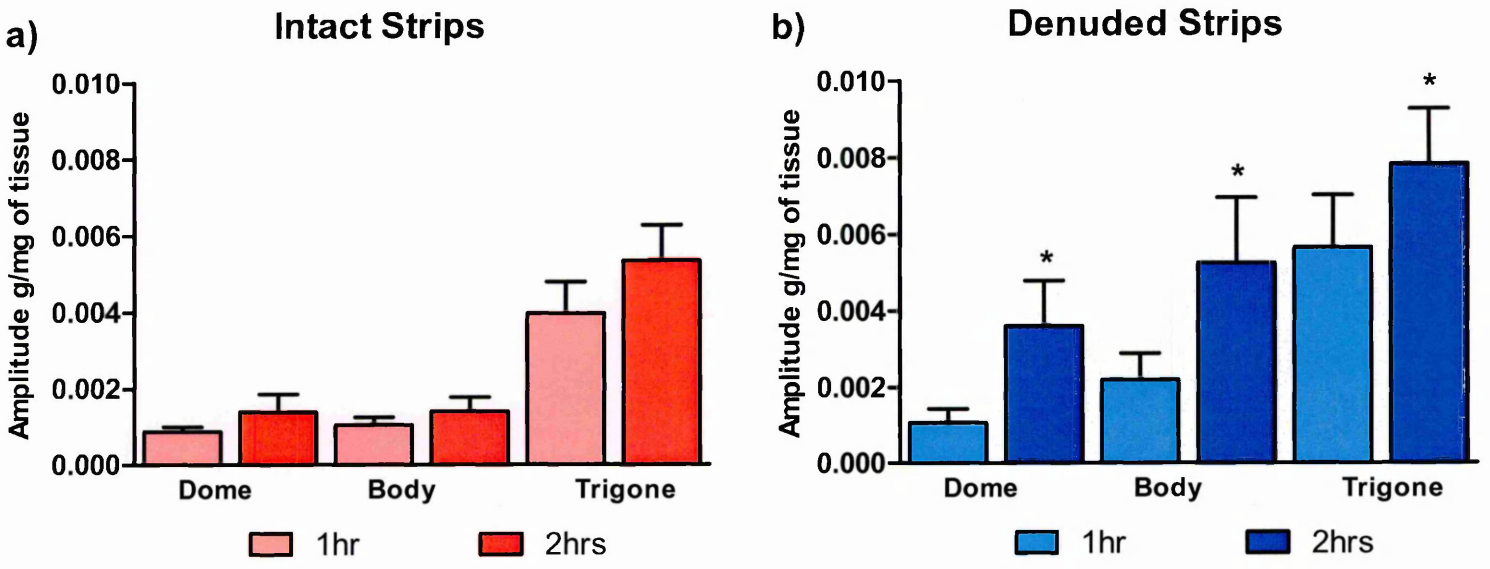


Figure 2.15 - Comparison of amplitude of PCs between the dome, body and trigone in a) intact and b) denuded strips over time. Data are normalized for tissue weight and presented as mean ± SEM. n= 9 (dome) n=10 (body and trigone); *p<0.05 vs. 1hr in denuded strips.

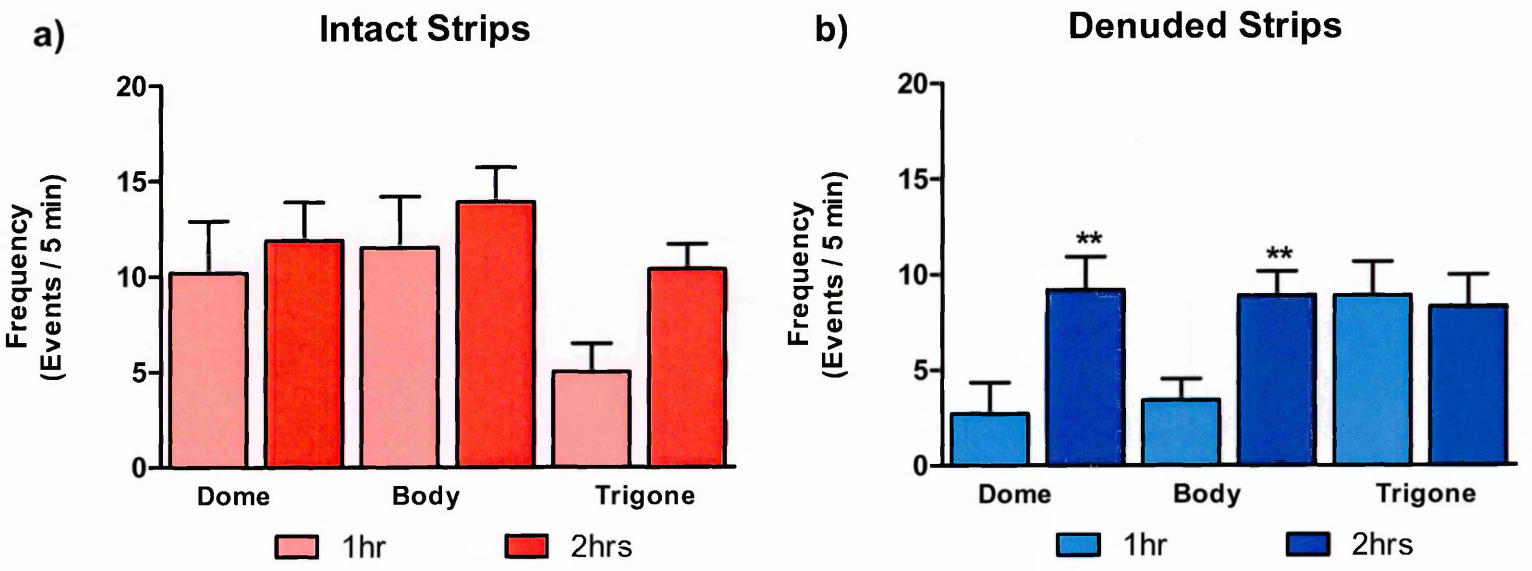


*Figure 2.16 - Comparison of frequency of PCs between the dome, body and trigone in a) intact and b) denuded strips over time. Data are normalized for tissue weight and presented as mean ± SEM. n= 9 (dome) n=10 (body and trigone); **p<0.01 vs. 1hr in denuded strips.*

2.4.4 *INVESTIGATION OF UROTHELIUM, SUB-UROTHELIUM AND DETRUSOR LAYER THICKNESS IN PIG BLADDER DOME, BODY AND TRIGONE REGIONS*

The different layers of the bladder wall were clearly distinguishable using the two different staining methods. For the H & E stain, nuclei and basophilic structures stained blue whereas the cytoplasm and acidophilic structures stained a light to dark red colour (Figure 2.17, Figure 2.18 a & b). In contrast, the MTS method stained cell nuclei black, connective tissue and collagen blue and the cytoplasm and muscle fibres red (Figure 2.17, Figure 2.18 c & d). Much better structural integrity was preserved using wax embedding, and thus histological measurements of the bladder layers were taken using sections of tissue prepared by this method. Figure 2.19 shows example MTS on 3 micron full thickness paraffin embedded slides from the three bladder regions showing the urothelium, sub-urothelium and detrusor muscle bundles in all the three bladder regions. A similar layer to the muscularis mucosae described by Heppner *et al.* (2011) was visible in sections from the dome and body but not the trigone.

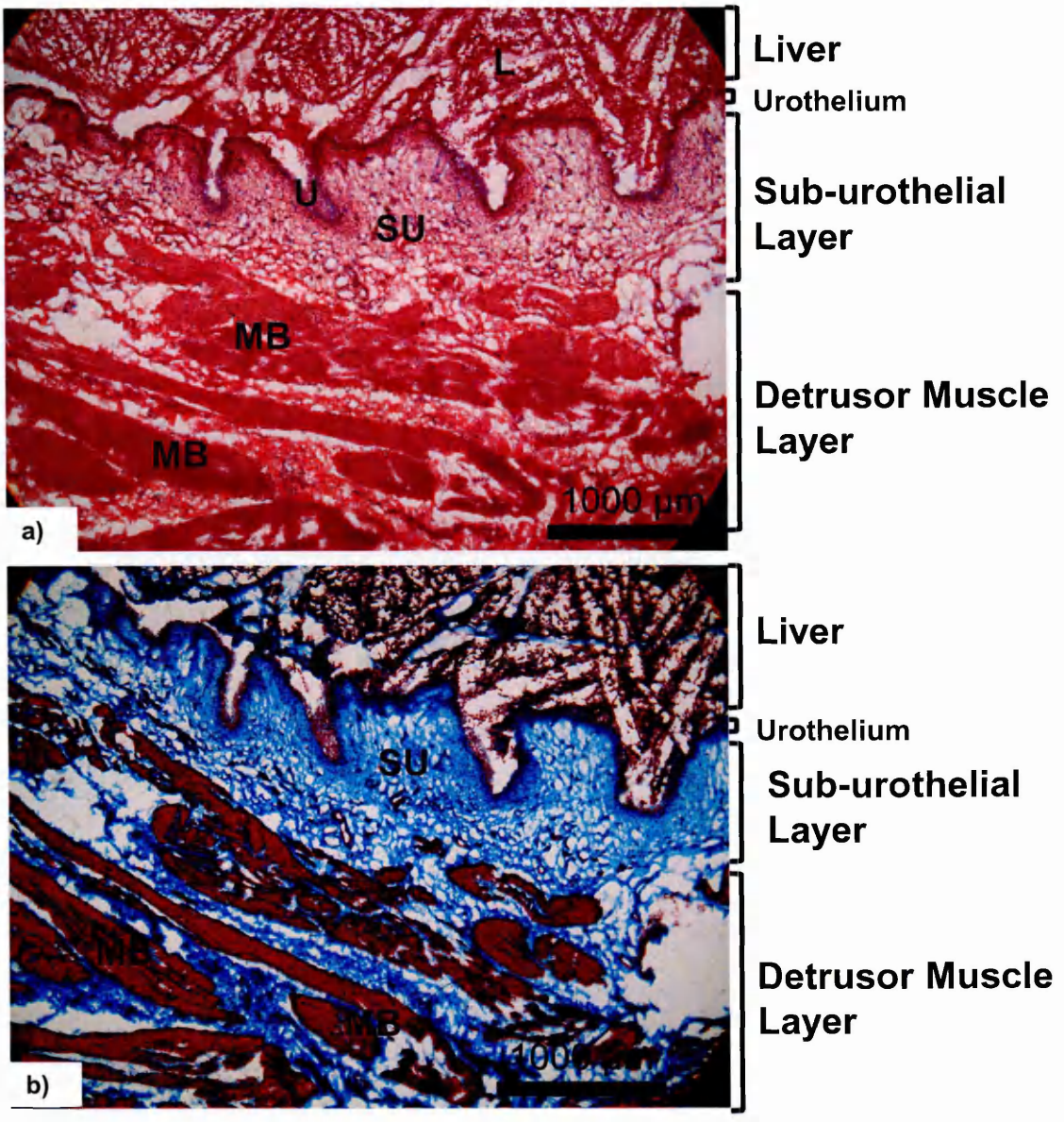


Figure 2.17 - Comparison of H & E vs. Masson's trichrome staining of liver-bladder-liver sandwich sections from the dome. A and B: H&E stain; C and D: MT stain on 10 micron thick sections. Left panel - 4X; Right panel - 10X magnification of highlighted region. U - urothelium, SU - suburothelium, MB - muscle bundle, L - liver.

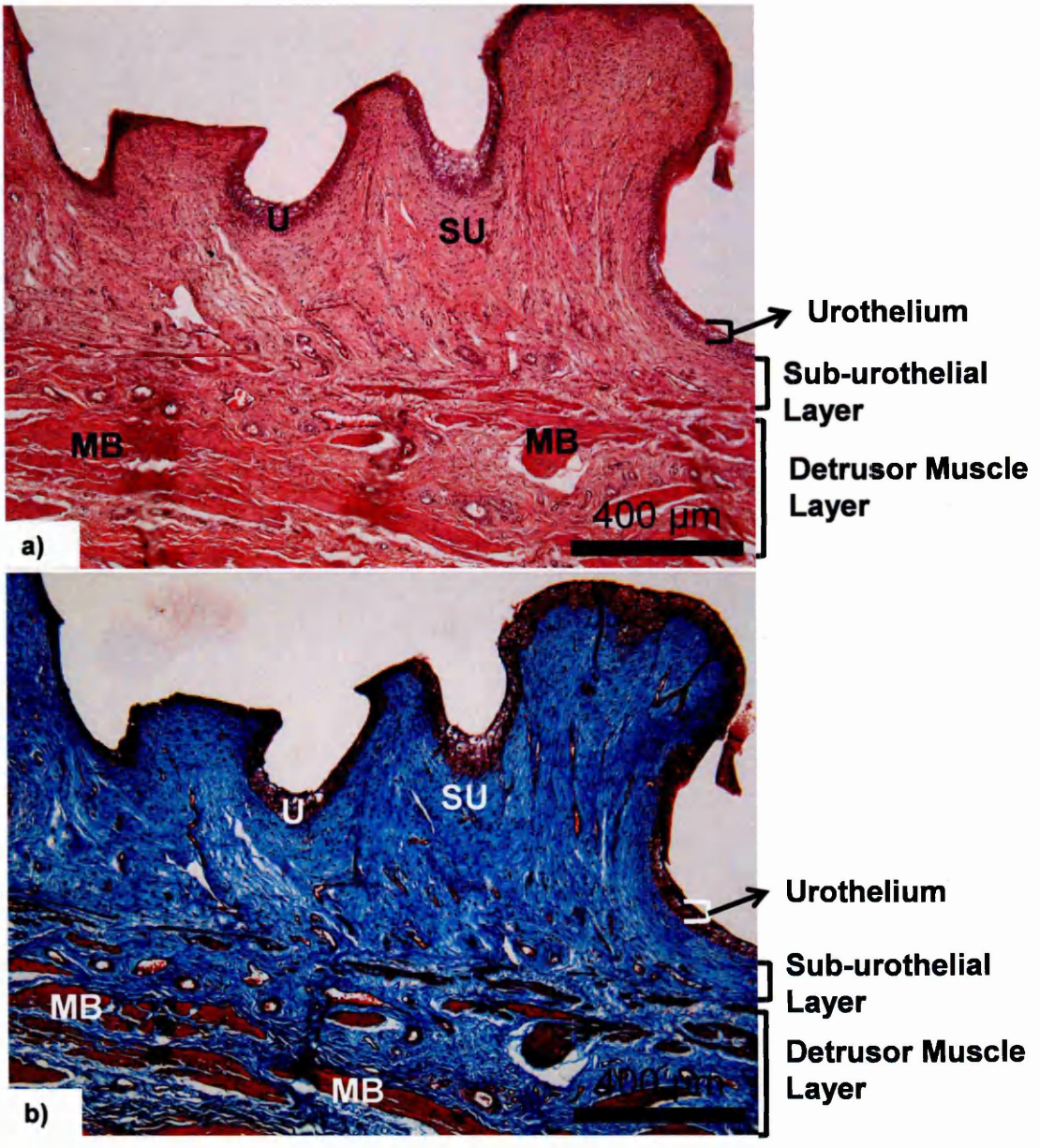


Figure 2.18 - Comparison of H & E vs. Masson's trichrome staining of paraffin embedded bladder sections from the dome. A and B: H&E stain; C and D: MT stain on 4 micron thick sections. Left panel - 10X; Right panel - 20X magnification of highlighted region. U - urothelium, SU - suburothelium, MB - muscle bundle, L - liver.

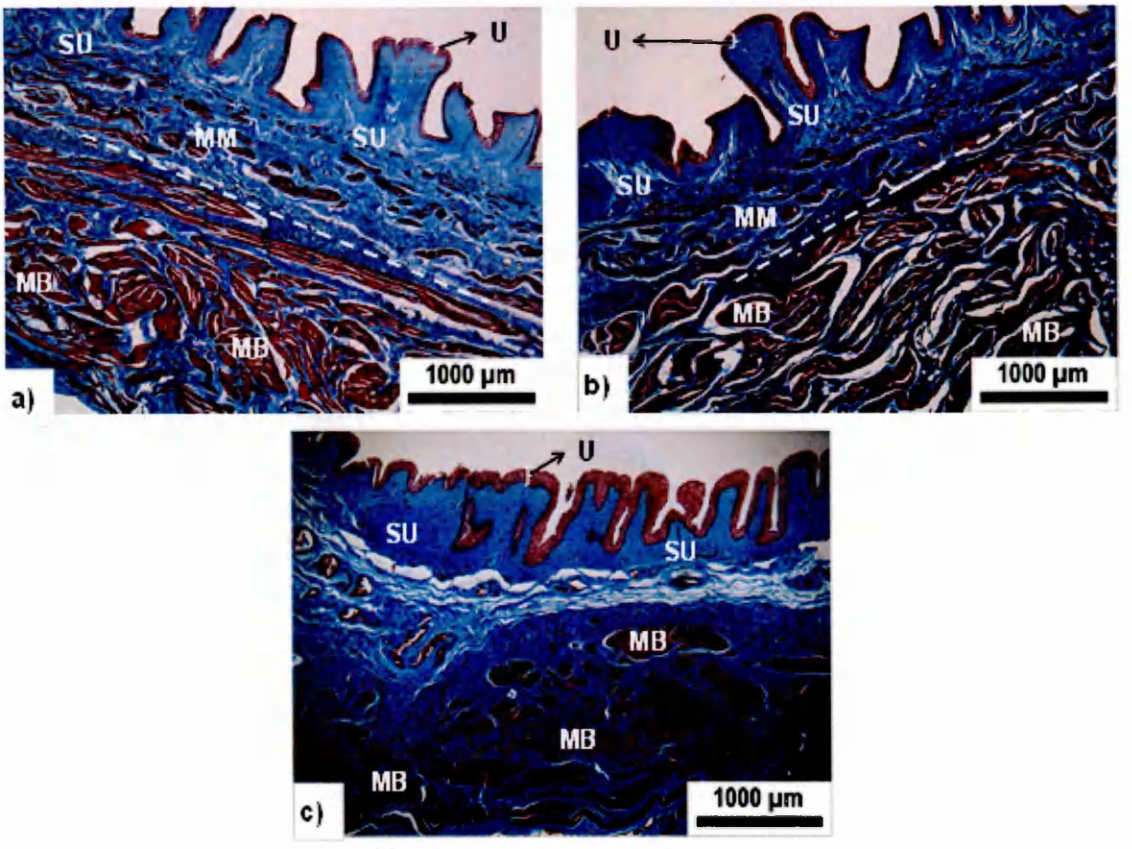


Figure 2.19 - Typical example of MTS on wax embedded sections from a) dome, b) body and c) trigone regions showing the urothelium (U), sub-urothelium (SU), muscularis mucosae (MM) and the detrusor muscle bundles (MB); 4X magnification.

A comparison of the measurements taken from the different bladder layers in the three bladder regions is shown in Figure 2.20. In the dome region (Figure 2.20a) bladder layer thickness increased from the urothelium to the detrusor. Using one way ANOVA, the sub-urothelium and detrusor layers of the dome were significantly thicker than the urothelium layer in the dome. There were no significant differences between the suburothelium and the detrusor layers of the dome. In the body region (Figure 2.20b) the detrusor layer was significantly thicker than both the urothelium and suburothelium, with no significant differences observed between the urothelium and suburothelium. For the trigone region, the suburothelium and detrusor layers were both significantly thicker than the urothelium (Figure 2.20c) with no significant differences noted between the suburothelium and detrusor layers.

Comparison of the individual layers between the three regions is shown in Figure 2.21. The thickness of the urothelium was similar in the dome, body and trigone regions (Figure 2.21a), whereas the suburothelium layer was significantly thicker in the trigone compared to the dome and body regions (Figure 2.21b). Comparison of the detrusor layer revealed that the detrusor of the body region was significantly thicker compared to the dome and trigone regions. No differences were noted in comparing the dome and trigone detrusor layers (Figure 2.21c).

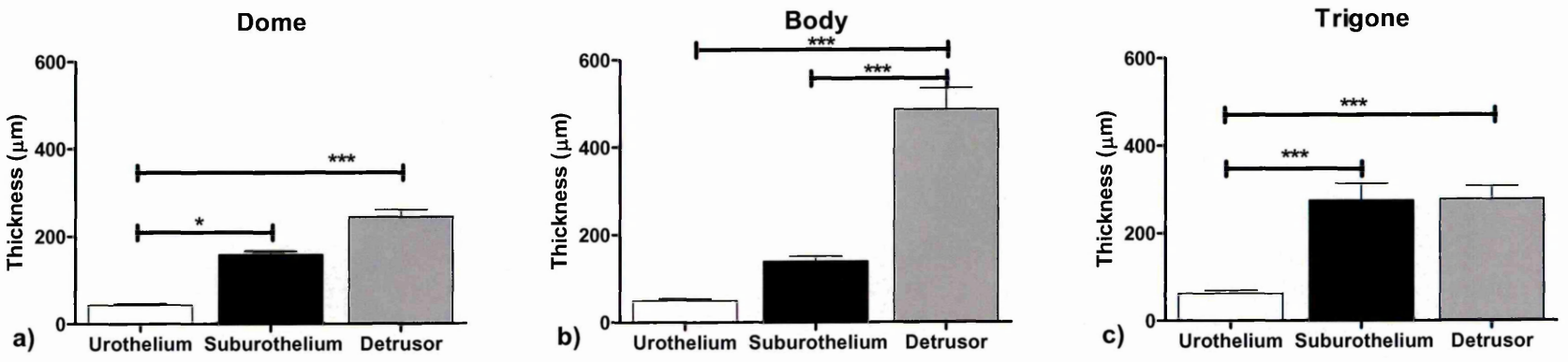


*Figure 2.20 - Comparison of bladder layer thickness in pig bladder a) dome, b) body and c) trigone regions. Data are presented as mean ± SEM. Statistical significance shown obtained via one way ANOVA with *p<0.05, ***p<0.001; n=6.*

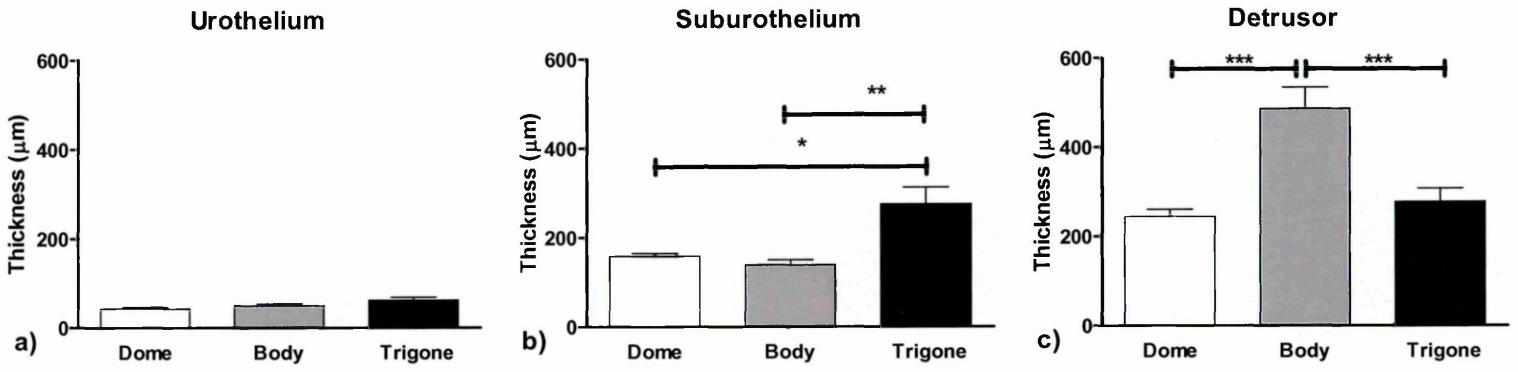


*Figure 2.21 - Comparison of individual pig bladder layers in the bladder wall between the three bladder regions - dome, body and trigone. A) urothelium, b) suburothelium and c) detrusor layers. Data are presented as mean ± SEM, statistical significance shown obtained via one way ANOVA with *p<0.05, **p<0.01 and ***p<0.001; n=6.*

2.5 Discussion

In vitro studies of isolated bladders have shown that phasic spontaneous contractions (PCs) develop without any stimulation, although little is known regarding the origin and physiological role of these PCs. These phasic contractions have been attributed to the release of mediators from the urothelium that may generate spontaneous action potentials that rapidly propagate throughout the detrusor causing bladder contractions (Hashitani *et al.,* 2001). In the present study, spontaneous phasic contractions did not develop in all bladder strips used. In the dome, 90% of the 20 strips used developed PCs while in the body and trigone regions, 100% of 20 strips developed PCs. This is in contrast to a study which reported that 19% of 191 strips from the pig bladder dome developed PCs and 89% of the 19 strips from the trigone developing PCs (Sibley 1984). The same study also reported that 20% of 139 strips and 71% of 7 strips from the human dome and trigone bladder regions respectively developed PCs (Sibley 1984). Roosen *et al* (2009) reported that 68% of 28 trigone strips from male guinea-pig bladders developed PCs compared to 38% of 21 strips from the dome (Roosen *et al.,* 2009). Differences in species, bladder size and bladder composition may account for the observed differences in development of PCs development. Age may also have an effect on the differences observed, although there is no evidence in the literature. .

Analysis of the time taken for development of PCs in intact (mucosa/urothelium present) and denuded (urothelium absent) strips revealed that denuded strips from the dome and body took significantly longer to develop PCs compared to intact strips, whereas in the trigone which no difference in PC development time was seen between intact and denuded strips. These findings are similar to those published by Akino *et al* (2008) who showed that the time required for PCs to develop was shorter in intact strips of the dome compared to their denuded counterparts. Similarly, that study also found that the intact and denuded strips of the trigone were similar in PC development time (Akino *et al., 2008*). Comparing between the three bladder regions, intact strips were similar in PCs development time although denuded strips from the dome and body were significantly different to the trigone.

From these results, it is possible to propose that there is an excitatory mechanism present in intact strips that allows for faster development of PCs that is lacking in denuded strips. The urothelium has been shown to contain a variety of receptors and ion channels and has the ability to release various mediators such as ATP (Ferguson *et al.,* 1997), ACh (Yoshida *et al.,* 2006) and NO (Birder 2006). There is also published literature on an unidentified urothelium derived inhibitory factor (UDIF) (Hawthorn *et al.,*

2000). The presence of receptors and the release of various transmitters by the urothelium, coupled with the presence of a network of myofibroblasts and interstitial cells (ICCs) in close proximity to nerves may implicate the urothelium in the initiation of action potentials that can propagate down the bladder detrusor causing spontaneous contractions. Take away the urothelium, and this development of PCs is significantly delayed. Exactly which type of cell is responsible is not clear, but what is clear is that this mechanism does not seem to be present in the trigone, suggesting a functional difference in generation of PCs in the bladder regions, which may reflect the different physiological roles of these regions.

When amplitude and frequency measurements of PCs between intact and denuded strips across the 3 bladder regions were compared, the denuded strips had PCs of greater amplitude than intact strips in the body and trigone, although this was not significant. These results are consistent with Akino *et al.* (2008) who showed no differences in amplitude between intact and denuded strips from the dome and trigone. However, differences in PCs in the trigone were again apparent with significantly greater amplitude compared to the dome and body in intact and denuded tissues.

Measurements of frequency of PCs showed intact strips of the trigone to have significantly lower frequency compared to the dome and body. Removal of the mucosa significantly reduced frequency of PCs in the dome and body but increased the frequency (non-significantly) in the trigone, again illustrating differences between the three regions in the PCs and the role of the mucosa. In the dome and body, the urothelium may release mediators that can mediate PCs, hence the intact strips exhibit a greater number of contractions than the denuded strips without the urothelium. These mediators may act on the underlying nerves, ICCs or the detrusor muscle. The process of denuding the bladder strips ultimately removes the urothelium and part of the sub-urothelium, and thus would subsequently affect mediator release and response by the myofibroblast and ICC network present in the suburothelium (Wiseman *et al.,* 2002). This may explain why denuded strips have fewer contractions per 5 min period than the intact strips. It is also possible that the tissue damage as a result of removal of the urothelium may be responsible for the decrease in the number of PCs and subsequent impairment in function. However, since removal of the urothelium did not to affect the trigone region, tissue damage does not appear to occur

Amplitude and frequency of PCs at 1hr and 2hr time points was also assessed between the dome, body and trigone. Comparison of PCs across the 3 regions showed no significant differences in amplitude of intact strips between 1hr and 2hr time

points. In denuded strips, the amplitude was significantly increased in all regions at the 1hr versus the 2hr time points. Frequency comparison between the regions in intact strips showed no significant differences between the 1hr and 2hrs time points. In denuded strips the frequency of PCs from the dome and body was significantly higher at 2hrs compared to the 1hr time point, whereas for the trigone, no difference was observed at the 1hr and 2hr time points.

The differences observed functionally between the bladder regions may lie in a different morphology and composition of the bladder wall in these regions and thus were investigated histologically. In order to accurately measure the different layers of the bladder wall histologically, it was imperative to ensure the tissue remained intact throughout the tissue processing (freezing, sectioning and staining procedures). In initial experiments using frozen bladder tissues, the urothelium layer became detached from the rest of the bladder during sectioning. This led to the use of the liver-bladder-liver sandwich method, in order to provide support for the delicate urothelium and help maintain its integrity for measurements. Staining of the bladder sections prepared in this way showed that in some regions of the bladder urothelium were not sufficiently supported and ultimately had separated from the bladder. Also tissue integrity was not optimal. Paraffin embedded tissue sections proved to be the better method to maintain tissue integrity and was therefore the method of choice for analysis of the different bladder layers. Comparison of the two most commonly used stains on the paraffin embedded section showed the MTS method to achieve the better contrast of the tissue structure and accurate demarcation of the different layers for measurements.

Previous studies comparing between the bladder trigone region and the main detrusor (body) showed no apparent differences in the composition of the urothelium (Romih *et al.,* 2005; Thomas *et al.,* 2005; Sun 2006). This supports the findings of the present chapter since no significant differences were observed in the thickness of the urothelial layer between the dome, body and trigone regions. Comparison of the sub-urothelium layer on the other hand showed the dome and trigone to have significantly thicker layer compared to the body. Drake *et al.* (2003) has shown the spread of bladder autonomous activity from the dome region to the rest of the bladder (Drake *et al.,* 2003).

In contrast, detrusor thickness was greatest in the body compared to the dome and trigone regions. As previously stated bladder SMCs need to be able to stretch to accommodate bladder filling. At the same time, once urine levels reach threshold levels, the sudden increase in bladder intravesical pressure results in neural overload and hence the sudden synchronized contraction of the bladder to allow for micturition

(Brading 1997b). The presence of a thicker detrusor layer in the body possibly due to a higher concentration of muscle bundles appears to makes sense in order to allow it to function in fulfilling the micturition cycle. Thus histological findings in the present study highlight structural differences in the bladder wall which may underlie the functional differences observed. Although, it is probable that the differences lie at the cellular level, in the role of the different cell types.

2.6 SUMMARY AND CONCLUSIONS

The experimental results presented in this chapter demonstrate that there are differences in functional characteristics of the pig bladder across the dome, body and trigone regions.

- Major differences are observed particularly between the trigone versus the dome and body implying functional heterogeneity between the trigone and the dome and body.
- Structurally, there are also marked differences in the different bladder layers across the three regions, particularly in the sub-urothelium. These differences may play a role in how these distinct regions function and will be further investigated in subequent chapters

CHAPTER 3

CHOLINERGIC MODULATION OF PHASIC ACTIVITY IN PIG BLADDER DOME, BODY AND TRIGONE.

3.1 INTRODUCTION

3.1.1 MUSCARINIC RECEPTORS IN THE BLADDER

In the bladder detrusor, there is a heterogeneous population of M_2 and M_3 muscarinic receptors as evidenced by molecular and binding studies. The ratio of M_2:M_3 receptors is 3:1 in human and pig bladders and has been shown to be as high as 9:1 in rats (Wang *et al.,* 1995; Yamaguchi *et al.,* 1996; Baselli *et al.,* 1999; Yamanishi *et al.,* 2000). Although the M_2 subtype outnumbers the M_3, it is the latter that has been credited with the direct contraction *in vitro* of the detrusor muscle. To date, this has been shown in mice (Choppin 2002; Canda *et al.,* 2009), humans (Chess-Williams *et al.,* 2001; Fetscher *et al.,* 2002), pigs (Sellers *et al.,* 2000), rats (Longhurst *et al.,* 1995; Wang *et al.,* 1995), guinea pig (Noronha-Blob *et al.,* 1989) and the rabbit (Choppin *et al.,* 1998). M_3 receptor gene knock-out studies performed on mice showed a significant reduction in bladder contraction (95%) with the remaining 5% being attributed to M_2 receptor function (Matsui *et al.,* 2002). M_2 and M_3 double knock-out viable mice showed absolutely no muscarinic receptor mediated contractions (Matsui *et al.,* 2002).

Muscarinic receptors in the bladder are also located on both parasympathetic and sympathetic pre-junctional nerve endings where they play a role in the regulation of neurotransmitter release. Inhibition of ACh release is attributed to either M_2 or M_4 receptors whereas enhancement of ACh release is due to M_1 receptors. This increase in ACh is via pathways involving L-type calcium channels and protein kinase C (Somogyi and de Groat 1999). Distinguishing between M_2 and M_4 subtypes and establishing the one that is responsible for inhibition of ACh release has proved to be challenging, as both subtypes are negatively coupled to adenylate cyclase and cause a decrease in cyclic adenosine monophosphate (cAMP) levels and the available antagonists do not discriminate between the M_2 and M_4 receptors (Chess-Williams 2002). In humans, the M_4 subtype may be responsible for the inhibition of ACh release (D'Agostino *et al.,* 2000) whereas in mice, the M_2 subtype is primarily responsible for the regulation of noradrenaline (NA) release (Trendelenburg *et al.,* 2003; Trendelenburg *et al.,* 2005).

Molecular studies have shown that muscarinic receptors are also present in the bladder urothelium of several species including human (Mansfield *et al.,* 2005; Tyagi *et al.,* 2006; Bschleipfer *et al.,* 2007) and mouse (Zarghooni *et al.,* 2007). In human studies, within the urothelial layers, the M_1 subtype is mainly expressed in the basal layer, with the M_2 receptor subtype being expressed in the umbrella cells and the M_3 - M_5 subtypes expressed heterogeneously in all layers of the urothelium (Bschleipfer *et al.,*

2007). Studies using immunohistochemistry to localise muscarinic receptors in the mouse bladder have demonstrated a similar distribution of the various receptor subtypes to the human bladder (Zarghooni *et al.,* 2007). M_2 and M_3 immunopositive reactive staining has been used to confirm presence of these receptors in human bladder detrusor (Mukerji *et al.,* 2006). Expression of muscarinic receptors on bladder afferent neurons has been confirmed by Nandigama *et al.* (2010) who reported the expression of M_2, M_3 and M_4 receptors on mouse bladder afferent neurons but were unable to detect the M_1 or M_5 subtypes. M_2 and M_3 receptors have also been shown to be expressed in nerve fibre bundles and in the dorsal root ganglion of small and medium sensory neurons in the sub-urothelium (Mukerji *et al.,* 2006). This finding strongly supports the theory that processing of sensory information may be mainly via cholinergic control (Nandigama *et al.,* 2010). Muscarinic receptors are also expressed on ICCs, as shown in immunohistochemical studies of guinea pig bladders, which revealed the expression of the M_3 receptor subtype on suburothelial ICCs (Grol *et al.,* 2009) and on myofibroblast-like cells in human bladder sub-urothelium (Mukerji *et al.,* 2006).

Although there is a lot of information on muscarinic receptors in the detrusor and how important a role they play in bladder function, relatively little is known about these receptors in the urothelium, nerves and ICCs and what their functions is at these other locations. Non-neuronal ACh is released from bladder urothelium and sub-urothelium layers (Yoshida *et al.,* 2006; Yoshida *et al.,* 2008) and it is not clear what it does in the bladder but it is possible that it may act on receptors located in the underlying nerves, muscles and ICCs and subsequently enhance bladder phasic activity especially in pathological conditions.

3.1.2 CHOLINERGIC MODULATION OF PCS

Investigation of the origin of bladder spontaneous contractions in neonatal and adult rats showed that these normal PCs that occur naturally in both isolated strips and whole bladders can be modulated cholinergically using low concentrations (50nM) of muscarinic agonist (Kanai *et al.,* 2007; Vahabi *et al.,* 2011a). It is not clear whether PCs that develop in the pig bladder can be modulated in a similar manner and is thus investigated in this chapter. In OAB (Andersson and Yoshida 2003) and ageing (Yoshida *et al.,* 2004), ACh release has been shown to be increased and may contribute to the enhanced PCs observed in these conditions and several other pathological conditions.

Muscarinic receptors have been investigated and implicated in a number of pathological conditions of the bladder. Various studies have shown that there is enhanced sensitivity of the bladder detrusor to muscarinic agonist in several disease states including denervation (Braverman *et al.,* 1998), BOO (Speakman *et al.,* 1987), diabetes (Mimata *et al.,* 1995), ageing (Yu *et al.,* 1997) and hyper-reflexic bladder (Martin *et al.,* 1997). In a study involving children with recurrent urinary tract infections, it was noted that there was increased selective sensitivity of bladders to CCh (Werkstrom *et al.,* 2000). In cases of chronic BOO, the detrusor supersensitivity experienced is thought to be due to parasympathetic denervation (Harrison *et al.,* 1987; Speakman *et al.,* 1987). In animal experiments using rats, it was observed that the M_2 receptor density in the bladder increased by 60% following denervation (Braverman *et al.,* 1998) an effect not observed in rats with obstructed bladder whose responses were still mediated by the M_2 receptor subtype (Krichevsky *et al.,* 1999).

Urothelial muscarinic receptor activation leads to a release of various factors including ATP, which is involved in bladder sensory mechanisms (Kullmann *et al.,* 2008; Burnstock 2009). Stimulation of urothelial muscarinic receptors by agonists also induces the production of an unidentified urothelium derived inhibitory factor (UDIF) which inhibits detrusor contraction (Hawthorn *et al.,* 2000). These responses mediated by muscarinic receptors may possibly be altered in other bladder disease states as evidenced by increased population of M_2 and M_3 receptors in the urothelium from rats with partial BOO (Kim *et al.,* 2008). Ruggieri *et al.* (2006) using rats showed that in bladder pathologies resulting from hypertrophy due to denervation led to the upregulation of the M_2 receptor subtype and it becomes the primary mediator of bladder contraction (Ruggieri and Braverman 2006). It is possible that increased ACh in pathological conditions may act on the muscarinic receptors present in the bladder thereby leading to increased PCs.

3.1.3 PHARMACOLOGICAL TOOLS FOR INVESTIGATION OF MUSCARINIC RECEPTORS

The use of muscarinic agonists and antagonists has been on the front line of identification of functional and pharmacologic responses of the different muscarinic receptor subtypes. This progress has been hampered mainly due to the lack of selectivity of the available antagonists for a single muscarinic subtype thereby hindering pharmacological characterization (Scarpero and Dmochowski 2003). The problem of lack of selectivity is compounded by the fact that tissues also express more than one subtype of receptors making it more challenging to determine the function of a particular subtype.

There are various muscarinic agonists and antagonists available that have been utilized in the pharmacological identification and characterisation of receptor selectivity profiles. Radioligand binding experiments involving ligands such as [^{3}H]pirenzepine and [^{3}H]N-methylscopalamine have been used in the determination of differences in antagonist equilibrium dissociation constants (K_d) in order to discriminate the various receptor subtypes, and functional experiments have been performed on cells or organs expressing the specific subtype of interest as a conclusive confirmation (Caulfield and Birdsall 1998).

Some of the main muscarinic agonists and antagonists used in functional investigation studies of receptor subtypes include pirenzepine, methoctramine, 4-Diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), tropicamide, oxotremorine, carbamycholine (carbachol - CCh), arecaidine and (+)-cis-dioxolane. Table 3.1 shows some of these muscarinic antagonists and their respective pK_B value range at the receptor subtypes. It is important to note that these antagonists are not exclusively selective but rather relatively selective for the different subtypes at the shown ranges.

Pirenzepine has a high affinity for M_1 receptors (7.8 - 8.5) and a low affinity for M_2 and M_3 receptors (6.3 - 6.7 and 6.7 - 7.1 respectively). It has a moderate (7.2 - 7.6) to high (7.7 - 8.1) affinity for M_4 receptors. Overall, pirenzepine is tenfold selective for the M_1 receptor over the other subtypes. Methoctramine has a high affinity for $_{M2}$ receptors (7.8 - 8.3) and is similarly tenfold more selective for the M_2 receptor over the others. 4-DAMP exhibits a high affinity for M_3 receptors (8.9 - 9.3) and a moderate affinity for M_2 (7.8 - 8.4). In contrast, 4-DAMP has an affinity tenfold lower at M_2 vs. M_3 receptors (Sellers and Chess-Williams 2012).

The affinity of pirenzepine for the M_4 receptor in conjunction with the high affinity of methoctramine and 4-DAMP for this receptor presents the problem of distinguishing M_2 or M_3 from the M_4 receptor (Caulfield and Birdsall 1998). Tropicamide, a slightly selective M_4 antagonist, has been used to distinguish M_4 from the other receptors (Lazareno and Birdsall 1993) but its affinity for the different subtypes (as shown in Table 3.1) makes it an inconclusive determinant of the M_4 receptor subtype (Sellers and Chess-Williams 2012).

The use of muscarinic agonists in the identification of receptor subtypes has also not been hugely successful. Similarly, this is due to the issue of lack of selectivity of the available agonists for specific receptor subtypes. Carbachol is a non-specific muscarinic agonist and has been widely used in *vitro* functional investigations in the bladder but it is not selective for any of the five subtypes (Sellers and Chess-Williams

2012). Oxotremorine methiodide (Oxo-M), (+)-cis-dioxolane and arecaidine are also high affinity muscarinic agonists lacking selectivity for any of the receptor subtypes. Arecaidine has been used in functional studies of the bladder due to its selectivity for muscarinic receptors over nicotinic receptors (Gillespie *et al.,* 2003). There is the added complication of potency when using agonists as well, which complicates things, as the response may differ in different tissues - so agonists are not normally used for receptor characterisation.

Antagonist	Receptor Subtype				
	M_1	M_2	M_3	M_4	M_5
Pirenzepine	7.8 - 8.5	6.3 - 6.7	6.7 - 7.1	7.1 - 8.1	6.2 - 7.1
Methoctramine	7.1 - 7.8	7.8 - 8.3	6.3 - 6.9	7.4 - 8.1	6.9 - 7.2
4-DAMP	8.6 - 9.2	7.8 - 8.4	8.9 - 9.3	8.4 - 9.4	8.9 - 9.0
Himbacine	7.0 - 7.2	8.0 - 8.3	6.9 - 7.4	8.0 -8.8	6.1 - 6.3
AF-DX 384	7.3 - 7.5	8.2 - 9.0	7.2 - 7.8	8.0 8.7	6.3
Darifenacin	7.5 - 7.8	7.0 - 7.4	8.4 - 8.9	7.7 - 8.0	8.0 - 8.1
Tropicamide	7.3	7.3	7.3	7.8	Unknown

Table 3.1- Antagonist affinity constants expressed as pK_B for the mammalian muscarinic receptors including human. Data is combined from radioligand binding and functional studies on cloned receptors and preparations of CHO cells transfected with and expressing a particular single receptor subtype. Adapted from (Lazareno and Birdsall 1993; Hegde *et al.*, 1997; Caulfield and Birdsall 1998; Sellers *et al.*, 2000; Wuest *et al.*, 2006; Stevens *et al.*, 2007; Sellers and Chess-Williams 2012).

3.2 CHAPTER AIMS

The aim of this chapter was:

- Investigate whether phasic contractions (PCs) of the pig bladder dome, body, and trigone can be modulated cholinergically.

3.3 Materials and Methods

Fresh pig bladders used in the experiments presented in this chapter were collected and prepared as described in chapter 2 section 2.3.1. Experiments were performed on intact and urothelium-denuded tissue strips of approximately 3x3x10mm from the dome, body and trigone regions of the pig bladder. Tissue strips were set up in tissue baths and allowed to equilibrate, as described in chapter 2 section 2.3.4 for 60 minutes under a load of 1g. Following equilibration, baseline measurements of amplitude and frequency of PCs were taken. Following this, the strips were incubated with low cumulative concentrations of carbamylcholine chloride (carbachol - CCh) at concentrations of 0.01, 0.05, 0.07, 0.1, 0.2, 0.3, 0.4 and 0.5μM. Strips were incubated with each concentration of CCh for 10 minutes prior to addition of the next concentration. Amplitude and frequency measurements of PCs were taken during the last 5 minutes with each concentration as described in chapter 2 section 2.3.5.

In separate experiments, the effect of muscarinic antagonists on basal phasic activity was investigated. Intact and denuded bladder strips from the dome, body, and trigone were set up in tissue baths as described above and allowed to equilibrate under a load of 1g for 30 minutes. The tissues were then incubated with different concentrations of muscarinic antagonists for 30 minutes and the amplitude and frequency of PCs was measured. Methoctramine was used at concentrations of 100nM, 300nM and 1μM whereas 4-Diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) was used at 3, 10 and 30nM concentrations. Each tissue was subjected to only one concentration of antagonist.

The effect of muscarinic antagonists on CCh-modulated PCs was also investigated. The tissues were incubated with the muscarinic antagonist for 30 minutes after which cumulative concentrations of CCh were added, as above. Measurement of the amplitude and frequency of PCs was then performed.

3.4 Data and Statistical Analysis

Amplitude and frequency measurements were recorded as described in chapter 2 section 2.3.5. Baseline measurements were taken during the last 5 minutes of the 60 minute equilibration period. To investigate the effect of CCh, measurements of amplitude and frequency were taken during the last 5 minutes with each concentration of the drug. To calculate the change in amplitude, the baseline measurements before addition of the drug were subtracted from the amplitude and frequency measurements in the presence of the drug. Statistical comparison for the effect of carbachol on PCs was via repeated measures one-way ANOVA with Dunnett's post-test comparing all data vs. control (baseline). A Student t-test was used to compare amplitude and frequency data between intact vs. denuded strips. Analysis of the effect of methoctramine and 4-DAMP on basal PCs was performed using a one-way ANOVA with a Bonferroni post hoc test. Inter-tissue comparison between the three bladder regions was performed via a 2-way ANOVA with a Bonferroni post hoc test.

Intra-tissue differences in the data from CCh modulated experiments were analysed using a one-way ANOVA, while inter-tissue comparisons were made using a 2-way ANOVA, both performed with Bonferroni post hoc tests. All data presented in this chapter is shown as mean ± SEM. Statistical significance was measured at 95% confidence interval with p values ≤ 0.05 considered significant. Experiments on dome and body regions were performed on n=7 strips for each bladder region whereas for the trigone n=5 strips ere used (each strip from a separate bladder).

3.5 RESULTS

3.5.1 EFFECT OF CCH ON THE AMPLITUDE OF BASAL PHASIC CONTRACTIONS

Isolated strips from all bladder regions developed PCs. This phasic activity was significantly increased by the addition of increasing concentrations of the muscarinic agonist CCh. Figures 3.1 - 3.3 show experimental traces of intact and denuded strips from the dome, body and trigone regions respectively. The effect of increasing concentrations of CCh on the amplitude of PCs is shown in figures 3.4 - 3.6. In intact strips from the dome region, CCh significantly increased the amplitude of PCs at 0.01 - 0.5μM CCh concentrations (Figure 3.4a) when compared to control, whereas in the denuded strips, CCh significantly increased the amplitude of PCs at 0.05 - 0.5μM CCh (Figure 3.4b). In the body region, CCh again significantly increased amplitude of PCs in intact strips at 0.2μM - 0.5μM CCh (figure 3.5a), whereas in their denuded counterparts, amplitude was significantly increased at 0.05 - 0.5μM CCh versus the control (Figure 3.5b). CCh did not significantly increase the amplitude of PCs in strips of intact trigone (Figure 3.6a). In denuded strips of trigone on the other hand CCh significantly increased amplitude at 0.01 - 0.1μM CCh, although this effect was not observed at the higher concentrations of CCh (Figure 3.6b).

3.5.2 EFFECT OF CCH ON THE FREQUENCY OF BASAL PHASIC CONTRACTIONS

The effect of CCh on the frequency of PCs is shown in figures 3.7 - 3.9. Analysis of intact strips from the three bladder regions showed that CCh had no significant effect on the frequency of PCs in the dome and trigone regions (Figure 3.7a and Figure 3.9a). On the other hand, in the body regions, CCh significantly increased the frequency of PCs only at 0.07μM and 0.2μM CCh concentration (Figure 3.8a). In denuded strips, CCh significantly increased the frequency of PCs in the dome (Figure 3.7b) between 0.03μM - 0.3μM CCh while in denuded strips of the body CCh significantly increased the frequency of PCs between 0.05μM - 0.2μM CCh (Figure 3.8b). CCh had no effect on the frequency of PCs in denuded strips from the trigone region.

Comparisons of the effect of CCh on PCs between intact vs. denuded strips showed that in the dome (Figure 3.10a) and trigone (Figure 3.10c) regions denuded strips had significantly greater amplitude of PCs than their intact counterparts. In the body (Figure 3.10b), the difference was not significant between intact and denuded strips. Comparisons of the effect of CCh on the frequency of PCs between intact and denuded strips showed that in the dome (Figure 3.11b) denuded strips had significantly greater frequency at 0.05μM, 0.07μM and 0.1μM CCh, although no significant differences were

observed between intact and denuded strips from the body and trigone (Figure 3.11 a & c respectively).

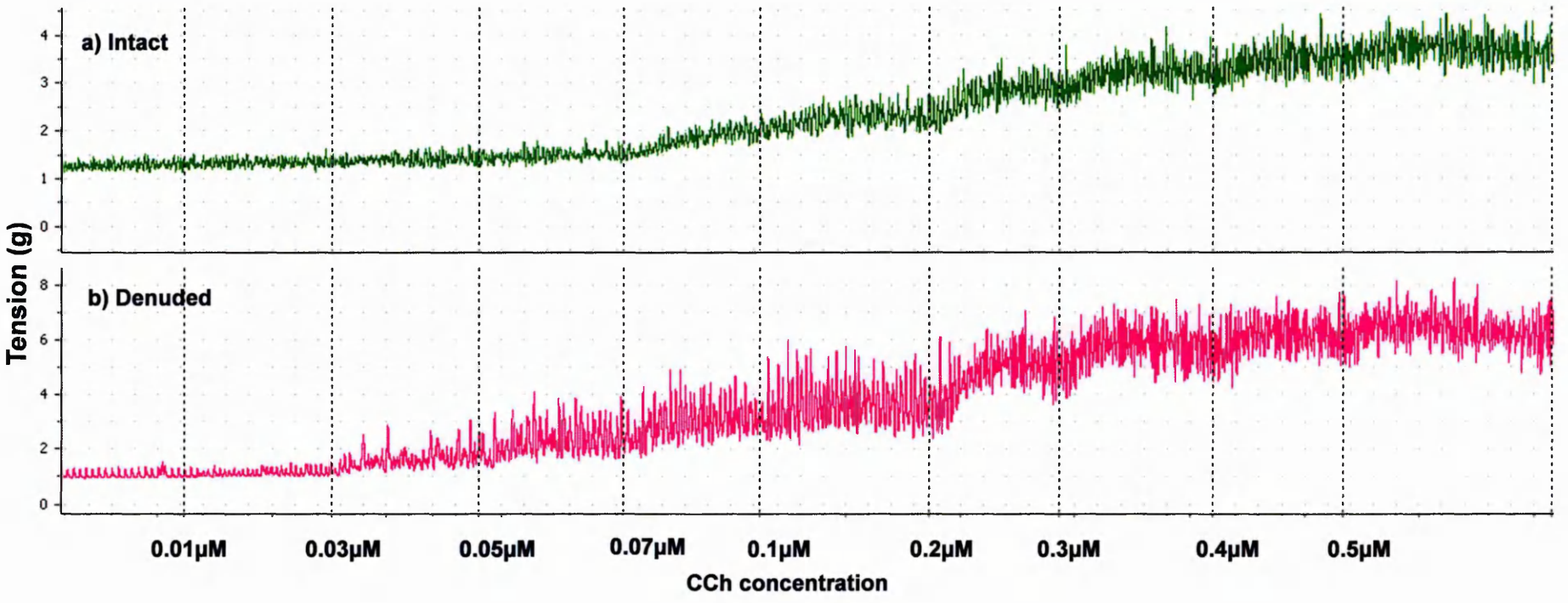


Figure 3.1- Experimental trace showing the effect of cumulative concentrations of CCh on PCs intact and denuded pig bladder strips from the dome region.

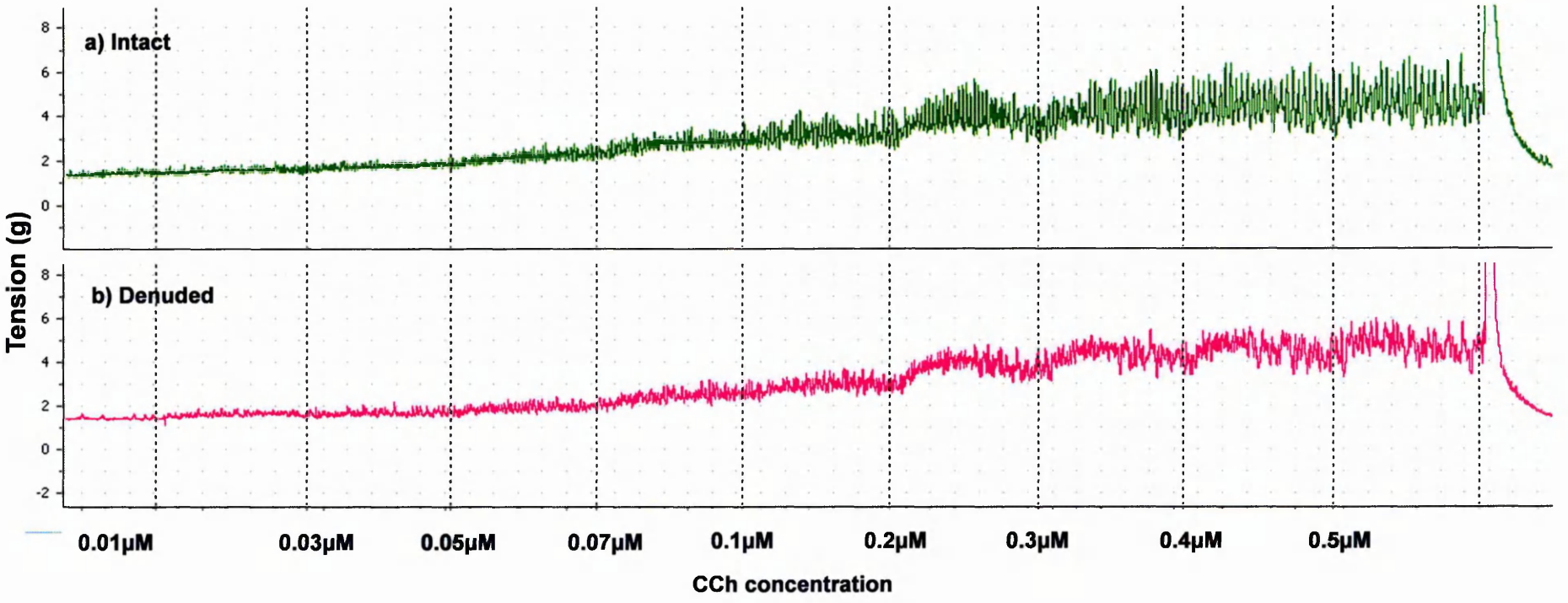


Figure 3.2 - Experimental trace showing the effect of cumulative concentrations of CCh on PCs in intact and denuded pig bladder strips from the body region.

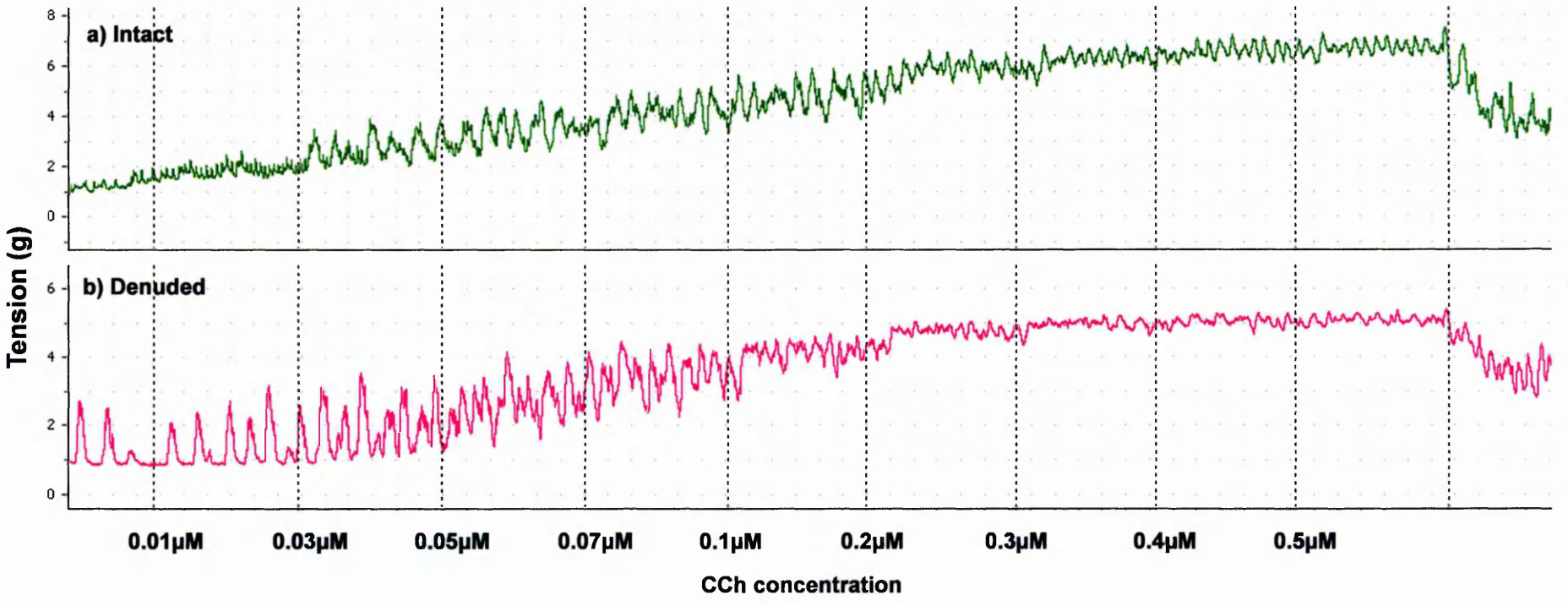
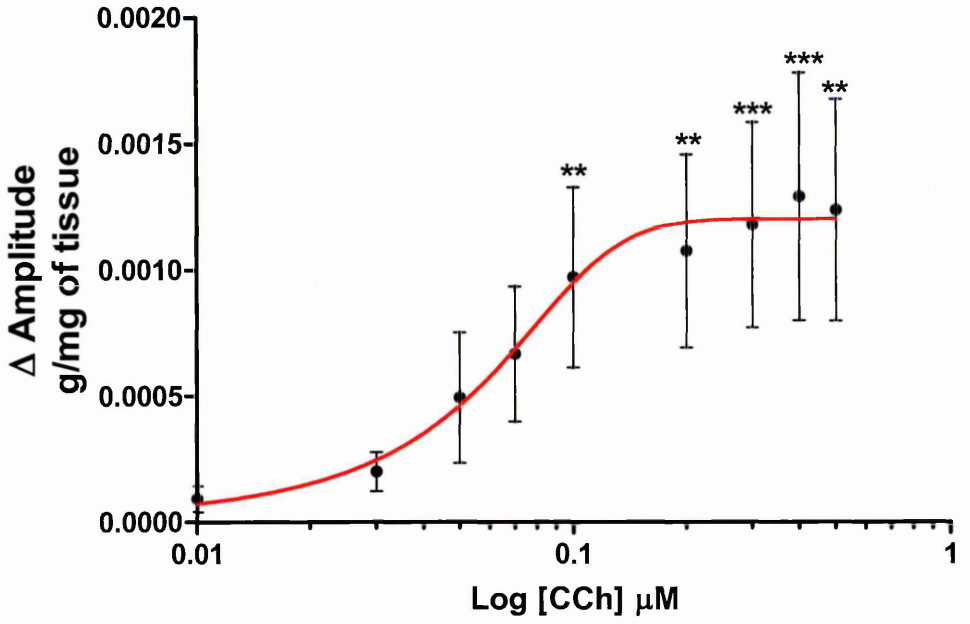
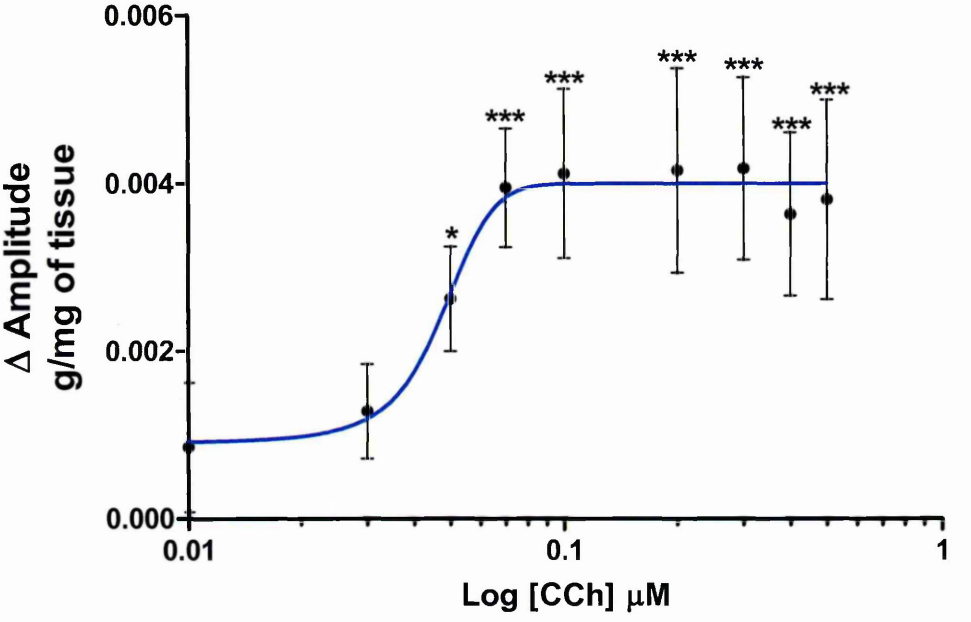


Figure 3.3- Experimental trace showing the effect of cumulative concentrations of CCh on PCs in intact and denuded pig bladder strips from the trigone region.

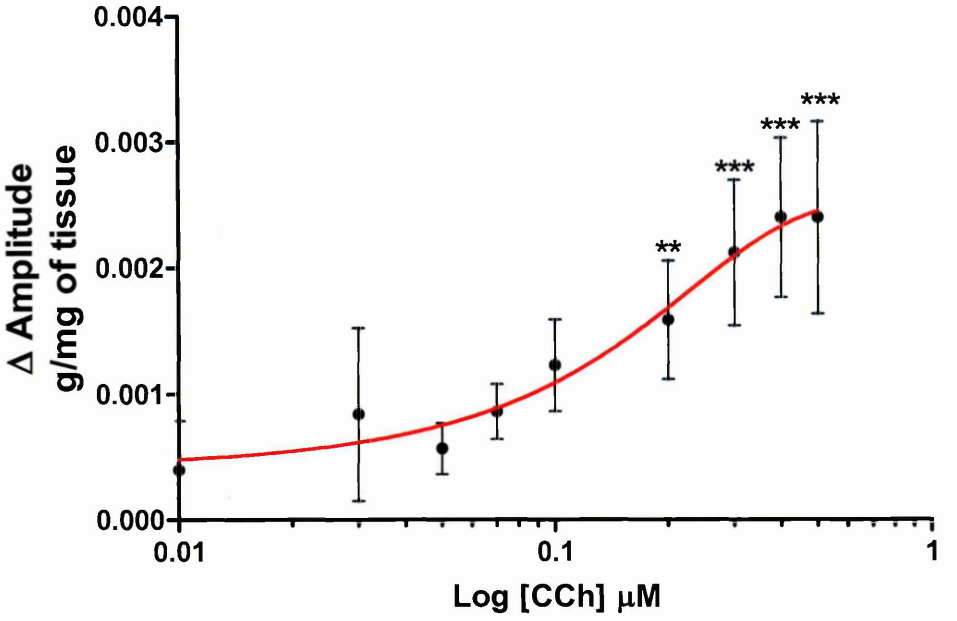


a)

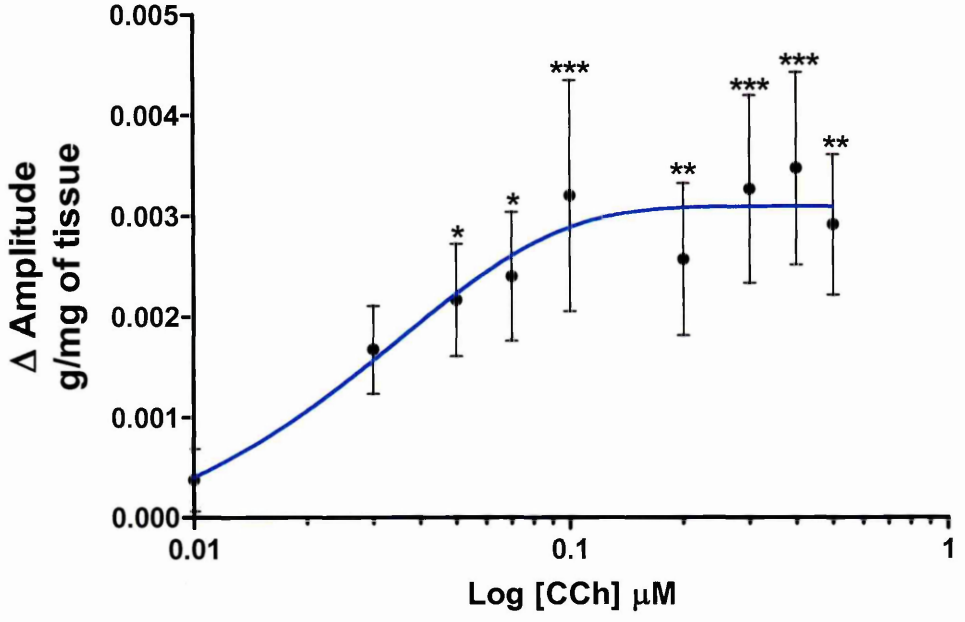


b)

Figure 3.4 - Effect of increasing concentrations of carbachol on the amplitude of phasic contractions in bladder strips from a) intact and b) denuded dome. All data are presented as mean ± SEM; *p≤0.05, **p≤0.01, ***p≤0.001 vs. control (0μM CCh); n=7.



a)



b)

Figure 3.5 - Effect of increasing concentrations of carbachol on the amplitude of phasic activity in bladder strips from a) intact and b) denuded body region. All data are presented as mean ± SEM; *p≤0.05, **p≤0.01, ***p≤0.0001 vs. control (0μM CCh); n=7.

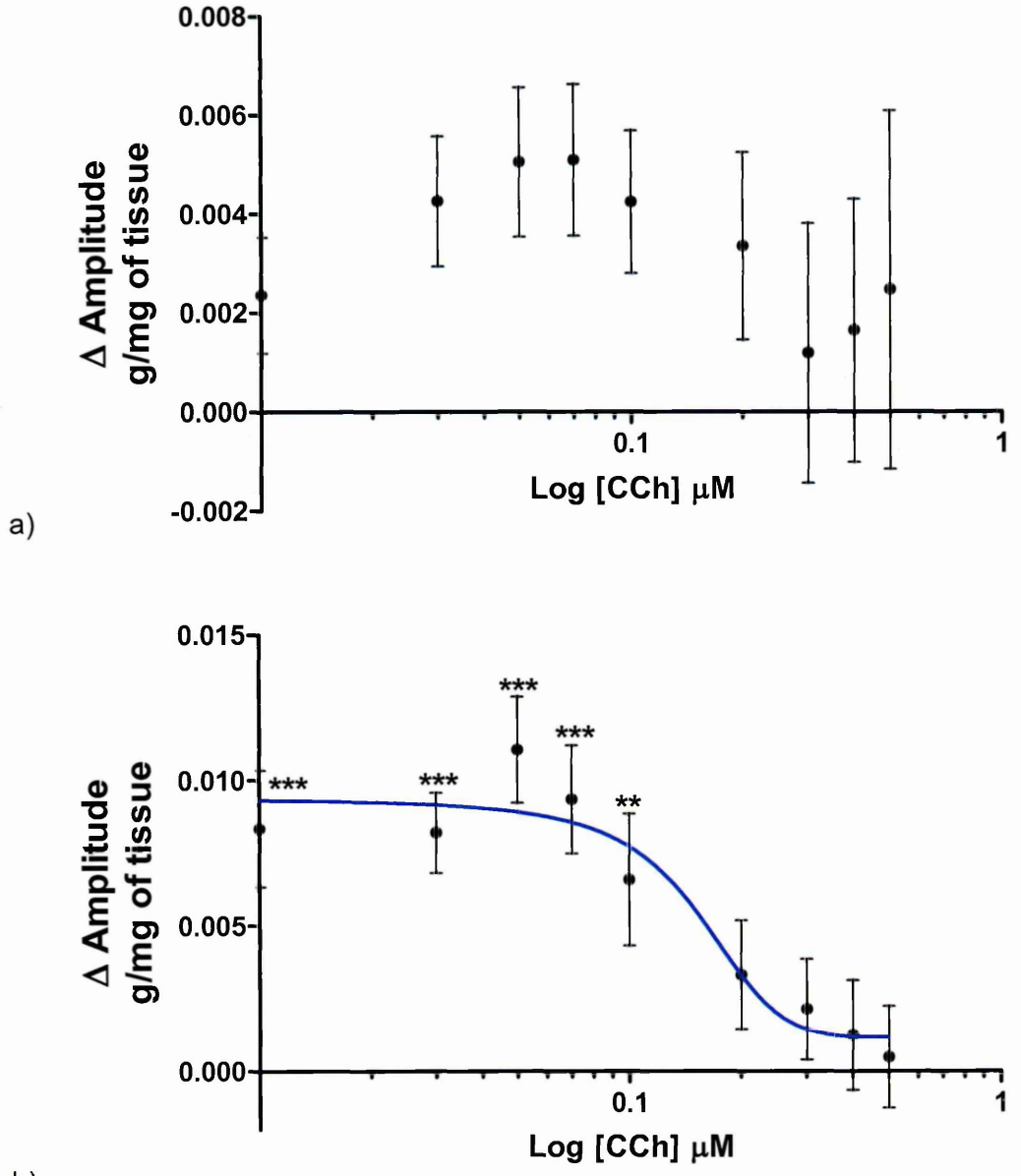
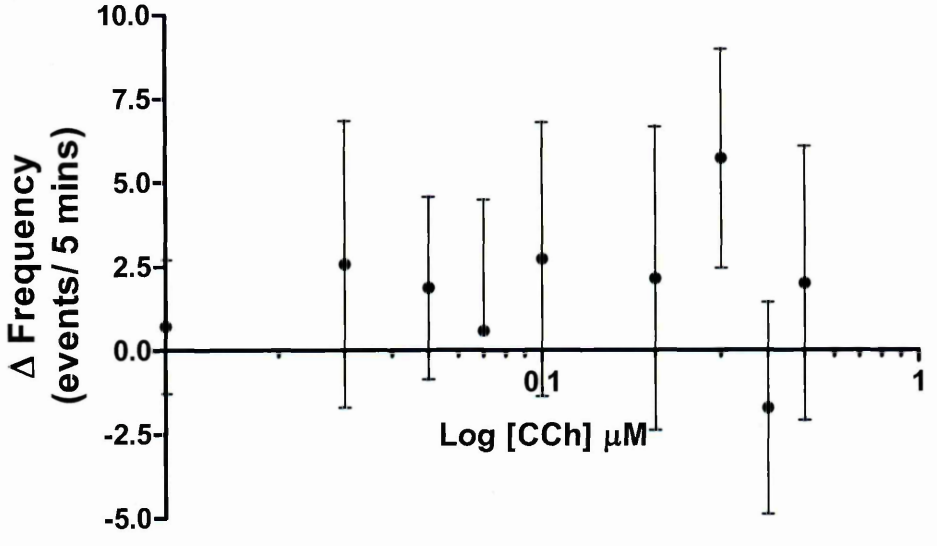


Figure 3.6 - Effect of increasing concentrations of carbachol on the amplitude of phasic activity in bladder strips from a) intact, b) denuded trigone region. All data are presented as mean ± SEM; **p≤0.01, ***p≤0.0001 vs. control (0μM CCh); n=5.

a)



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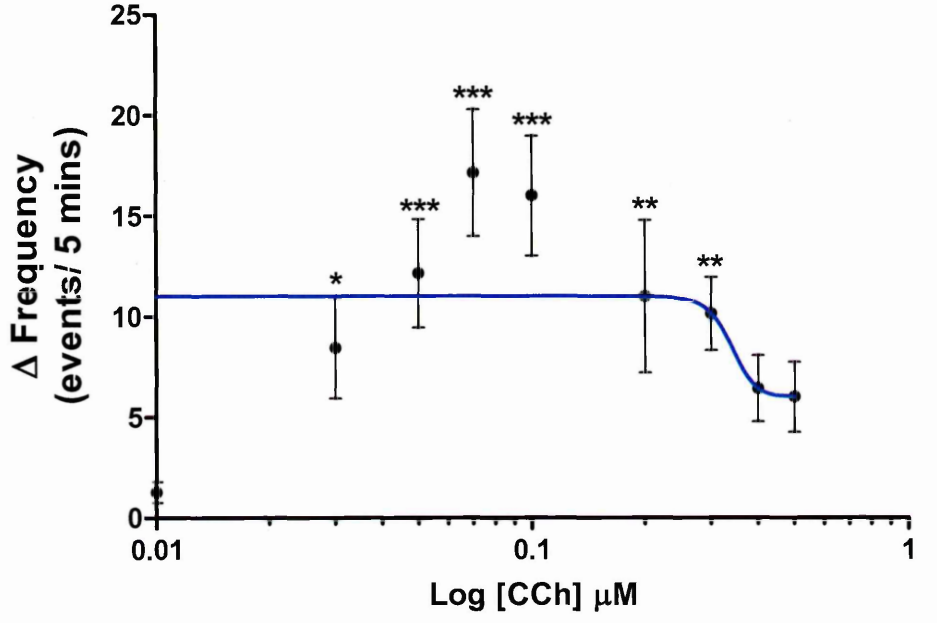


Figure 3.7 - Effect of increasing concentrations of carbachol on the frequency of phasic contractions on bladder strips from a) intact and b) denuded dome region. All data are presented as mean ± SEM; *p≤0.05, **p≤0.01, ***p≤0.001 vs. control (0μM CCh); n=7.

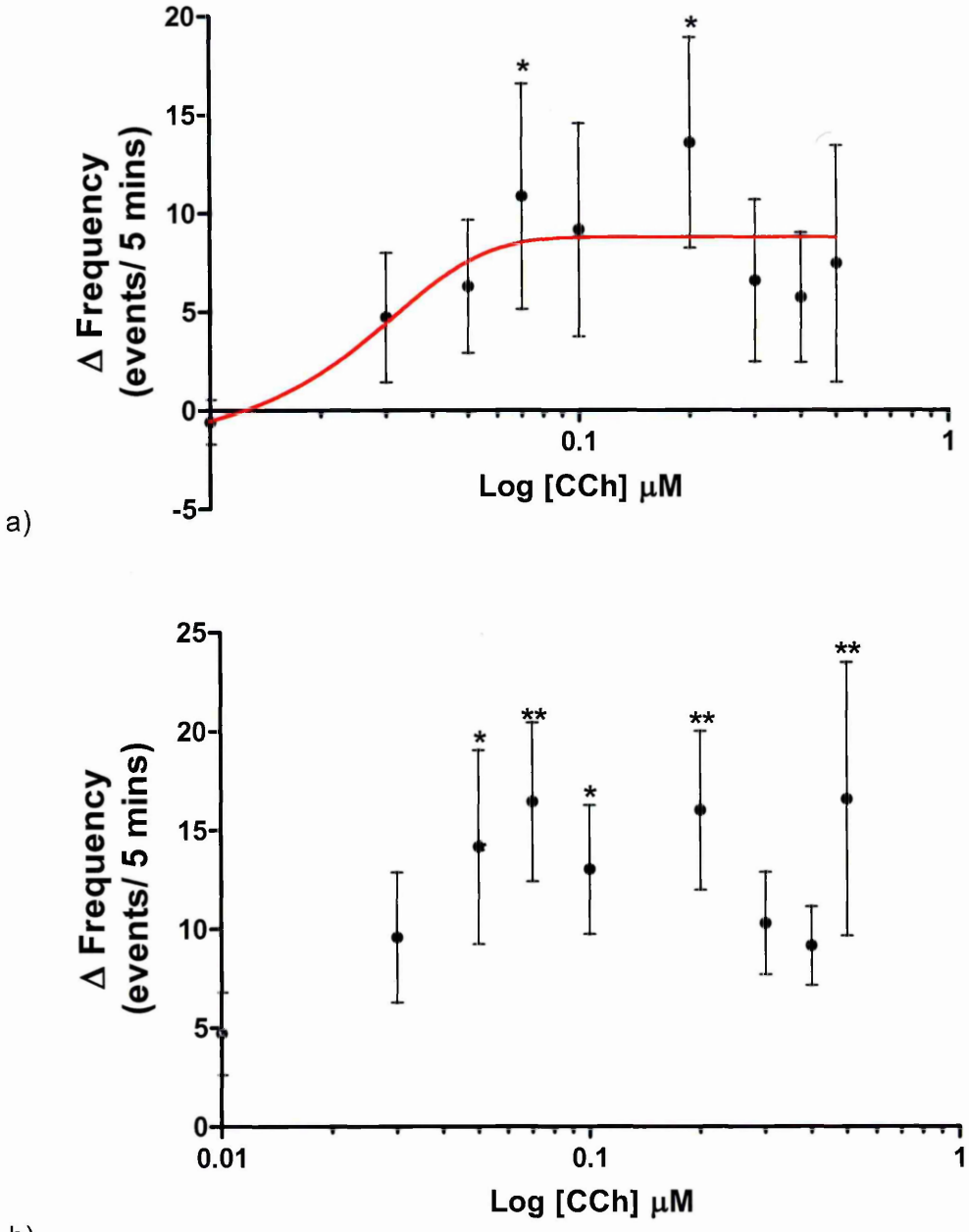


Figure 3.8 - Effect of increasing concentrations of carbachol on the frequency of phasic contractions in bladder strips from a) intact and b) denuded body region. All data are presented as mean ± SEM; *p≤0.05, **p<0.01 vs. control (0μM CCh); n=7.

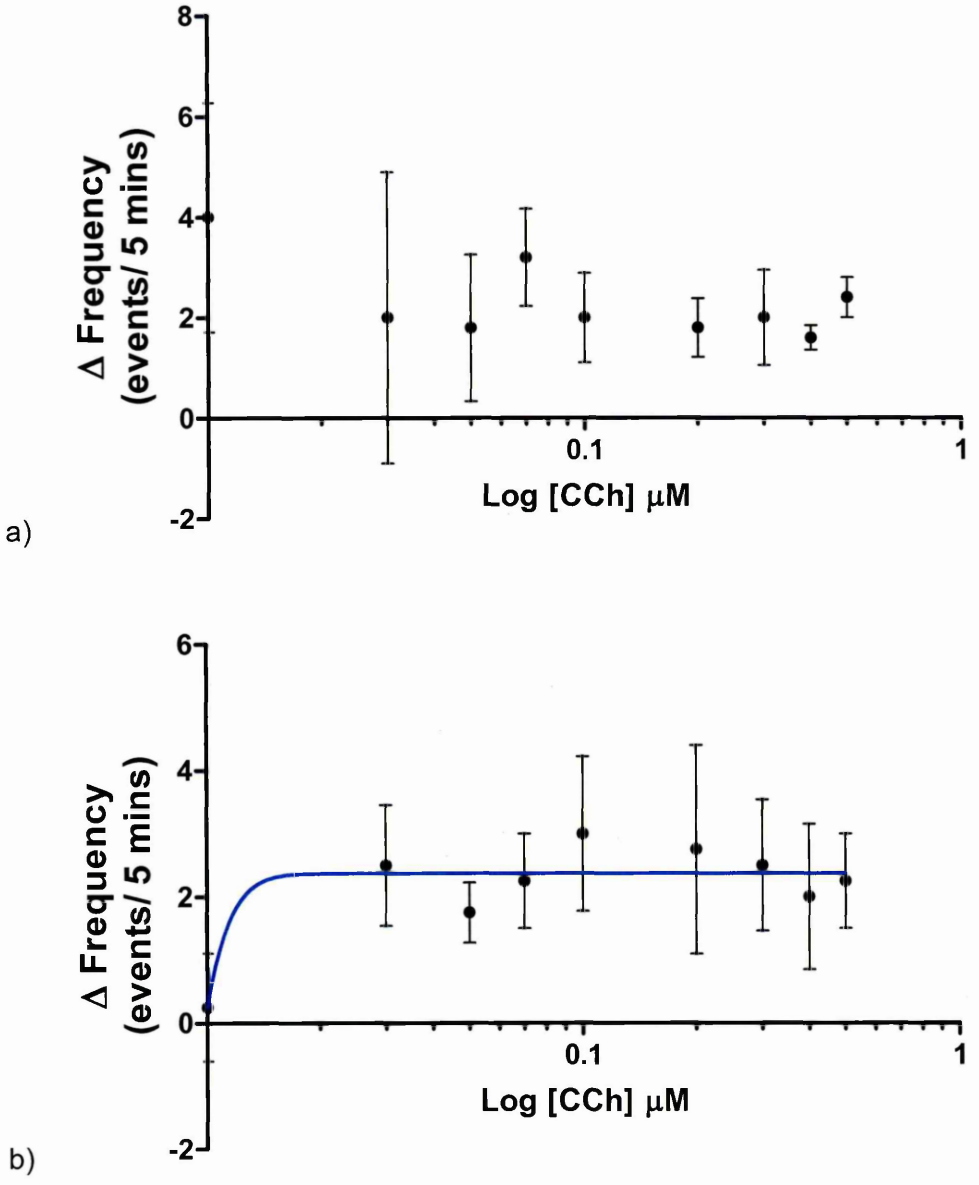
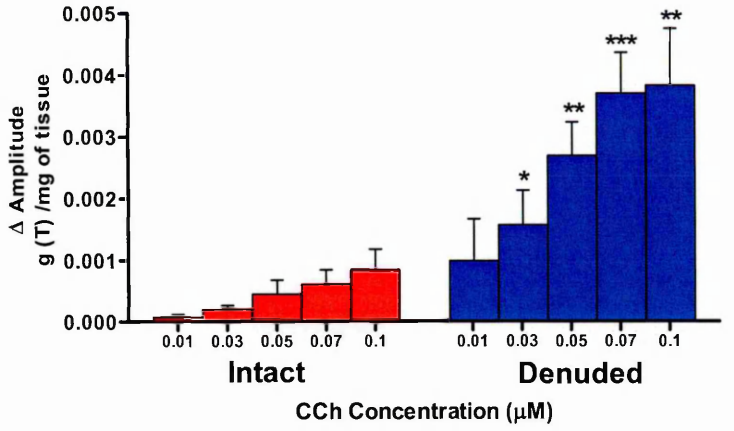
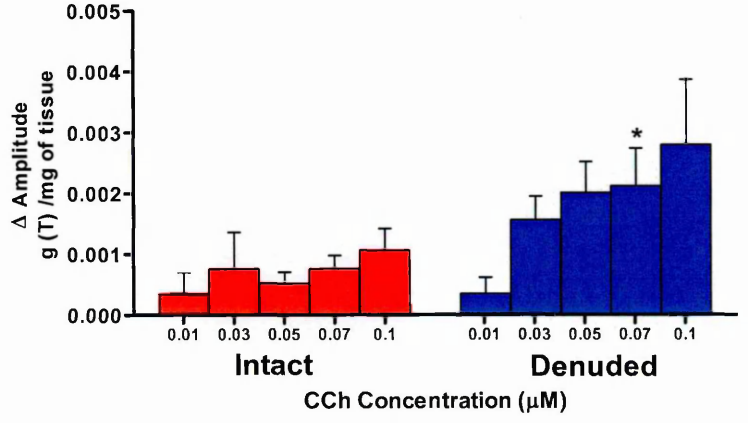


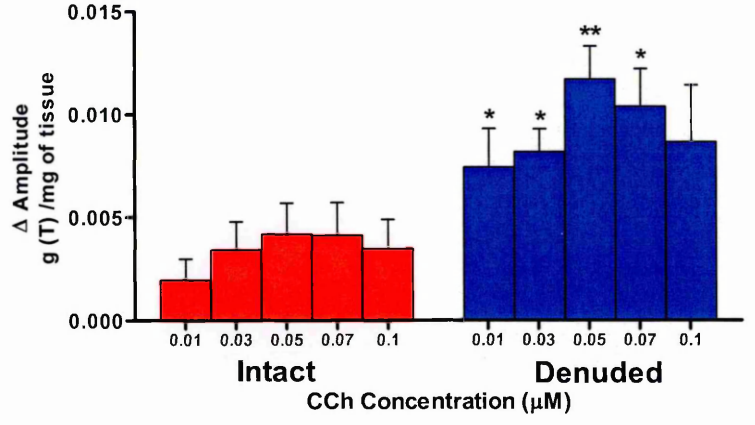
Figure 3.9 - Effect of increasing concentrations of carbachol on the frequency of phasic contractions in bladder strips from a) intact and b) denuded trigone region. All data are presented as mean ± SEM, n=5.



a)

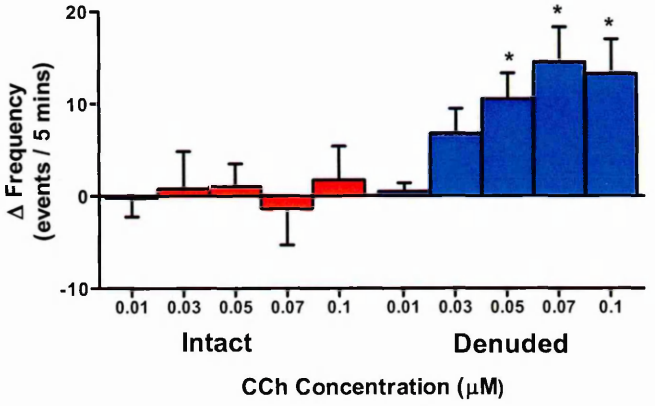


b)

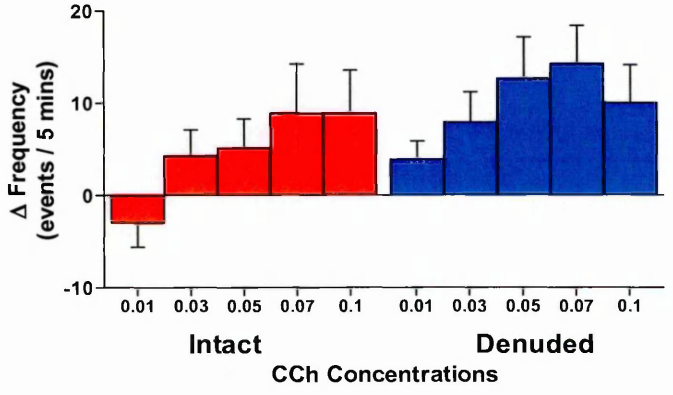


c)

Figure 3.10 - Effect of increasing concentrations of carbachol on the amplitude of PCs in intact and denuded strips from the pig bladder a) dome, b) body and c) trigone regions. Data are presented as mean ± SEM, n=6, *p<0.05, **p<0.01 and ***p<0.0001 vs. corresponding concentration of CCh in intact strips; Note the differences in scales used.



a)



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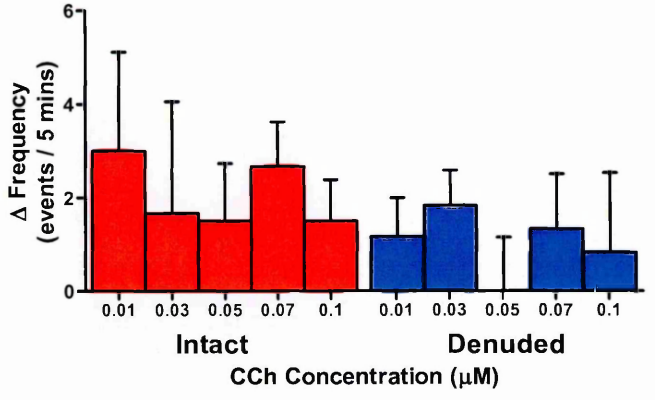


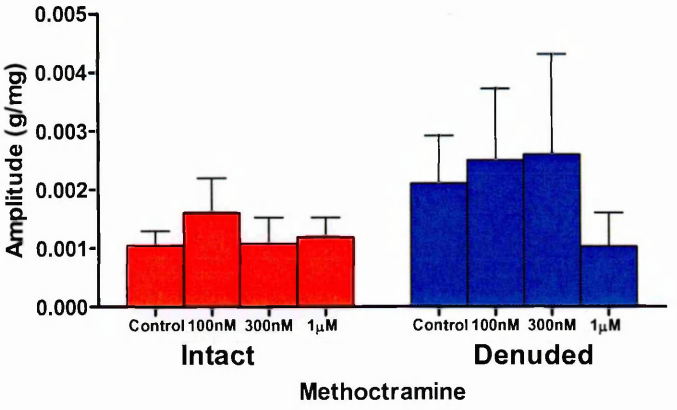
c)

Figure 3.11 - Effect of increasing concentrations of carbachol on the frequency of PCs in intact and denuded strips from the pig bladder a) dome, b) body and c) trigone regions. Data are presented as mean ± SEM, n=6, *p<0.05 vs. corresponding concentration of CCh in intact strips. Note the differences in scales used.

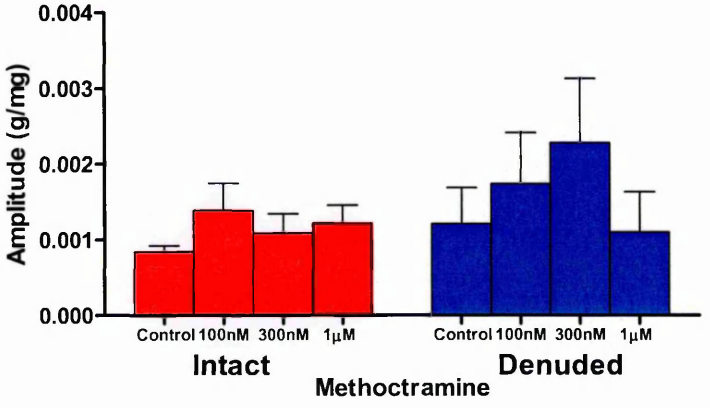
3.5.3 Effect of muscarinic antagonists on the amplitude and frequency of basal PCs

Figures 3.12 and 3.13 respectively show the effect of methoctramine on the amplitude and frequency of basal PCs in intact and denuded strips from the three bladder regions. Incubation with methoctramine had no significant effect on either the amplitude or the frequency of basal PCs in any of the different regions of the bladder, at all concentrations investigated.

The effect of 4-DAMP on the amplitude and frequency of basal PCs in the different bladder regions is presented in figures 3.14 and 3.15 respectively. Analysis of the amplitude data showed that no significant differences were observed in the presence vs. the absence of 4-DAMP in both intact and denuded tissue strips (Figure 3.14). Similarly, 4-DAMP also had no effect on the frequency of PCs in both intact and denuded strips from the three bladder regions (Figure 3.16).



a)



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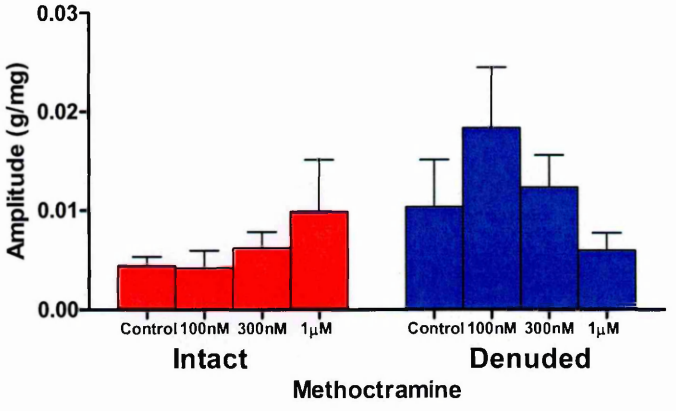
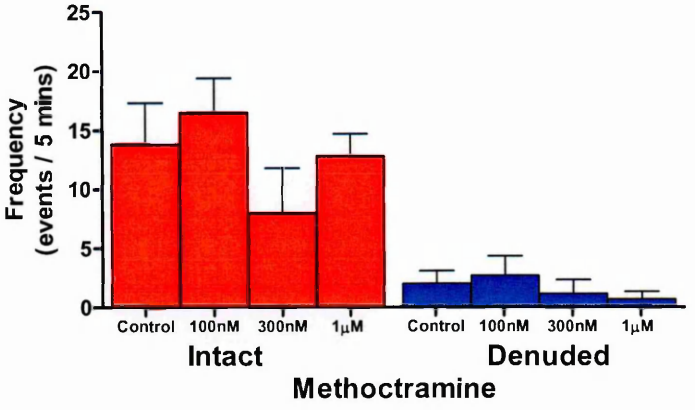
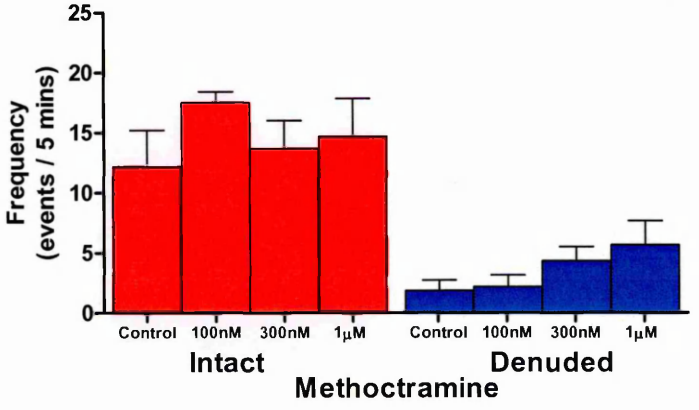


c)

Figure 3.12 - Effect of increasing concentrations of methoctramine on the amplitude of PCs in intact and denuded strips from the pig bladder a) dome, b) body and c) trigone regions. Controls have been treated with vehicle only, n=6, data are presented as mean ± SEM. Note the differences in the scales used.



a)



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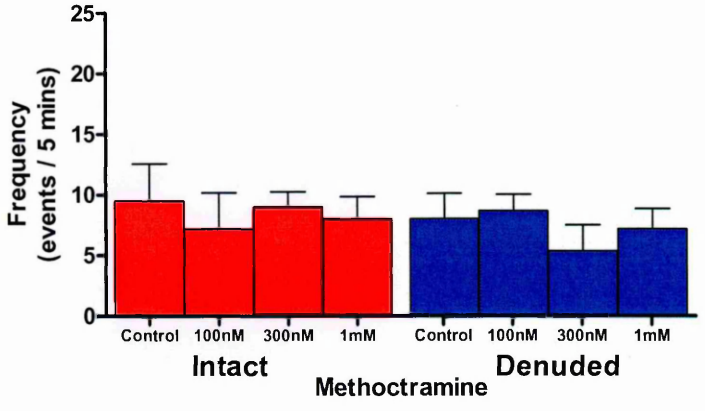
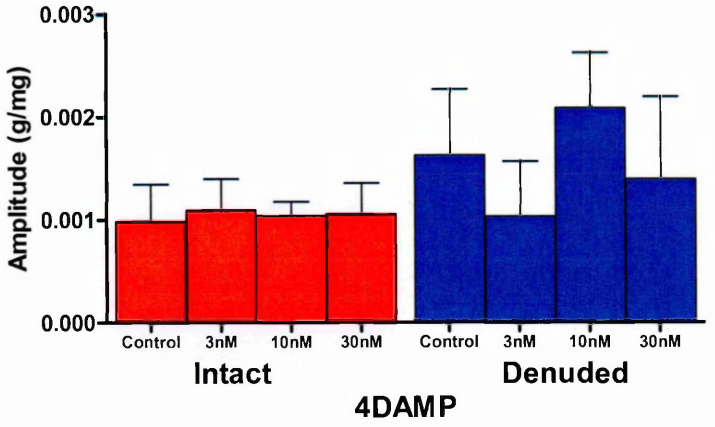
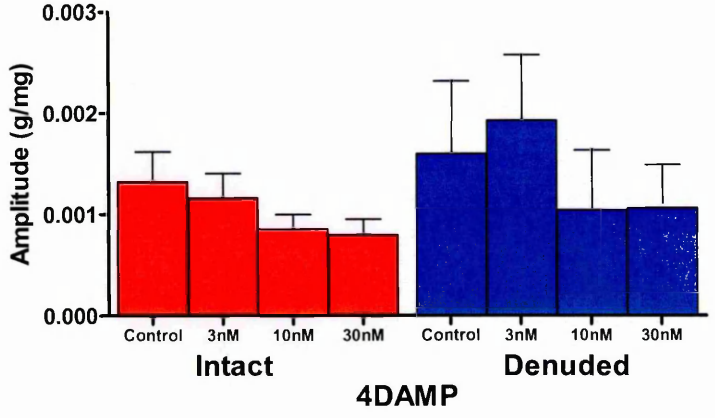


c)

Figure 3.13 - Effect of increasing concentrations of methoctramine on the frequency of PCs developing in intact and denuded strips from the pig bladder a) dome, b) body and c) trigone regions. Controls have been treated with vehicle only, n=6, data are presented as mean ± SEM



a)



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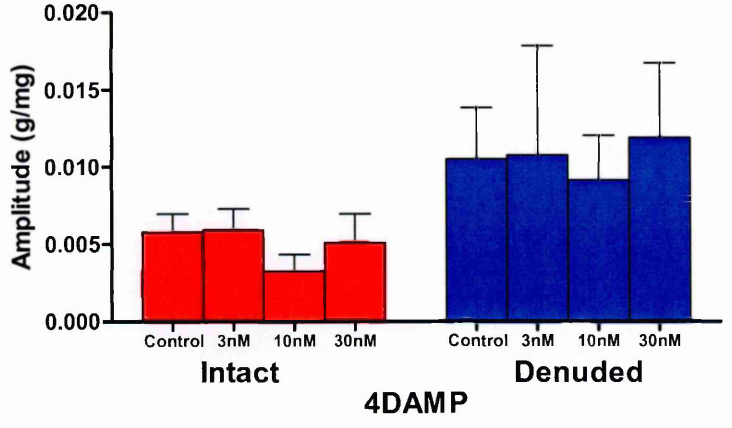
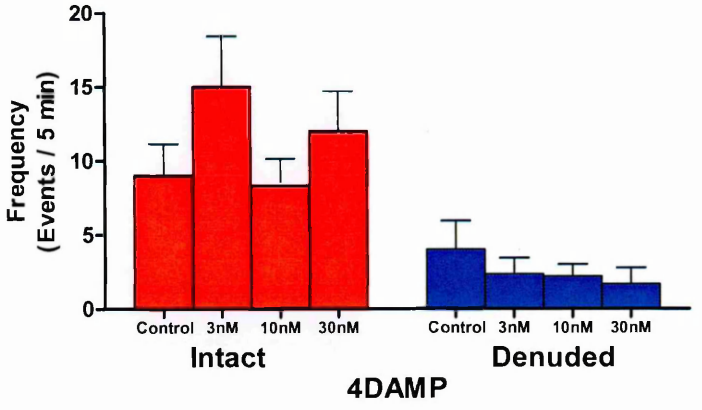
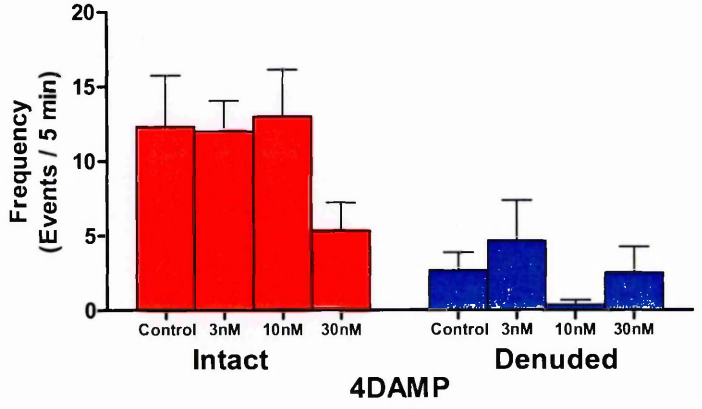


c)

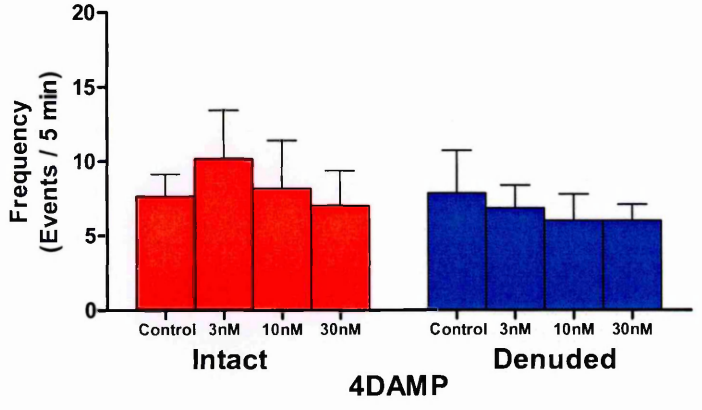
Figure 3.14 - Effect of increasing concentrations of 4DAMP on the amplitude of PCs in intact and denuded strips from the pig bladder a) dome, b) body and c) trigone regions. Controls have been treated with vehicle only, n=6, data are presented as mean ± SEM.



a)



b)



c)

Figure 3.15 - Effect of 4-DAMP on the frequency of basal PCs in intact and denuded strips from the pig bladder a) dome, b) body and c) trigone regions. Controls have been treated with vehicle only, n=6, data are presented as mean ± SEM.

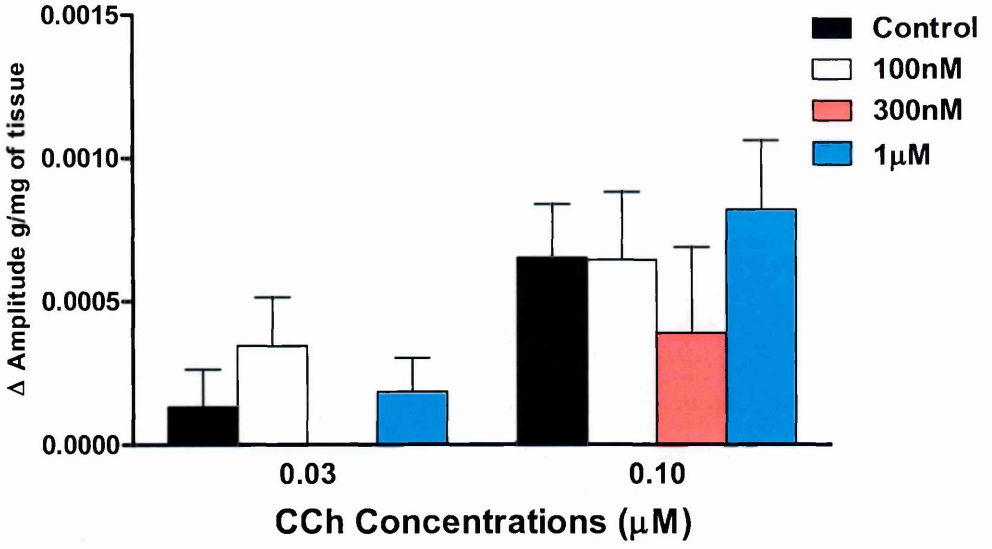
3.5.4 *Investigation of the muscarinic receptor subtypes mediating the effect of CCh on phasic contractions.*

The effect of methoctramine on the amplitude of CCh-modulated phasic contractions in the different bladder regions is presented in figures 3.16 - 3.18. No significant differences were observed in the effects of CCh on amplitude of PCs in the absence and presence of different concentrations of methoctramine in intact strips from the dome, body and trigone (figures 3.16a, 3.17a & 3.18a respectively). This was also the case for denuded tissue strips (figures 3.16b, 3.17b & 3.18b) from the three regions.

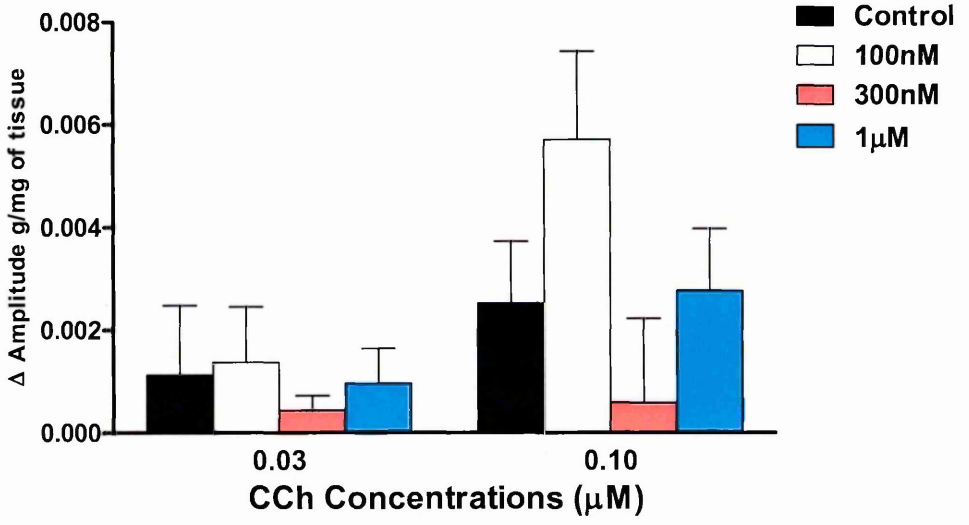
Methoctramine also had no effect on the frequency of CCh-modulated phasic contractions of intact strips from the body (Figure 3.21a) and trigone (Figure 3.22a) regions. In intact strips of dome, methoctramine significantly decreased the frequency of PCs when used at 300nM concentration (0.03μM CCh). Methoctramine similarly showed no effect on the frequency of CCh-modulated PCs of denuded strips from the dome (Figure 3.20b) and body (Figure 3.21b) regions, whereas in the denuded strips of the trigone, there was a significant decrease with 300nM methoctramine (0.1μM CCh - Figure 3.22b).

Figures 3.22 - 3.24 show the effect of 4-DAMP on the amplitude of CCh-modulated phasic contractions. 4-DAMP had no effect on the amplitude of PCs in intact strips from the dome, body or trigone (figures 3.22a, 3.23a & 3.24a respectively). 4-DAMP also had no significant effect on the amplitude of PCs in denuded strips from the body and trigone regions (figures 3.23b & 3.24b). In contrast, 4-DAMP significantly increased the amplitude of PCs in strips from the dome at 3nM 4-DAMP (0.1μM CCh - Figure 3.22b).

Analysis of the effect of 4-DAMP on frequency of CCh-modulated PCs are shown in figures 3.25 - 3.27. In the dome region (Figure 3.26a), the frequency of PCs in intact strips was significantly decreased by 10nM 4-DAMP (0.1μM CCh), although in the body (Figure 3.26a) and trigone (Figure 3.27a) there was no effect. There was also no significant effect observed with 4DAMP on the frequency of PCs in denuded strips from the body and trigone (Figure 3.26b & Figure 3.27b respectively) with strips from the dome showing a significant decrease when treated with 30nM 4-DAMP (Figure 3.25b).

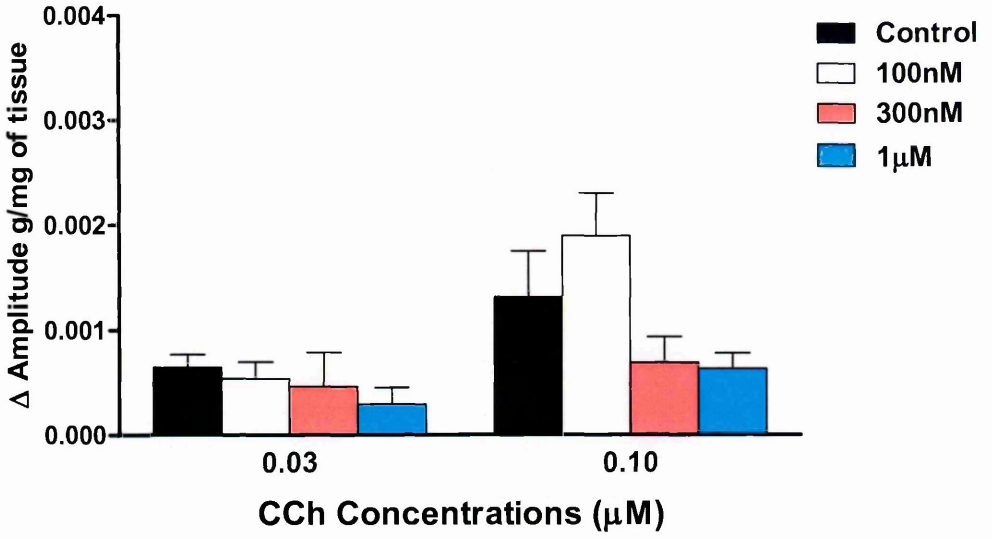


a)

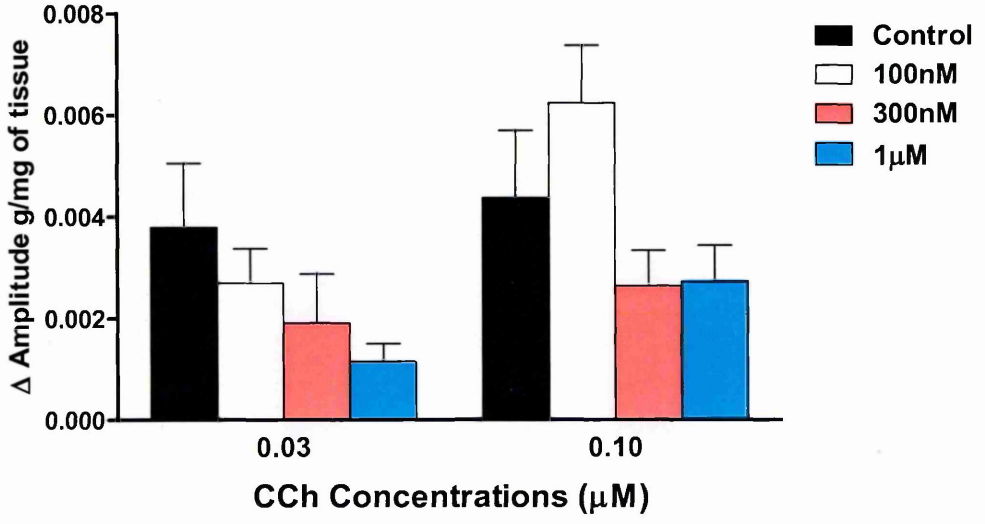


b)

Figure 3.16 - Effect of increasing concentrations of methoctramine on the amplitude of CCh-modulated PCs in a) intact and b) denuded strips from the pig bladder dome. Data are presented as mean ± SEM, n=6. Note the difference in scales used. The effect of antagonist in the following graphs only show two concentrations of CCh for simplicity representing approximately 50% and 100% of the response.

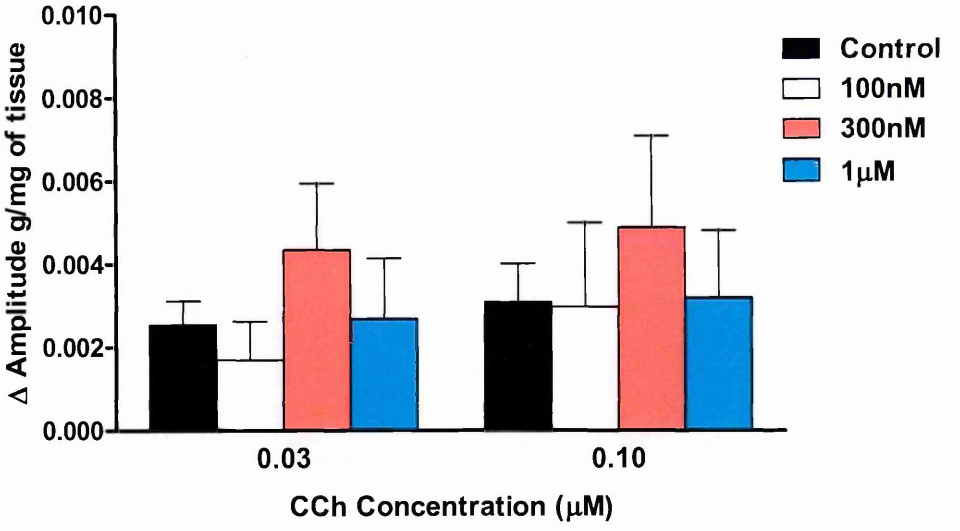


a)

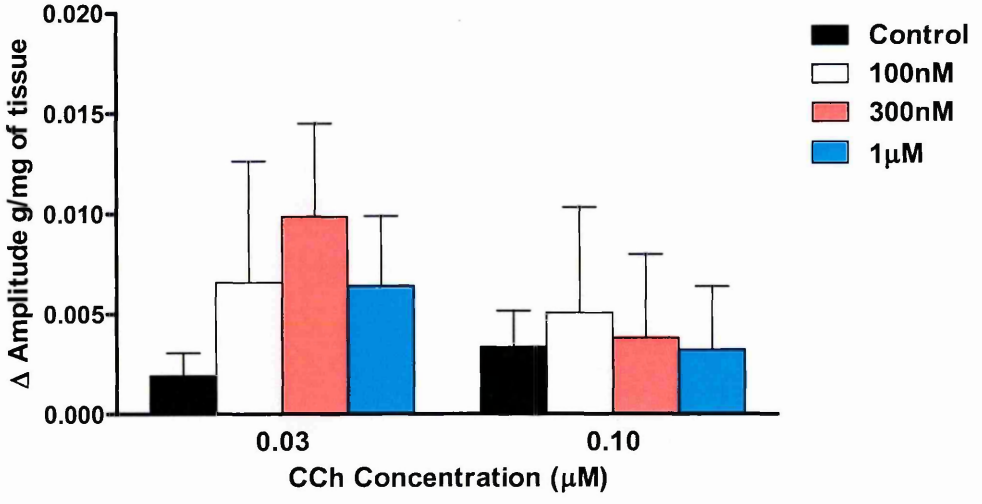


b)

Figure 3.17 - Effect of increasing concentrations of methoctramine on the amplitude of CCh-modulated PCs in a) intact and b) denuded strips from the pig bladder body. Data are presented as mean ± SEM, n=6. Note the difference in scale used.

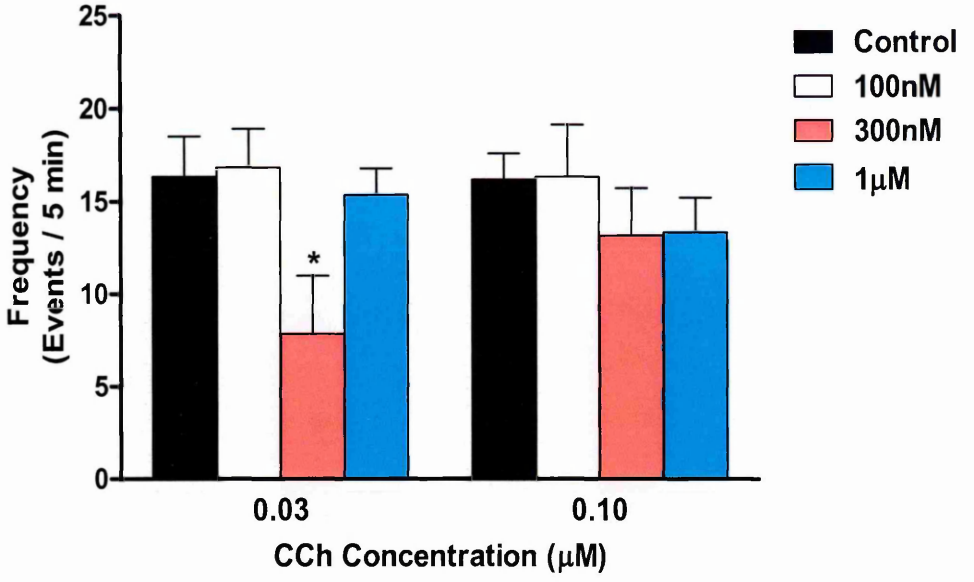


a)

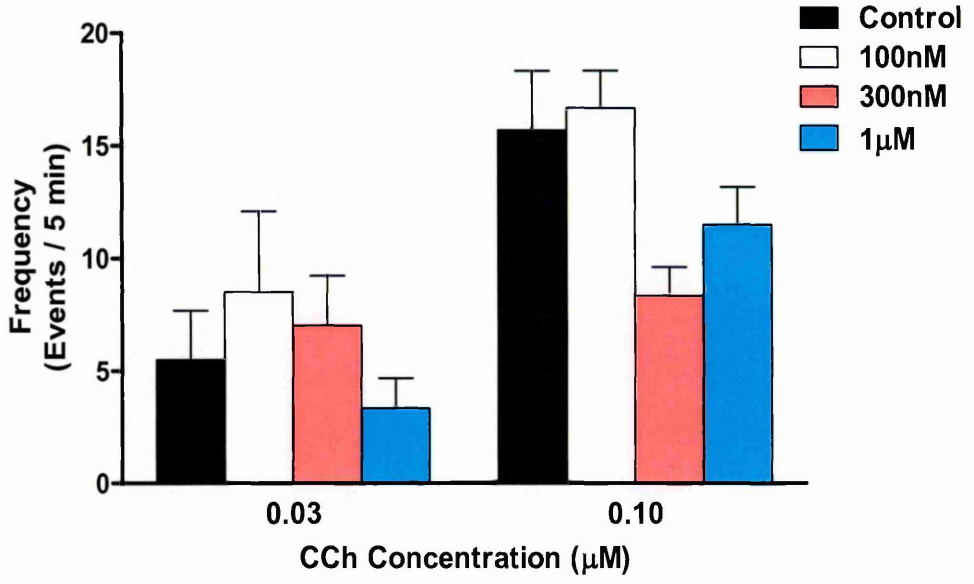


b)

Figure 3.18 - Effect of increasing concentrations of methoctramine on the amplitude of CCh-modulated PCs in a) intact and b) denuded strips from the pig bladder trigone. Data are presented as mean ± SEM, n=6. Note the difference in scale between intact and denuded.

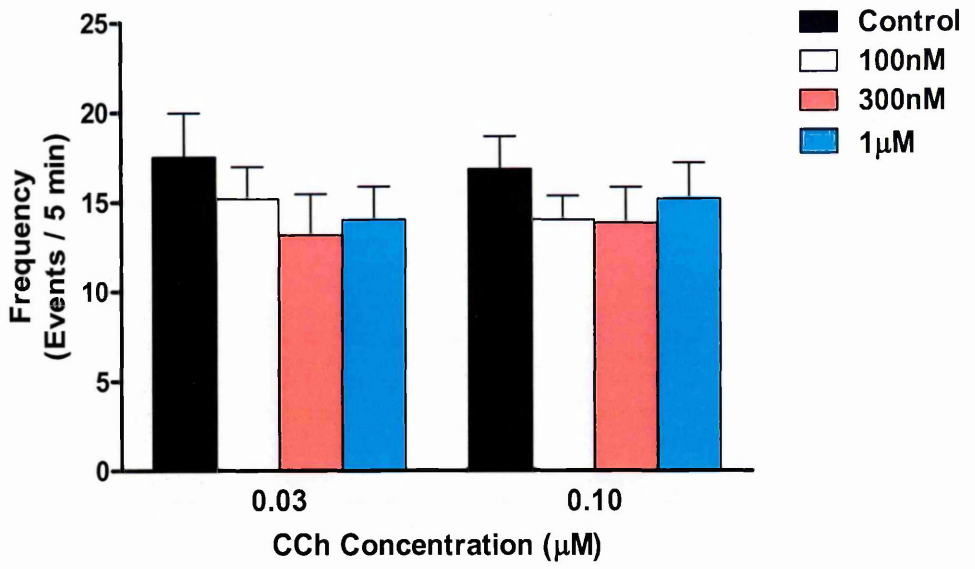


a)



b)

Figure 3.19 - Effect of increasing concentrations of methoctramine on the frequency of CCh-modulated PCs in a) intact and b) denuded strips from the pig bladder dome. Data are presented as mean ± SEM, n=6, *p<0.05 vs. control.



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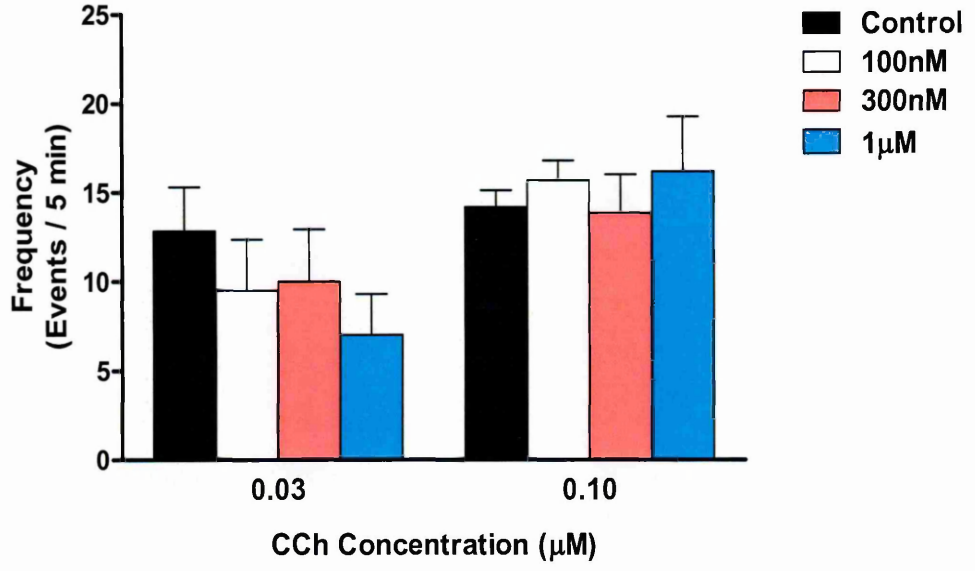
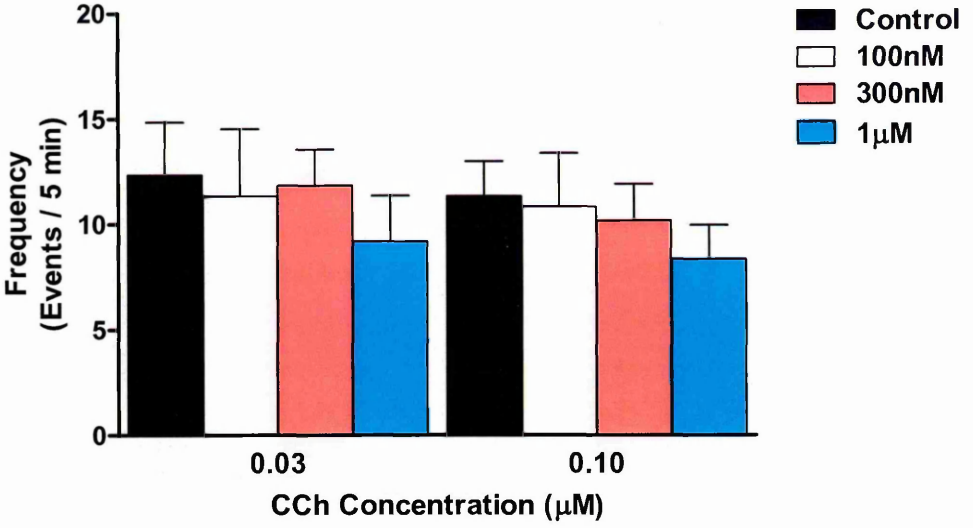
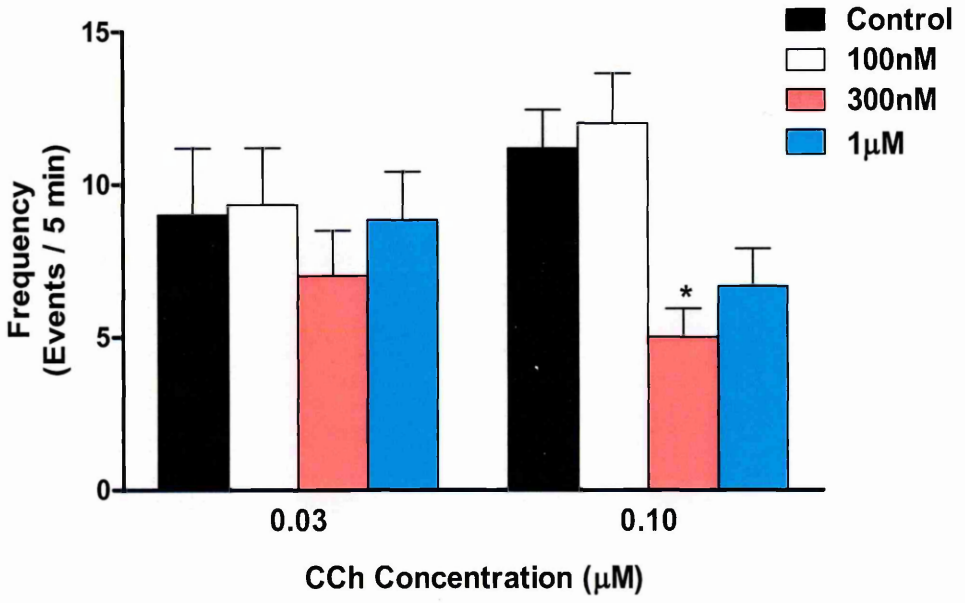


b)

Figure 3.20 - Effect of increasing concentrations of methoctramine on the frequency of CCh-modulated PC in a) intact and b) denuded strips from the pig bladder body. Data are presented as mean ± SEM, n=6.

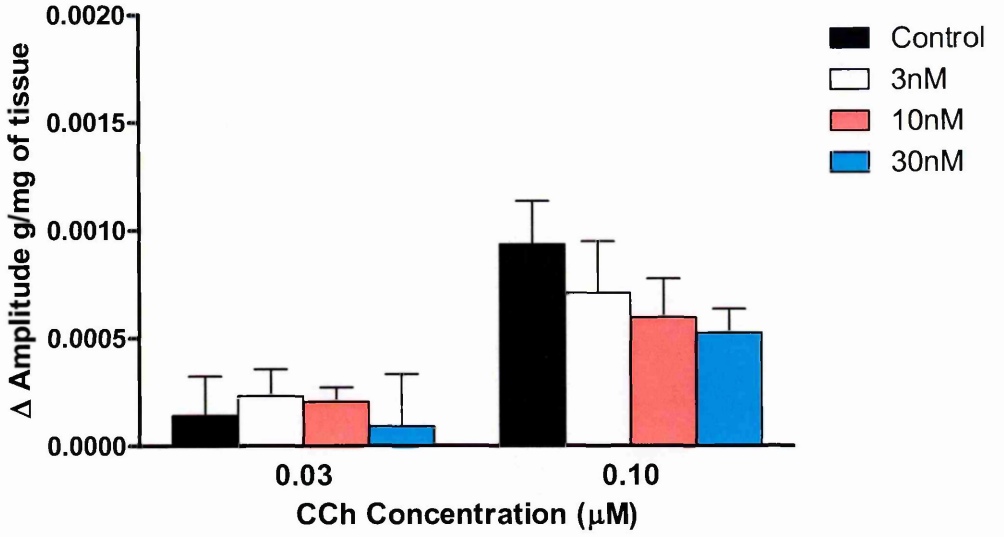


a)

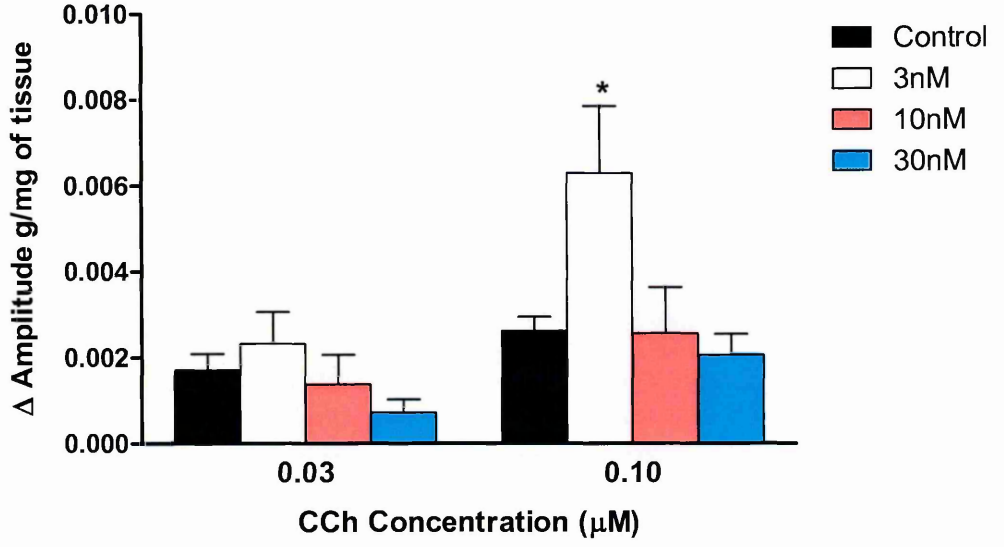


b)

Figure 3.21 - Effect of increasing concentrations of methoctramine on the frequency of CCh-modulated PCs in a) intact and b) denuded strips from the pig bladder trigone. Data are presented as mean ± SEM, n=6, *p<0.05 vs. control.

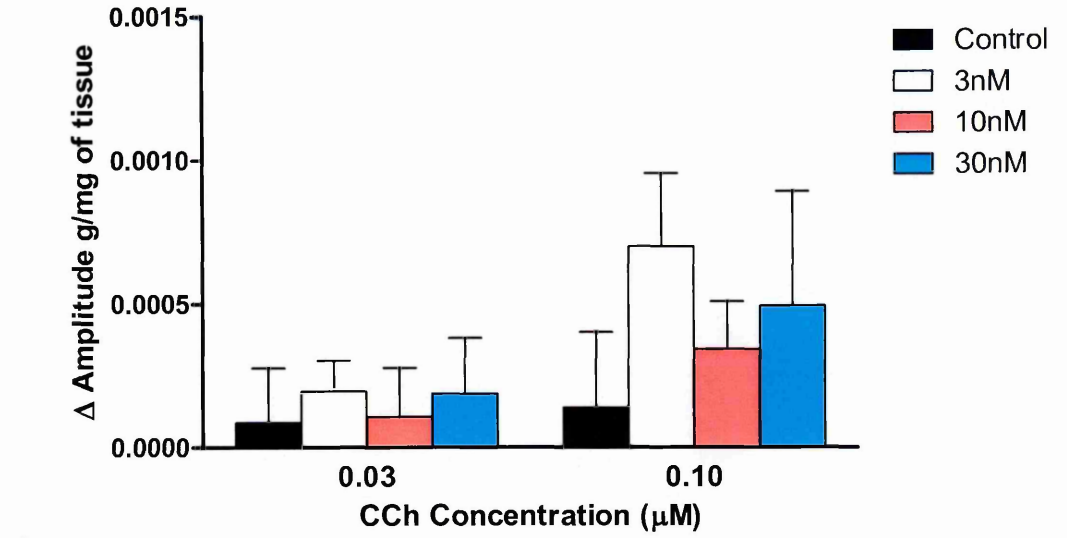


a)

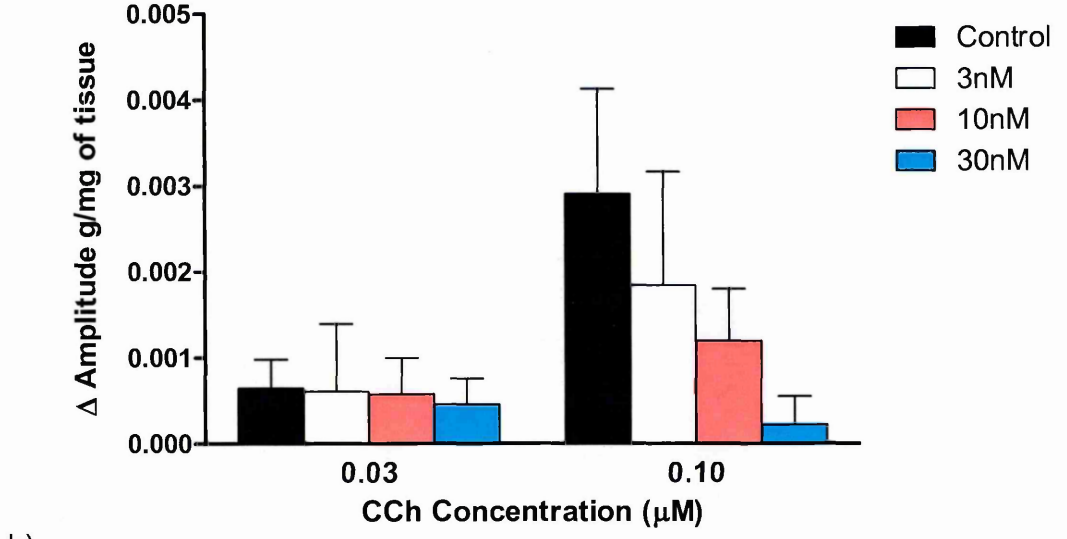


b)

Figure 3.22 - Effect of increasing concentrations of 4DAMP on the amplitude of CCh-modulated PCs in a) intact and b) denuded strips from the pig bladder dome. Data are presented as mean ± SEM, n=6, *p<0.05 vs. control (0.1μM CCh)

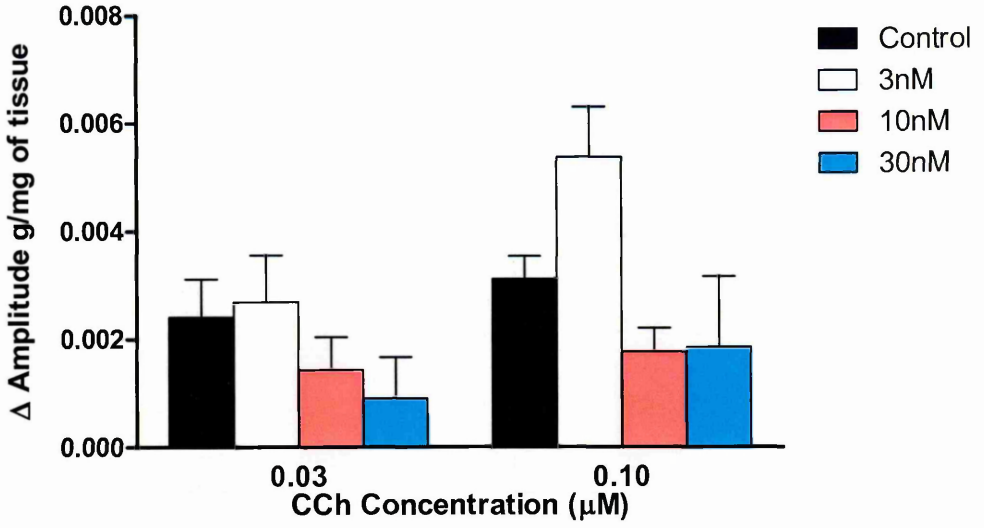


a)

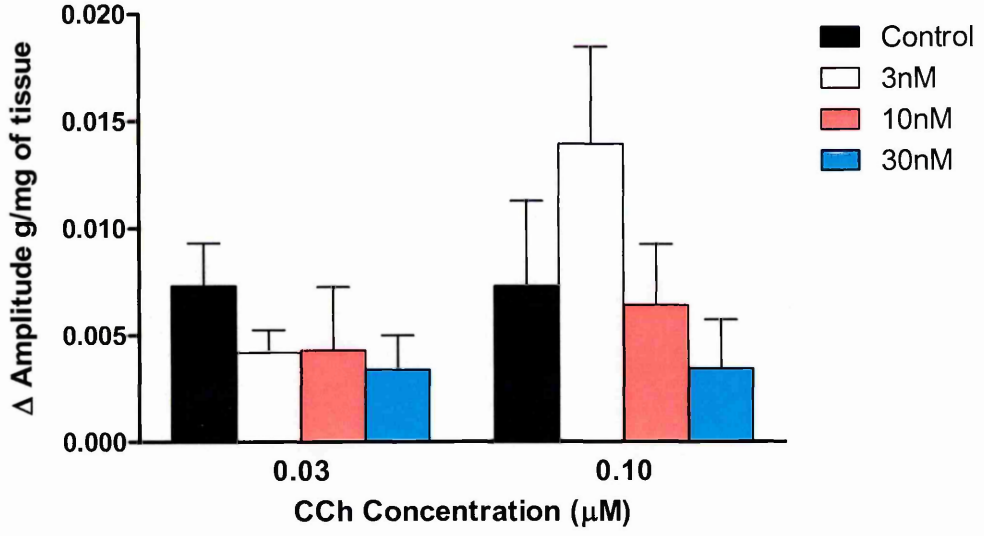


b)

Figure 3.23 - Effect of increasing concentrations of 4DAMP on the amplitude of CCh-modulated PCs in a) intact and b) denuded strips from the pig bladder body. Data are presented as mean ± SEM, n=6.

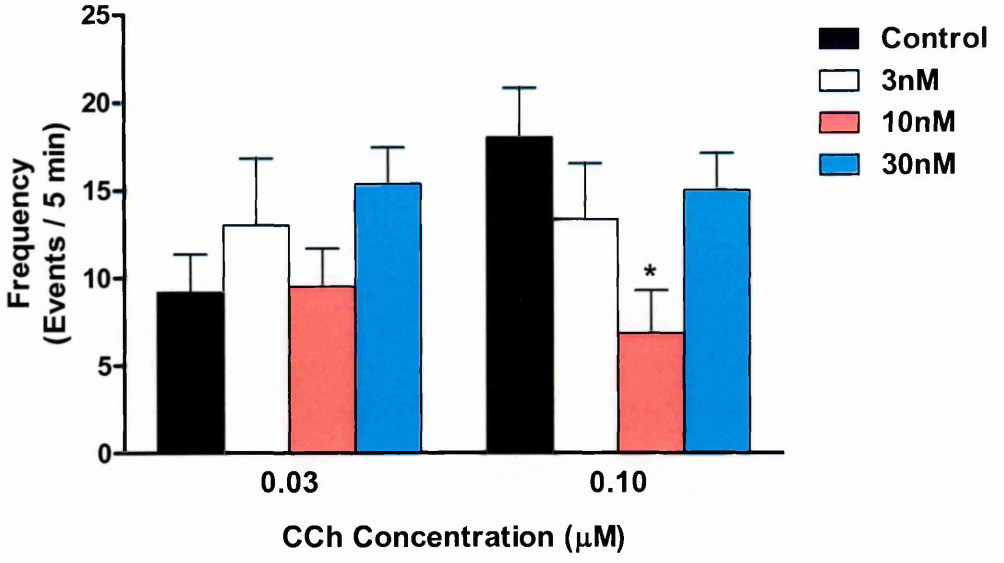


a)

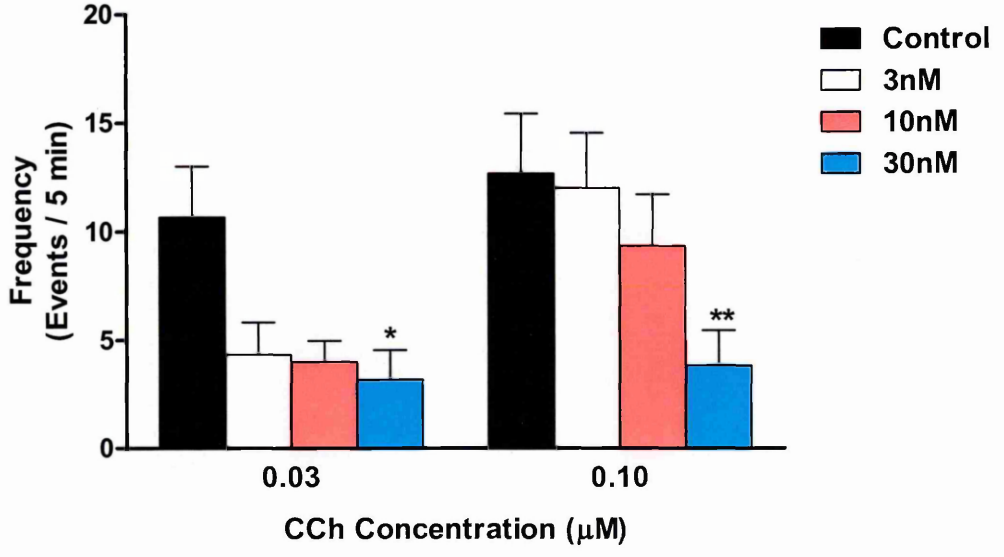


b)

Figure 3.24 - Effect of increasing concentrations of 4DAMP on the amplitude of CCh-modulated PCs in a) intact and b) denuded strips from the pig bladder trigone. Data are presented as mean ± SEM, n=6.

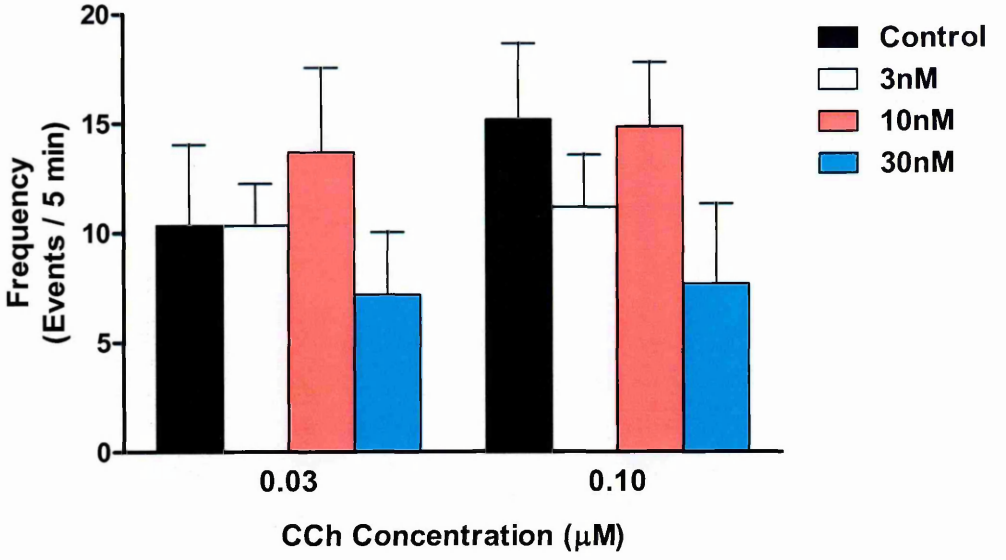


a)

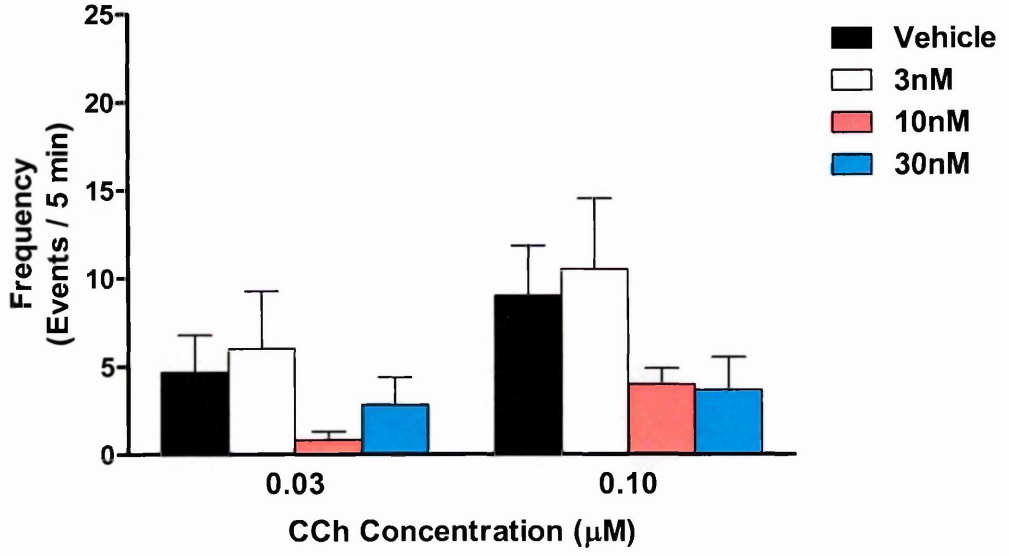


b)

Figure 3.25 - Effect of increasing concentrations of 4DAMP on the frequency of CCh-modulated PCs in a) intact and b) denuded strips from the pig bladder dome. Data are presented as mean ± SEM, n=6, *p<0.05, **p<0.01 vs. control.

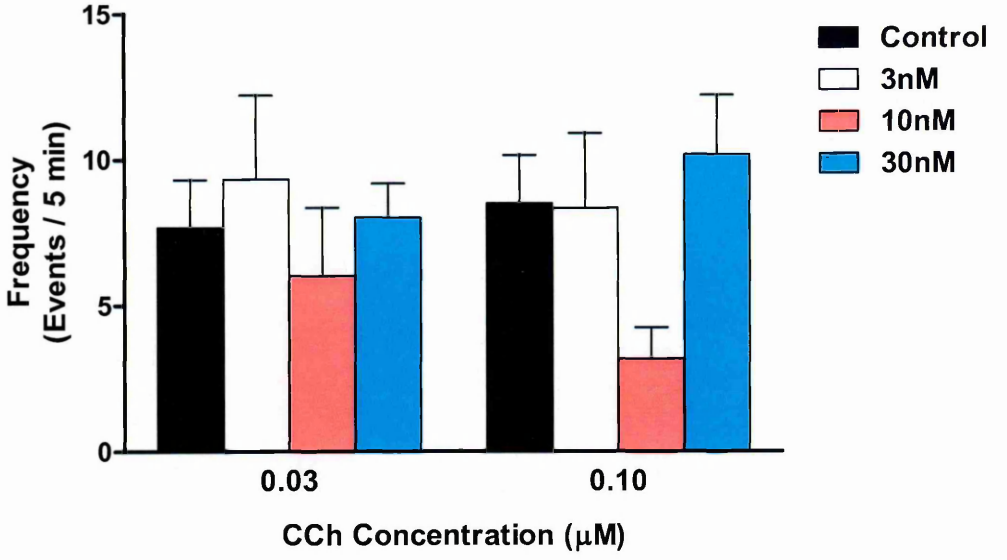


a)

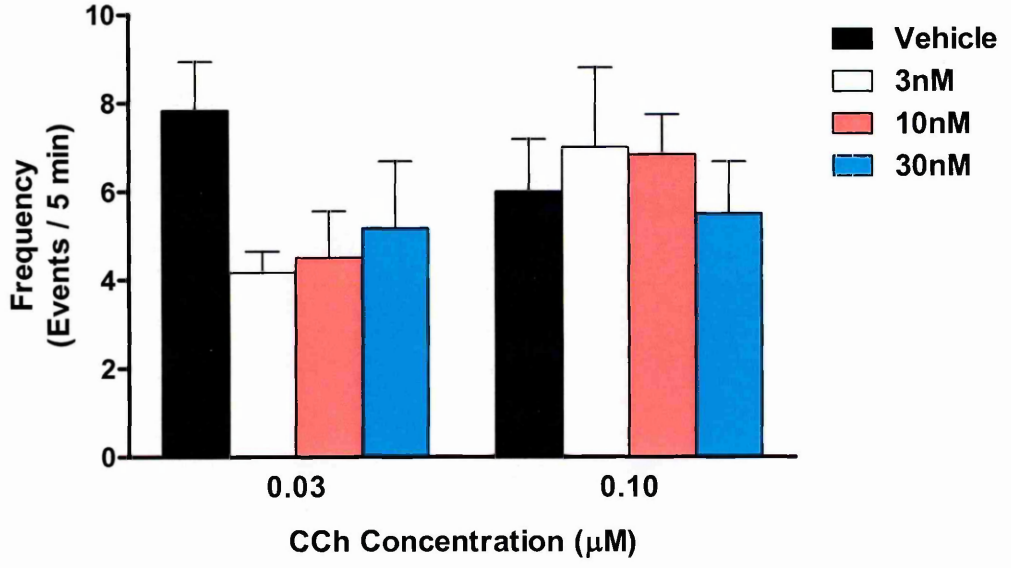


b)

Figure 3.26 - Effect of increasing concentrations of 4DAMP on the frequency of CCh-modulated PCs in a) intact and b) denuded strips from the pig bladder body. Data are presented as mean ± SEM, n=6.



a)



b)

Figure 3.27 - Effect of increasing concentrations of 4DAMP on the frequency of CCh-modulated PCs in a) intact and b) denuded strips from the pig bladder trigone. Data are presented as mean ± SEM, n=6.

3.6 Discussion

The urinary bladder has been shown to contain a heterogeneous population of M_2 and M_3 muscarinic receptors. There is a lot of information on the function of these receptors in the detrusor compared to the other areas of the bladder where they are found. In normal conditions, bladder contractility is under parasympathetic nervous system control whereby the primary input is via acetylcholine-induced excitation of post-junctional muscarinic receptors. Determination of the muscarinic receptors responsible for these contractions in the bladder has been mainly due to use of relatively subtype selective muscarinic agents (Abrams *et al.,* 2006). All of the muscarinic receptor subtypes are present in the detrusor of several species including humans but the M_2 and M_3 receptor subtypes are predominant with the M_2 outnumbering the M_3 receptor at a 3:1 ratio (Longhurst *et al.,* 1995; Wang *et al.,* 1995; Yamaguchi *et al.,* 1996; Baselli *et al.,* 1999; Yamanishi *et al.,* 2000). The minority M_3 subtype is responsible for the direct detrusor contraction *in vitro* (Sellers *et al.,* 2000; Chess-Williams *et al.,* 2001; Fetscher *et al.,* 2002).

Pharmacological characterization of the five muscarinic receptor subtypes has been undermined due to the lack of agonist and antagonist selectivity for any single receptor subtype. Antagonist affinities obtained using radioligand binding techniques combined with affinity constants obtained from functional experiments have been used to define and differentiate the different muscarinic receptor subtypes. M_1 receptors have a high affinity for pirenzepine and a low affinity for methoctramine. M_2 receptors on the other hand have a high affinity for methoctramine and low affinity for pirenzepine and 4-DAMP. M_3 receptors exhibit a high affinity for 4-DAMP and low affinity for methoctramine. M_4 receptors have a relatively high affinity for 4-DAMP and methoctramine thereby making it difficult to distinguish these receptors from M_2 and M_3. M_5 receptors are characterized by having a moderate affinity to 4-DAMP and a low affinity to methoctramine (Caulfield 1993).

Receptor subtypes responsible for detrusor contractions have been determined using some of the antagonists listed in table 3.2. In this study, investigation of whether naturally occurring basal PCs in the pig bladder could be modulated cholinergically was performed in addition to determination of the receptor subtypes responsible for these bladder PCs. This was performed using methoctramine, and 4-DAMP on CCh modulated PC. Isolated pig bladder strips from the dome, body and trigone regions developed intrinsic PC when set up in the tissue baths. The PC amplitude was

modulated by low concentrations of CCh, non-selective muscarinic agonist which activated the ACh receptor thereby causing smooth muscle contraction.

In the dome and body regions, in intact and denuded strips, CCh significantly increased the amplitude of PCs in a concentration-dependent manner. The amplitude of CCh-modulated PCs in the dome and body is less than in the trigone. The trigone strips especially denuded strips exhibited 50% greater amplitude than those from the dome and body. CCh modulation had a greater effect on the frequency of PCs in denuded strips compared to their respective intact strips in the dome and body regions whereas CCh had no effect on frequency in the trigone region.

The data obtained from amplitude and frequency measurements of CCh-modulated PCs showed that removal of the urothelium resulted in greater amplitude and frequency. This may be due to the fact the urothelium normally may be releasing a substance that inhibits PCs. Published data has shown that the urothelium releases an unidentified urothelium derived inhibitory factor (UDIF) that inhibits bladder contractions (Hawthorn *et al.,* 2000). Removal of the mucosa subsequently eliminated the possibility of UDIF release and therefore could explain the greater amplitude and frequency of PCs. The bladder urothelium has also been shown to have a higher metabolic rate than the detrusor muscle (Hypolite *et al.,* 1993) and its removal significantly increases the responses to a range of contractile agents (Dveksler *et al.,* 1987; Maggi *et al.,* 1987; Pinna *et al.,* 1992; Levin *et al.,* 1995).

It is now well established that there are two main sources of ACh in the bladder : a neuronal tetrodotoxin (TTX) sensitive source and TTX-resistant non-neuronal source (Yoshida *et al.,* 2004; Yoshida *et al.,* 2008). Using guinea pigs and rats, Zagorodnyuk *et al* (2009) showed that in whole bladder and isolated intact bladder strips treated with physostigmine - an acetylcholinesterase inhibitor - powerful phasic contractions were recorded and not affected by TTX. The same was observed using denuded strips and frequency recorded was similar to intact strips. Further treatment with hyoscine - an antimuscarinic - significantly inhibited the frequency of contraction by up to 83 ± 6% suggesting that the on-going TTX resistant PC recorded is due to endogenous non-neuronal ACh not released from the urothelium (Zagorodnyuk *et al.,* 2009). This could very well possibly be the same in the denuded tissue strips whose frequency is decreased in the presence of muscarinic antagonists. The antagonist experimental data collected suggests that the basal PCs that developed may not have been due to endogenous ACh release, but this may not necessarily be true. One explanation for this is that the lack of muscarinic receptor agonists with selectivity and antagonist with

high selectivity for a single receptor (Scarpero and Dmochowski 2003). Tissue also express more than one subtype which further makes the determination of the function of a single receptor subtype very difficult (Caulfield and Birdsall 1998).

A major drawback for the experiments performed in this chapter is the lack of confirmation that the basal phasic contractions were due to ACh release. This could have been overcome by performing confirmatory experiments using atropine which is a non-selective antagonist. The presence of continuing PCs validate presence of ACh and a possible role in PCs. A different approach would have been to add an acetylcholinesterase inhibitor such as neostigmine or physostigmine to prevent the breakdown of ACh in the tissue bath. If ACh was indeed being released, an increase in PCs would have confirmed this.

The effect of methoctramine and 4DAMP on CCh-modulated PCs showed no significant effect on the amplitude of CCh-modulated PC in all three bladder regions. The PCs observed in these experiments when blocked by the muscarinic antagonist may be due to different pathways such as CCh acting on other muscarinic subtypes present in the bladder. There are numerous sites at which CCh may be acting on making a specific determination complex. All five muscarinic receptor subtypes have been shown to be present in the bladder urothelium (Bschleipfer *et al.,* 2007), in addition to the muscarinic receptors present in the detrusor. There is also a population of ICC and afferent nerves that express various receptors at which CCh can exert an effect. It is this heterogeneous population expressing both excitatory and inhibitory receptors throughout the bladder wall that complicates the task of identifying a single subtype responsible for PCs.

3.7 SUMMARY/CONCLUSIONS

These experiments have shown that:-

- PCs in the pig bladder can be modulated cholinergically and similar to previous chapter results, there were some functional differences observed particularly in the trigone compared to the dome and body regions.
- The muscarinic antagonist experiments suggest that basal PCs in the pig bladder are not due to endogenous ACh but this observation requires further investigation.
- Data obtained in this chapter reveals that it is not clear to determine which receptor subtype(s) are responsible in mediating the effects of CCh on pig bladder PCs.

CHAPTER 4

INVESTIGATION OF C-KIT POSITIVE CELLS IN THE PIG URINARY BLADDER

4.1 INTRODUCTION

The discovery of interstitial cell of Cajal (ICCs) is credited to the Spanish neuroanatomist and Nobel laureate Ramon y Cajal whom these cells were aptly named after (Cajal 1893). He described these ICCs as fibroblast-like in appearance and histological staining with silver nitrate and methylene blue led to the assumption that ICCs were primitive neurons. Advances in the fields of light and electron microscopy proved that these cells were neither neurons nor Schwann cells (Rolle *et al.,* 2007). ICCs have been morphologically described as being spindle or stellate shaped cells connected via gap junctions and nerves and containing numerous mitochondria and intermediate filaments (Blyweert *et al.,* 2004). The discovery that ICCs express the proto-oncogene c-Kit led to the development of antibodies against c-Kit and subsequent rapid accumulation of knowledge with regards to their function and localization in various tissues.

4.1.1 ICCs AND THEIR FUNCTION IN THE GASTROINTESTINAL TRACT

ICCs were first identified in the gastrointestinal (GI) tract and early developmental studies using mice showed that ICCs were of great importance in the function and maintenance of this system. Torihashi *et al.* (1997) and Kluppel *et al.* (1998) both showed that ICCs and smooth muscle cells (SMCs) arise from the same mesenchymal precursors (Torihashi *et al.,* 1997; Klüppel *et al.,* 1998) but the normal development of ICCs is dependent on expression of the c-Kit proto-oncogene, which encodes for the protein KIT - a tyrosine kinase receptor. Experiments performed using mice have proven that expression and signalling of ICCs via the KIT receptor is essential for the normal development and onset of GI electrical rhythmicity that maintains intestinal peristaltic waves (Torihashi *et al.,* 1997). This pacemaker function of ICCs is strongly supported by their localization in tissue analysed by electron microscopy. This is mainly due to their close association with nerve terminals and gap junctions within the GI smooth muscle cells (SMCs) suggesting a connection between nerves and muscle (Faussone Pellegrini *et al.,* 1977; Yamamoto 1977). ICCs have also been shown to act as non-neural stretch receptors in the GI muscle and this subsequently affects slow wave frequency and smooth muscle excitability (Won *et al.,* 2005).

Several subtypes of ICCs have been described in the GI tract with the largest density of ICCs occurring within the myenteric plexus (ICC-MP). This group of ICCs is composed of interconnected multi-polar cells whose main function is to act as pacemakers initiating slow wave activity in adjacent circular and longitudinal smooth muscles (Brading and McCloskey 2005; Komuro 2006). There are also intramuscular

ICCs (ICC-IM) present in the intestinal muscle layer of the GI tract and these can be differentiated into two groups - ICCs of the circular muscle (ICC-CM) and ICCs of the longitudinal muscle (ICC-LM). Both of these classes are bipolar cells distributed around the muscle cells and along nerve bundles (Komuro 2006). They are mainly involved in transmission of signals from enteric neurons to SMCs (Brading and McCloskey 2005). Other types of ICCs have been described in the GI sub-mucosa (ICC-SM) and sub-mucosal plexus (ICC-SMP), in the deep muscular plexus (ICC-DMP) and within the sub-serosal layer (ICC-SS). All of these different types of ICCs have been demonstrated along the length of the digestive tract and their distribution patterns are entirely dependent on their anatomical location (Faussone-Pellegrini and Cortesini 1985; Hagger *et al.,* 1998; Torihashi *et al.,* 1999).

4.1.2 INTERSTITIAL CELLS IN THE URINARY BLADDER

Besides GI ICCs, urinary tract ICC have been the second most widely studied. Bladder ICCs were first identified by Smet *et al.* (1996) in the human and guinea pig bladder. This finding was confirmed by Gillespie *et al.* (2004). ICC identification was possible due to the specific immunoreactivity of these cells to anti-c-Kit antibodies. Investigation of urinary bladder properties has led to the identification of a novel class of cells resembling the ICCs of the gut, though their physiological function in the bladder is not fully understood. The nomenclature or terminology used for these cells outside of the GI tract has been controversial. This controversy has mainly stemmed from the fact that there are a number of subpopulations of these cells, leading to a heterogeneous population of this class of cells in the bladder wall, localized in different layers and with different morphologies. These cells have been referred to as interstitial cells (IC), interstitial-like cells, ICCs and myofibroblasts. During the "Vth International Symposium on ICC" held in Ireland in July 2007 the consensus was to refer to them as ICCs (McCloskey 2010), although within the literature this is still not consistent, and in this thesis bladder interstitial cells will be referred to as ICC. The definition of ICC encompasses cells found in various tissues, including the bladder, urethra, ureter and blood vessels, possessing physiological and morphological characteristics of ICC but not necessarily the "typical" physiological function of ICC in the GI tract where they initiate contractile activity (McCloskey 2010).

ICC were originally visualized using the technique of silver staining but since then, the rapid accumulation of information pertaining to this novel class of cells has led to the development of several immuno-histological techniques to identify them (Brading and McCloskey 2005). Determination of ICC localization in the urinary bladder has been

possible due to the availability of antibodies directed against a number of proteins and molecules expressed in these cells including c-Kit, vimentin, connexin-43, and cyclic guanidine monophosphate (cGMP) (Smet *et al.,* 1996; Sui *et al.,* 2002; Wiseman *et al.,* 2003). Investigation into the targets of cGMP signalling in human and guinea pig bladders following nitric oxide stimulation by Smet *et al* (1996) indicated a population of cells similar to gut ICCs, due to their morphological appearance. The same study also showed immunopositive staining for vimentin - an intermediate filament - typically present in ICC and other cells of mesenchymal origin, leading to speculation of ICC presence in the bladder (Smet *et al.,* 1996). These findings have since been confirmed by Gillespie et al (2004) (Gillespie *et al.,* 2004). Immunostaining for connexin-43 in conjunction with desmin has also been used to identify ICC in the basal and urothelial layers of the bladder (Sui *et al.,* 2002). The use of antibodies has helped establish c-Kit as a selective marker for ICC since it is exclusively expressed by ICC but not by SMCs or fibroblasts (Maeda *et al.,* 1992; Hirst and Ward 2003). Various studies of guinea pig bladder (McCloskey and Gurney 2002; Davidson and McCloskey 2005) and human bladder (Johnston *et al.,* 2010) have shown ICC to be located throughout the bladder wall and classed into two main groups - detrusor ICC (ICC-DT) and sub-urothelial ICC (ICC-SU).

Detrusor ICCs are located along the boundary of smooth muscle cells and run in parallel to the muscle bundles (Davidson and McCloskey 2005). They can also be found between the muscle bundles and have close interaction with cholinergic nerves in this area (Kubota *et al.,* 2011). This morphological finding supports the hypothesis that these ICC are ideally placed to function as pacemakers in the detrusor as well as convey cell-to-cell communication similar to those in the GI tract. This hypothesis has not been widely accepted because isolated muscle bundles have been shown to independently develop PCs.

Sub-urothelial ICC are sometimes referred to as myofibroblasts due to their characteristic spindle and stellate morphology, with several branches emanating from a central soma, and the fact that they form a well-connected network linked via gap junctions (Sui *et al.,* 2002). This network is thought to be involved in bladder signalling pathways from the urothelium and may modulate the sensory processes involved in the initiation of the micturition reflex (Sui *et al.,* 2002; Grol *et al.,* 2009) see Figure 4.1. As previously stated in chapter 2, the urothelium is capable of releasing various mediators, which may act on bladder ICC and their different receptors. Sub-urothelial ICC have been shown to possess muscarinic M_3 receptors (Grol *et al.,* 2009), making them an ideal site for urothelially released mediators such as ACh. Within the sub-urothelium

layer is an extensive microvasculature network and within this network, a novel class of perivascular ICC has been identified in the human bladder. Dual labelling of human bladder biopsies with phalloidin-tetramethylrhodamine, which binds filamentous actin, and anti-Kit showed close connection between ICC, small arterioles and venules, in which Kit positive cells ran parallel along the blood vessels on the outer surface (Johnston *et al.,* 2010). The action of mediators on ICC combined with the network of nerves in and amongst smooth muscle bundles, may allow for rapid relay of information and subsequent development of bladder contractions. Thus ICC may also play a role in the development of bladder PCs.

The location of ICC in the bladder is for the most part consistent between different species. In the guinea pig bladder three populations of ICC have been described, located in the connective tissue between muscle bundles, adjacent to the detrusor bundle boundary and scattered among and within smooth muscle cells (Hashitani *et al.,* 2004). cGMP-positive ICCs have also been shown to be present beneath the urothelium and on the outer muscle layers of guinea pig bladders (Gillespie *et al.,* 2004; Gillespie *et al.,* 2005). In the normal human bladder, ICCs have been shown to be present in between muscle bundles and in the sub-urothelium layer (Piaseczna Piotrowska *et al.,* 2004; Van der Aa *et al.,* 2004). Mouse bladder ICCs have been shown in between muscle bundles and in the outer part of the detrusor layer but not in the urothelium or sub-urothelium layers (Lagou *et al.,* 2006).

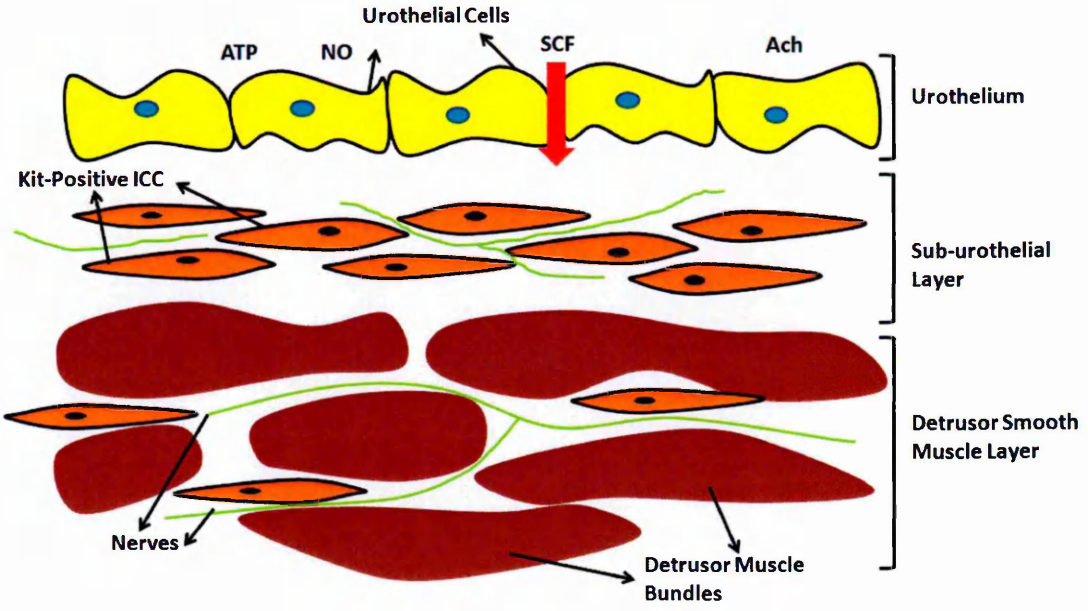


Figure 4.1 - Localization of ICC in the sub-urothelium and detrusor layers of the guinea pig urinary bladder wall and the relationship with nerve fibres and detrusor. ATP - adenosine triphosphate, NO - nitric oxide, SCF - stem cell factor, ACh - acetylcholine. Adapted from (Kubota *et al.*, 2011).

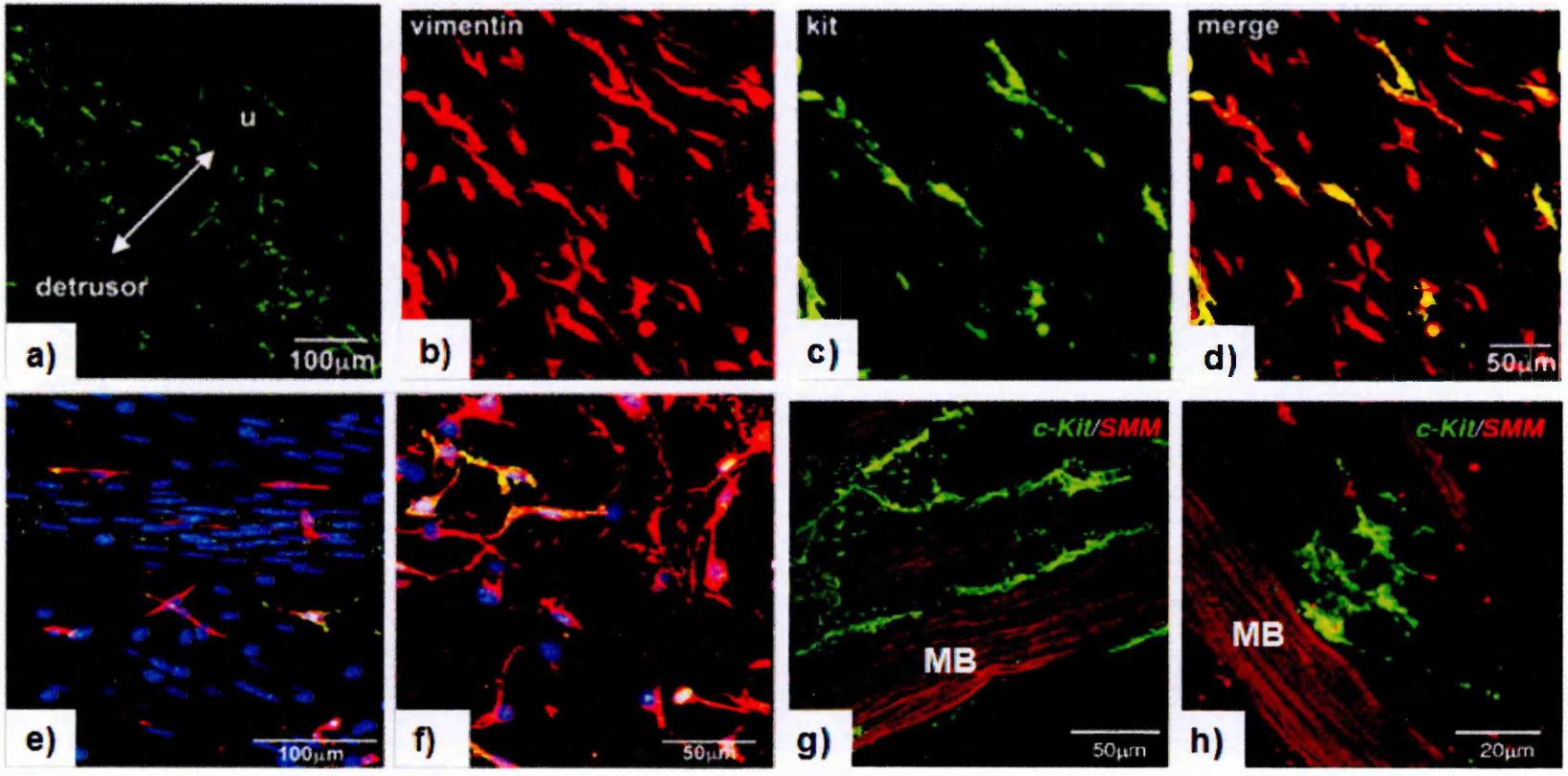


Figure 4.2 – immunohistochemisty images showing the location and morphology of interstitial cells. A) Kit labelling from a guinea pig bladder showing the urothelium (u) and the sub-urothelium (arrows); b - d confocal projection of guinea pig mucosa labelled with vimentin (b), Kit (c) and the co-localization of both vimentin and Kit (d); e) dual stain of guinea pig detrusor smooth muscle bundles labelled with Kit (green), vimentin (red) and counterstained with DAPI; f) magnified dual stain of guinea pig detrusor intermuscle bundles labelled with Kit and vimentin; g,h) human detrusor muscle bundles dual labelled with c-kit (green) and smooth muscle actin (red) showing c-kit along muscle bundles (MB). Images taken from (Davidson and McCloskey 2005; Johnston *et al.*, 2010).

4.1.3 c-KIT STRUCTURE AND FUNCTION

The c-Kit proto-oncogene encodes for a type III receptor tyrosine kinase, *Kit*, and has been shown to be structurally similar to platelet-derived growth factor receptors (PDGFRs) (Kitamura and Hirotab 2004). It is primarily made up of an extracellular ligand binding domain composed of five immunoglobulin-like repeats, a hydrophobic trans-membrane domain, a negative regulatory juxtamembrane domain and finally a tyrosine kinase domain split into two regions, namely TK-1 and TK-II domains separated by a variable length insert sequence (figure 4.3) (Kitamura and Hirotab 2004; Webster *et al.,* 2006). In humans, there are four isoforms of c-Kit that have been described, due to alternative RNA splicing, and these are characterised by the presence or absence of a serine residue in the kinase insert region (Lennartsson and Ronnstrand 2006). Stem cell factor (SCF) is the activating ligand for c-Kit and it is expressed by endothelial and fibroblast cells throughout the body. c-Kit expression on the other hand is restricted and primarily located on mast cells, hematopoietic cells, melanocytes, ICCs, vascular endothelial cells, testis, breast glandular epithelial cells, sweat glands and the brain (Lennartsson and Ronnstrand 2006). SCF exists as two splice forms - a membrane associated form containing a transmembrane domain and a soluble form. The membrane associated isoform has been shown to induce prolonged c-Kit phosphorylation (Huang *et al.,* 1992; Miyazawa *et al.,* 1995). Binding of the SCF to c-Kit activates the receptor and results in the dimerization of two receptor monomers (Blume-Jensen *et al.,* 1991) thereby bringing two c-Kit tyrosine kinase domains close to each other leading to autophosphorylation of tyrosine residues primarily outside the kinase domains but also within on residues 823 and 900 (Lennartsson *et al.,* 2003; Voytyuk *et al.,* 2003; Maulik *et al.,* 2004). Autophosphorylation leads to two main events - increased kinase activity and creation of high affinity interaction sites for proteins (Pawson 1995). Interaction of the proteins with the activated receptor results in phosphorylation and initiation of signal transduction of numerous pathways (Lennartsson and Ronnstrand 2006).

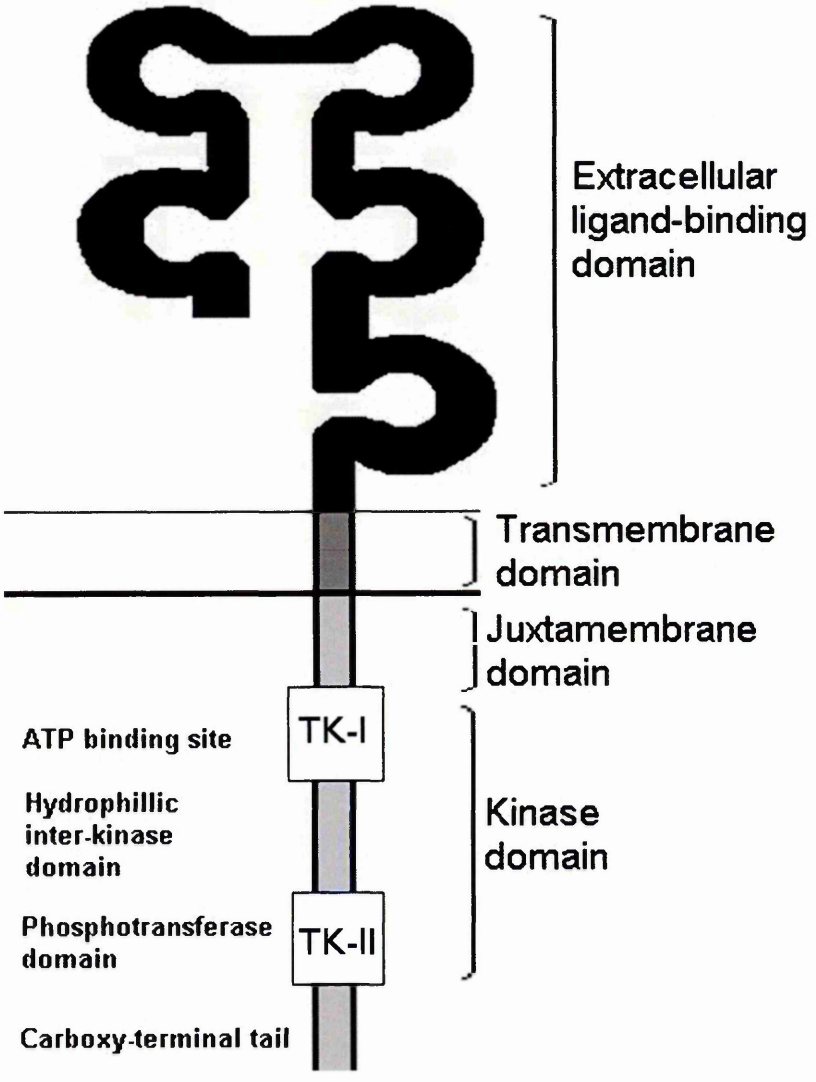


Figure 4.3 - Structure of the receptor tyrosine kinase c-Kit showing the two kinase domains TKI and TKII - Adapted from (Webster *et al.*, 2006).

4.1.4 ICC IN BLADDER PATHOLOGICAL CONDITIONS

The extensive research that has been performed on ICCs of the GI tract has attributed various motility disorders such as gastroparesis, chronic idiopathic intestinal pseudo-obstruction and gastrointestinal stromal tumours (GISTs) to the loss of ICC function in different regions of the digestive tract (Sanders 2006). Building on the knowledge of ICCs in the GI tract has led to a rapid advancement and collection of information on bladder ICC and although the precise function of these cells in normal bladder function is still not clear, it has been suggested that these cells may have a greater role in pathological conditions of the bladder, such as overactive bladder. Biers *et al.* demonstrated that bladders from patients with OAB exhibited significantly more c-Kit positive ICC in comparison to normal bladders, thereby suggesting that bladder ICC may play some kind of role in the pathophysiology of OAB (Biers *et al.,* 2006). Using a guinea pig bladder outlet obstruction (BOO) model, Kubota *et al.* (2008) showed that animals with BOO displayed increased voiding frequency and an increased incidence of non-voiding contractions. The sub-urothelium thickness was also increased and immunohistochemical staining confirmed an increased expression of ICC in the sub-serosal layer of the BOO bladders compared to normal bladders. It is thought that the altered distribution of ICC may contribute to bladder overactivity pathophysiology (Kubota *et al.,* 2008).

In OAB patients, there is increased urgency and frequency which may be explained by increased electrical coupling between SMCs via ICC gap junctions, leading to the enhanced excitability of the detrusor muscle as shown in Figure 4.1 (Brading 1997a). Subsequently, spontaneous excitation due to phasic activity may propagate down the muscle bundles by a longer distance, causing synchronous contraction thereby increasing intravesical pressure within the bladder and the sudden urgency (Hashitani 2006).

Imatinib mesylate (Gleevec®, Glivec®, Novartis Pharmaceuticals) - a 2-phenylaminopyrimidine derivative - commonly known as imatinib is a specific tyrosine kinase enzyme inhibitor which is used in the treatment of c-Kit positive gastrointestinal stromal tumours (GISTs) and Philadelphia chromosome-positive chronic myeloid leukaemia (CML) (Kubota *et al.,* 2004). Imatinib is specific for tyrosine kinase domains in the Abelson proto-oncogene (*abl*), c-Kit and PDGFRs. The active sites of tyrosine kinases have a binding site for ATP and are actively involved in a process known as tyrosine phosphorylation. This process entails the transfer of an ATP terminal phosphate to tyrosine residues on its substrates. The mechanism of action of imatinib

involves its binding to the ATP-binding site and inhibition of the tyrosine kinase's ability to phosphorylate its substrates thereby inhibiting the enzymatic activity (Takimoto and Calvo 2008). Imatinib has been shown to suppress phasic contractility of smooth muscle in various organs including human uterus and small intestines (Popescu *et al.,* 2006), guinea pig bladder (Kubota *et al.,* 2004; Kubota *et al.,* 2006) and the guinea pig gall bladder (Lavoie *et al.,* 2007)). In a study by Vahabi *et al.*, imatinib decreased the amplitude and frequency of carbachol induced PCs in both normal and streptozotocin-induced diabetic rat bladder strips in a concentration dependent manner (Vahabi *et al.,* 2011c). In human bladders, imatinib also inhibited EFS evoked smooth muscle contraction and spontaneous activity in overactive detrusor with the effect being less significant in normal detrusor tissue (Biers *et al.,* 2006).

4.1.5 IMMUNOHISTOCHEMISTRY

Immunohistochemistry (IHC) is a histological technique used to determine the distribution and localization of various biomarkers expressed in different regions of histological tissue. This technique employs the principle of antigen-antibody interaction and is now widely used in various medical diagnoses including detection of abnormal cells in tissue with cancerous tumours. The visualization of the antigen-antibody reaction is made possible by the use of fluorophores bound to the antibodies which can be viewed using a fluorescent microscope. There are two main IHC methods that are commonly used – direct and indirect IHC.

4.1.5.1 Direct Method

This is a one-step staining method that utilizes a single antibody linked to a fluorophore such as Fluorescein Isothiocyanate (FITC). The antibody recognizes the target molecule (antigen) and binds to it and the attached fluorophore is detected by microscopy.

4.1.5.2 Indirect Method

The indirect method differs from the direct method due to the fact that it uses two antibodies making it more sensitive since the fluorescent signal is amplified. The primary antibody is unlabelled and binds to the antigen of interest. The secondary antibody, which is raised against the IgG of the animal species in which the primary antibody has been raised in, is labelled with the fluorophore and reacts with the primary antibody to form a complex that can be viewed under the fluorescent microscope.

4.1.5.3 Confocal Scanning Laser Microscopy (CSLM)

High resolution optical images with depth selectivity can be obtained using confocal scanning laser microscopy (CSLM). This microscopy technique employs a process known as optical sectioning that makes it possible to obtain in-focus images from selected depths. As the name suggests, a laser beam is passed through a light source aperture and an objective lens is used to focus this beam into a small focal volume on the surface of a specimen which in this case is fluorescent (Figure 4.4). The light reflected from the specimen is collected by the objective lens and directed onto a beam splitter which directs a portion of this light into a detection apparatus. In the case of fluorescent microscopy, the detection apparatus contains a filter which effectively blocks the original excitation wavelength and allows the fluorescent wavelength across through a pinhole. This light intensity is detected by a photomultiplier tube thereby transforming the light signal into an electrical signal which is effectively recorded and viewed on a computer as one pixel. As the laser scans over the plane of interest – in this case the slide containing the stained sample tissue – a whole image is thus obtained pixel by pixel (Claxton *et al.,* 2011). The images presented in this chapter were obtained using a Zeiss LSM 510 scanning laser microscope.

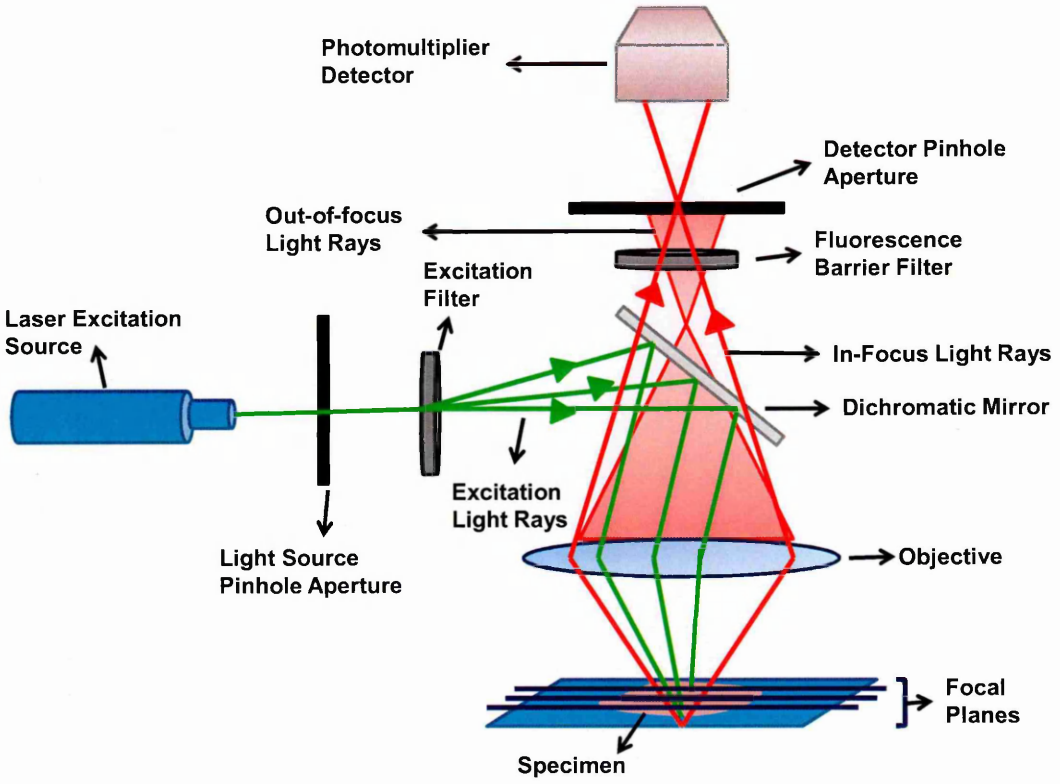


Figure 4.4 - Schematic representation of the principal components and optical pathway utilised in a scanning laser confocal microscope. Adapted from (Claxton *et al.*, 2011).

4.2 CHAPTER AIMS

The aims of this chapter were:-

- Investigate the role of ICC in phasic contractions of the dome, body and trigone regions of the pig bladder.
- To identify and localise c-kit positive cells in the pig urinary bladder

4.3 Materials and Methods

4.3.1 Functional investigation of the role of c-Kit positive cells in the pig bladder

Bladders for these experiments were collected and prepared as described in Chapter 2 section 2.3.1 and placed in cold (4°C) Krebs' buffer prior to experiments being performed. Experiments were performed on intact and denuded strips from the dome, body and trigone regions of the pig urinary bladder.

Tissue strips of approximately 3 x 3 x 10mm were suspended in tissue baths using non-absorbable surgical suture silk and allowed to equilibrate for a period of 60 minutes. Strips were bathed in Krebs' buffer maintained at 37°C and continuously bubbled with 95% O_2/5% CO_2. Tissues were washed with fresh Krebs' buffer every 15 minutes for the duration of the equilibration period. After 60 minutes, the tissue strips were incubated with cumulative concentrations of imatinib mesylate (imatinib) at concentrations of 1, 5, 10, 20 and 50μM. Tissues were incubated with each concentration for 20 minutes before addition of the next concentration. In control experiments, an equal volume of vehicle (dH_2O) was added in place of the drug. After the final concentration (50μM), the tissues were washed and left for 2-3 minutes and then demounted and the wet tissue weight recorded.

In separate experiments carbachol (CCh) was used to increase phasic contractions (PCs) and tissues were then incubated with increasing cumulative concentrations of imatinib as above. Experimental traces from both protocols are shown in figure 4.5.

4.3.2 Immunohistochemistry of the pig bladder

4.3.2.1 Tissue Preparation for immunohistochemistry

Tissue pieces of approximately 2 x 2cm isolated from the dome and body region and 1 x 1cm from the trigone region were prepared as described in chapter 2 section 2.3.6.1 and shown in figure 2.4. Prior to sectioning on the cryostat, tissues were removed and stored in -20°C, and then the frozen liver-bladder-liver sandwich tissue blocks were placed in a cryostat maintained at -20°C for 30 minutes. 10 and 12 micron thick sections were cut and placed on polylysine coated slides and stored in tissue slide chambers at -20°C until used for immuno-histochemical staining.

4.3.2.2 Optimisation of Immunohistochemistry Protocol

IHC optimisation was performed to ensure the immuno-detection of the specific antigens of interest in the bladder tissue. Optimal conditions were determined for

tissue section thickness, method of fixation and antibody incubation times and concentrations for each individual antibody.

10 and 12 micron thick sections were used for the IHC optimisation. Two different fixation protocols were also used. Briefly, method 1 involved immersing slides in cold acetone for 2 minutes and air drying prior to IHC staining. Method 2 involved fixing slides using 4% PFA for 15 minutes then washing slides in 1X PBS for 10 minutes. Slides were subsequently placed in ice cold methanol (4 minutes), ice cold acetone (2 minutes) and finally washed in 1X PBS and allowed to air dry at RT prior to IHC staining (See section 2.3.6 for full protocol).

For antibody optimisation, 1hr and overnight incubation times were investigated at different concentrations for each antibody. For the smooth muscle actin antibody optimisation, concentrations of 1:100 and 1:200 were used, with concentrations of 1:150 and 1:300 for both c-Kit and vimentin.

4.3.2.3 Immunohistochemistry protocol

Frozen sections were removed from the freezer (-20°C) and left to warm up to room temperature (RT) for 20 minutes. The slides were fixed in ice cold (-20°C) acetone for ten minutes and left to air dry. A hydrophobic delimiting barrier pen was used to outline around the tissue to minimize the amount of reagents used. Once completely dry, the slides were blocked with a 5% donkey serum (DS) in 1% bovine serum albumin (BSA) solution for 45 minutes at RT. Primary antibodies (Abcam, Cambridge, U.K - Table 4.1) were diluted in phosphate buffered saline (PBS) with 0.3% DS and 180µL per tissue section was added on to the sections immediately after blocking and incubated overnight at 4°C in a humidified chamber. The slides were then washed 3 times in 1X PBS for 5 minutes each wash and the secondary antibodies (Invitrogen U.K - Table 4.1), diluted in 1% BSA in 1X PBS were added at 180µL per tissue section for 1 hour at RT in the humidified chamber. Two more washes were performed with 1X PBS and the slides were subsequently immersed in 1% Sudan Black B (SBB) for 5 minutes in the dark at RT and then washed 8-10 times with 1X PBS until the washes ran clear. The slides were covered in foil and mounted with cover slips using vectorshield hard set mounting medium with DAPI (Vector laboratories, Peterborough, U.K.). Finally the slides were sealed using clear nail varnish, placed in a slide holder wrapped in foil and stored at 4°C ready for confocal laser microscopy.

Primary Antibodies						
Immunogen	**Isotype**	**Concentration**	**Working Dilution**	**Reactivity**	**Host**	**Clonality**
Anti-alpha smooth muscle actin (ab5694)	IgG	100µg at 0.2mg /ml	1:200	Human, Mouse, Rat, Chicken, Guinea pig, Cow and Pig	Rabbit	Polyclonal
Anti-Vimentin [V9] (ab8069)	IgG1	100µg at 1mg/ml	1:300	Cat, Chicken, Cow, Dog, Horse, Human, Pig and Rat	Mouse	Monoclonal
Anti-c-Kit (ab5506)*	IgG	100µg at 0.25mg/ml	1:300	Human, Mouse	Rabbit	Polyclonal
Anti-c-Kit [57A5D8] (ab49212)*	IgG1	100µg at 1mg/ml	1:200 - 1:400	Human	Mouse	Monoclonal
Secondary Antibodies						
Antibody		**Isotype**	**Concentration**	**Working Dilution**	**Reactivity**	**Host**
Alexa fluor 488 donkey anti-rabbit (A 21206)		IgG	2mg/ml	1:500	Rabbit	Donkey
Alexa fluor 568 donkey anti-rabbit (A 10042)		IgG	2mg/ml	1:500	Rabbit	Donkey
Alexa fluor 488 donkey anti-mouse (A 21202)		IgG	2mg/ml	1:500	Mouse	Donkey

Table 4.1– Summary of primary and secondary antibodies used for immunohistochemical staining. * Anti-c-Kit antibody used with predicted reactivity to porcine antigens.

4.4 Data and Statistical Analysis

Amplitude and frequency of PCs were recorded and measured as described in chapter 2 section 2.3.5. Measurements for these experiments were taken during the last 5 minutes of the equilibration period for baseline, and then during the last 5 minutes with each concentration of imatinib. All data are presented as mean ± SEM. Amplitude is shown as absolute tension per mg of tissue and frequency as the number of contractile events within the 5 minute measurement period. Intra-tissue statistical analysis of baseline vs. effects of imatinib in the different bladder regions was performed using a Student's t test. A Student's t test was also performed to compare responses in intact vs. denuded tissue strips. Comparison of responses in the different bladder region was performed via repeated measures one way ANOVA with a Bonferroni post hoc test. P values ≤ 0.05 were considered significant.

4.5 RESULTS

4.5.1 FUNCTIONAL INVESTIGATION OF THE ROLE OF C-KIT POSITIVE CELLS IN THE PIG BLADDER

The effect of imatinib on the phasic contractions of isolated pig bladder strips was investigated. Representative experimental traces showing the effect of imatinib on naturally occurring PCs in the different bladder regions are presented in figure 4.5. At all concentrations investigated, Imatinib exhibited no effect on the amplitude of PCs in both intact and denuded strips of the dome, body and trigone regions (figures 4.6, 4.7 and 4.8 respectively). Statistical comparison of intact strips treated with cumulative concentrations of imatinib (1 - 50μM) vs. control between all the 3 bladder regions showed no significant differences observed. This was also mirrored in comparisons performed on denuded tissue strips between all 3 regions as well.

Analysis of the effect of imatinib on the frequency of PCs in intact strips from the dome, body and trigone is presented in Figure 4.9a, Figure 4.10a and Figure 4.11a. No significant effect of imatinib was observed in intact strips from the three bladder regions. Similarly, analysis of frequency measurements performed on denuded tissue strips from the dome, body and trigone (Figure 4.9b, Figure 4.10b and Figure 4.11b respectively) also showed that imatinib had no significant effect on the number of contractile events measured.

Figure 4.12 shows sample experimental traces of the effect of imatinib on CCh modulated PCs across the three bladder regions. Imatinib had no effect on neither the amplitude (figures 4.13 - 4.15) nor the frequency (figures 4.16 - 4.18) of CCh modulated PCs from both intact and denuded bladder strips of the dome, body and trigone regions.

Experiments performed to evaluate the effect of the vehicle (data not shown) revealed that in both intact and denuded tissue strips from all three bladder regions, there was no significant effect on the amplitude and frequency of PCs and CCh modulated PCs.

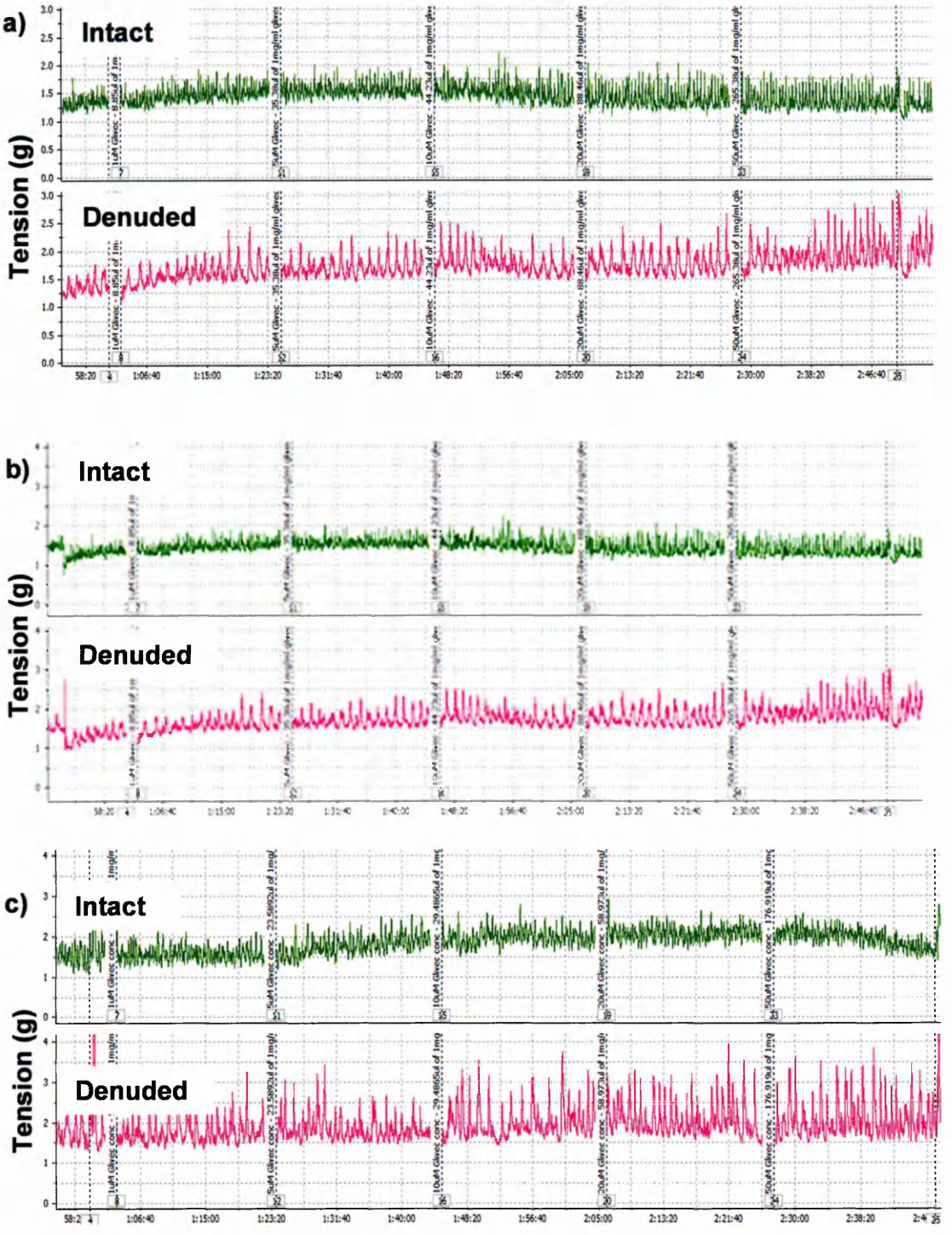
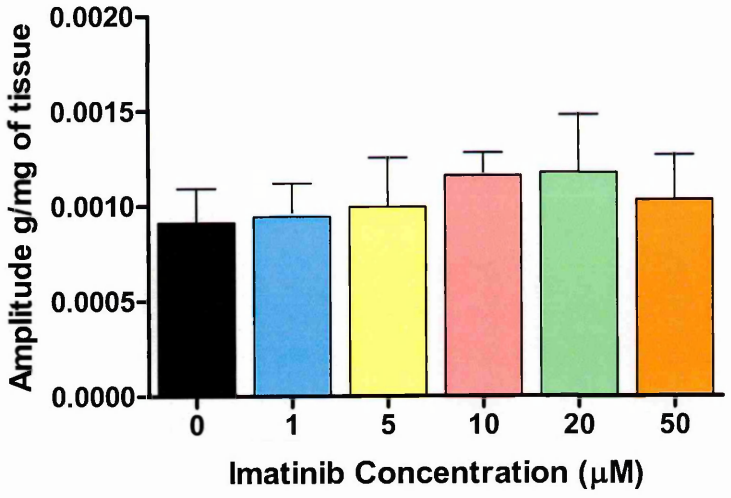
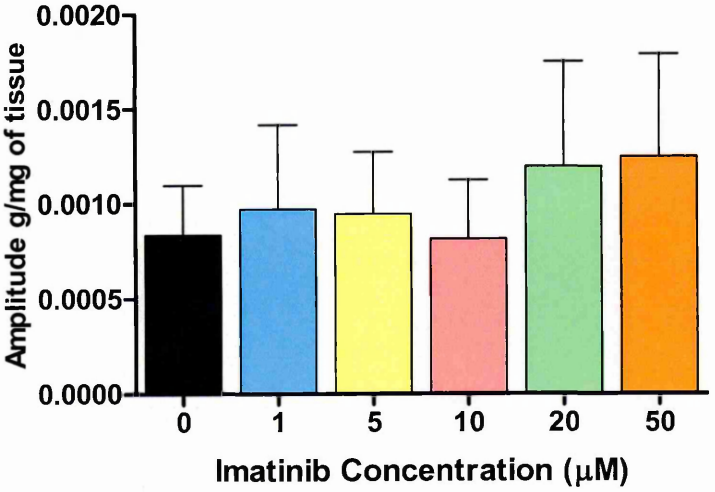


Figure 4.5 - Representative traces showing the effect of imatinib on phasic contractions from intact and denuded a) dome, b) body and c) trigone bladder regions.

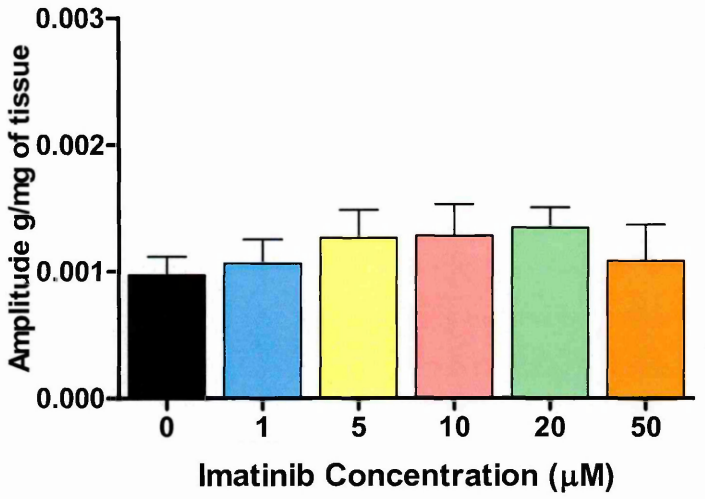


a)

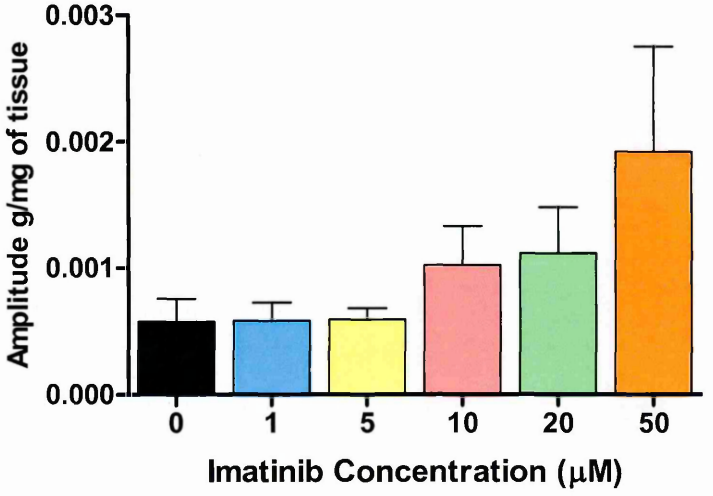


b)

Figure 4.6 - Effect of imatinib on the amplitude of PCs (normalised for tissue weight) in a) intact and b) denuded strips of pig bladder dome. All data are presented as mean ± SEM, n=6.

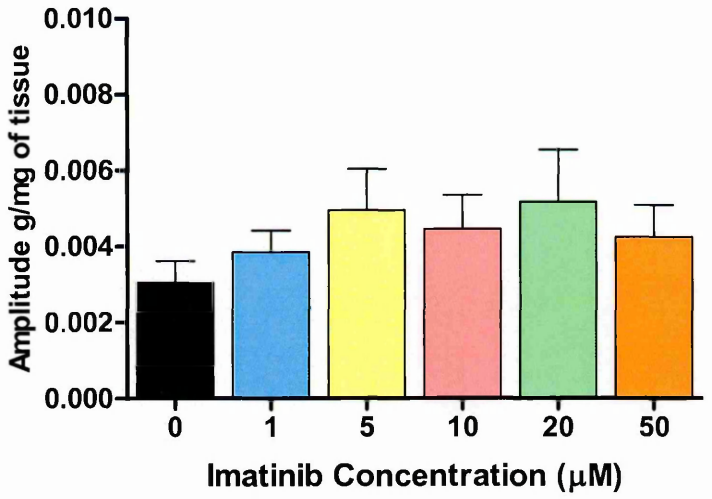


a)

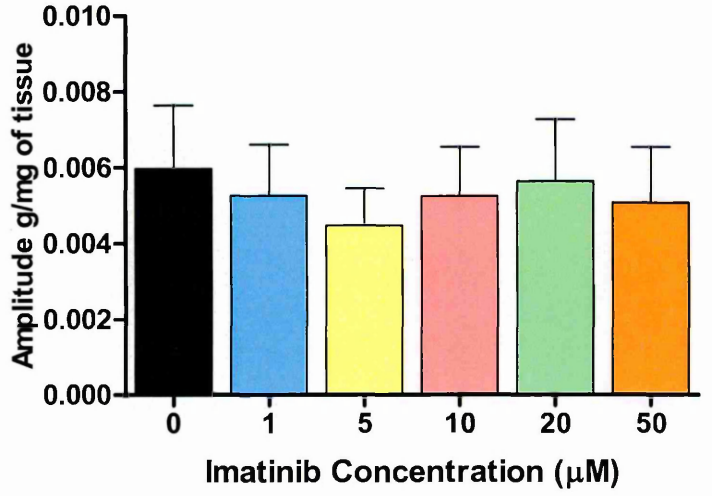


b)

Figure 4.7 - Effect of imatinib on the amplitude of PCs (normalised for tissue weight) in a) intact and b) denuded strips of pig bladder body. All data are presented as mean ± SEM, n=6.

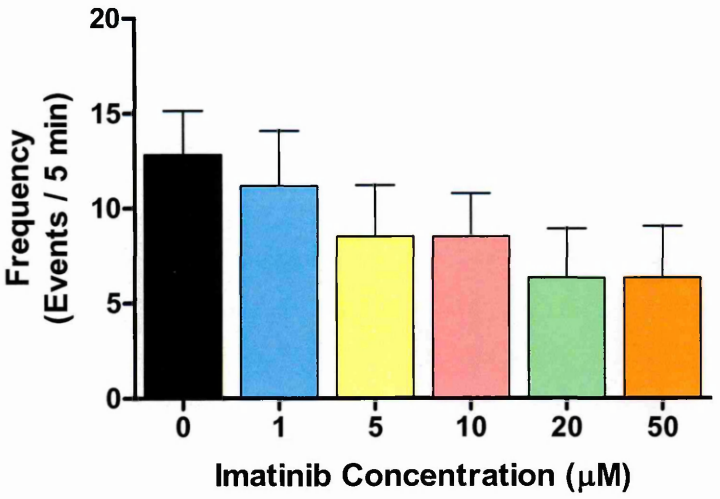


a)

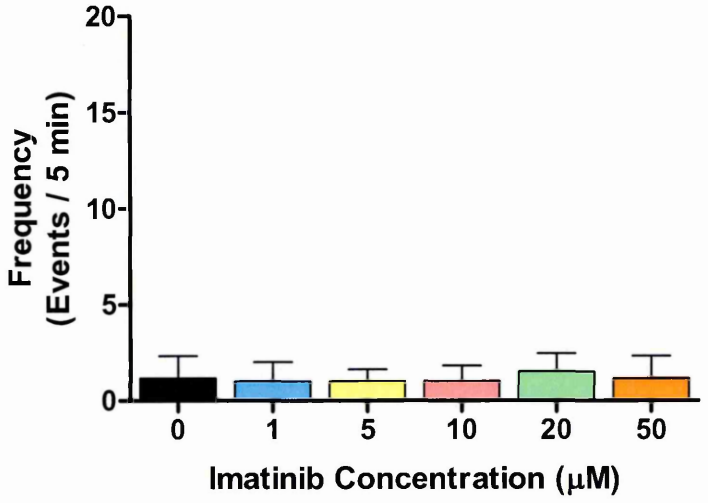


b)

Figure 4.8 - Effect of imatinib on the amplitude of PCs (normalised for tissue weight) in a) intact and b) denuded strips of pig bladder trigone. All data are presented as mean ± SEM, n=6.

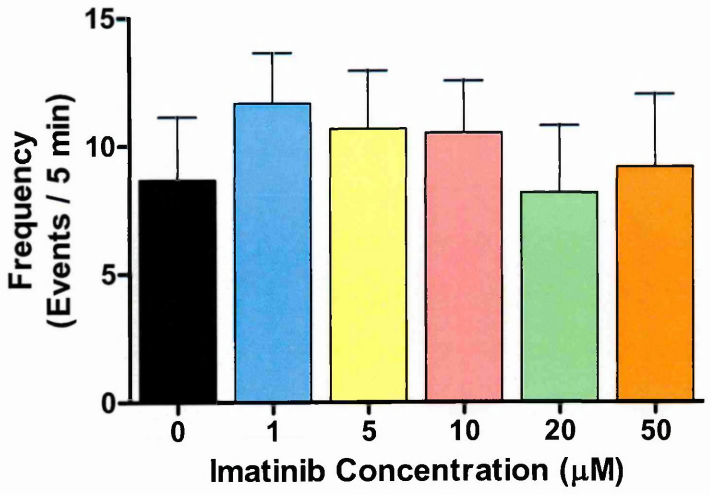


a)

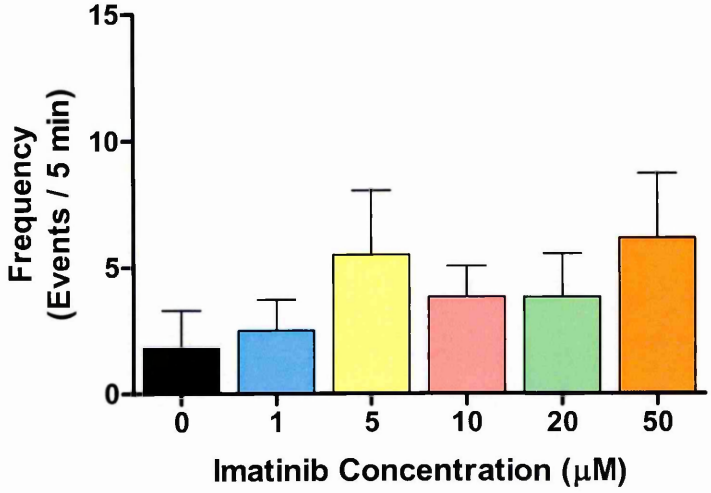


b)

Figure 4.9 - Effect of imatinib on the frequency of PCs (normalised for tissue weight) in a) intact and b) denuded strips of pig bladder dome. All data are presented as mean ± SEM, n=6.

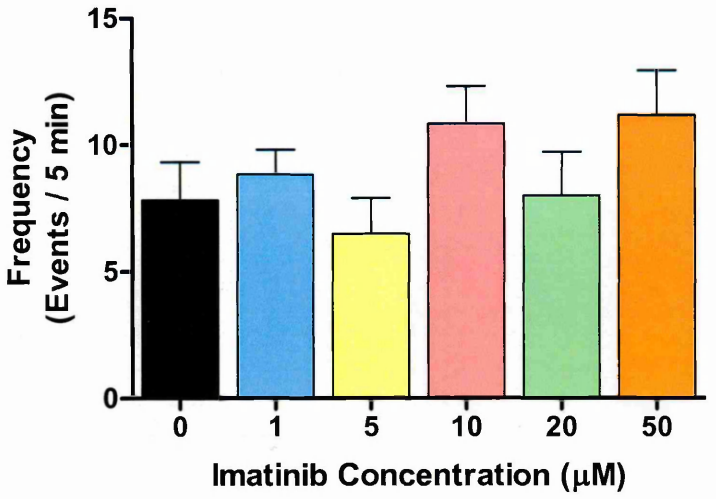


a)

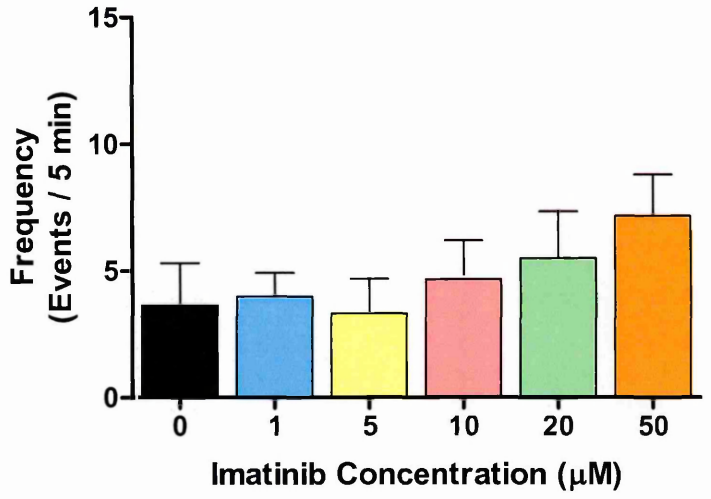


b)

Figure 4.10- Effect of imatinib on the frequency of PCs (normalised for tissue weight) in a) intact and b) denuded strips of pig bladder body. All data are presented as mean ± SEM, n=6.



a)



b)

Figure 4.11 - Effect of imatinib on the frequency of PCs (normalised for tissue weight) in a) intact and b) denuded strips of pig bladder trigone. All data are presented as mean ± SEM, n=6.

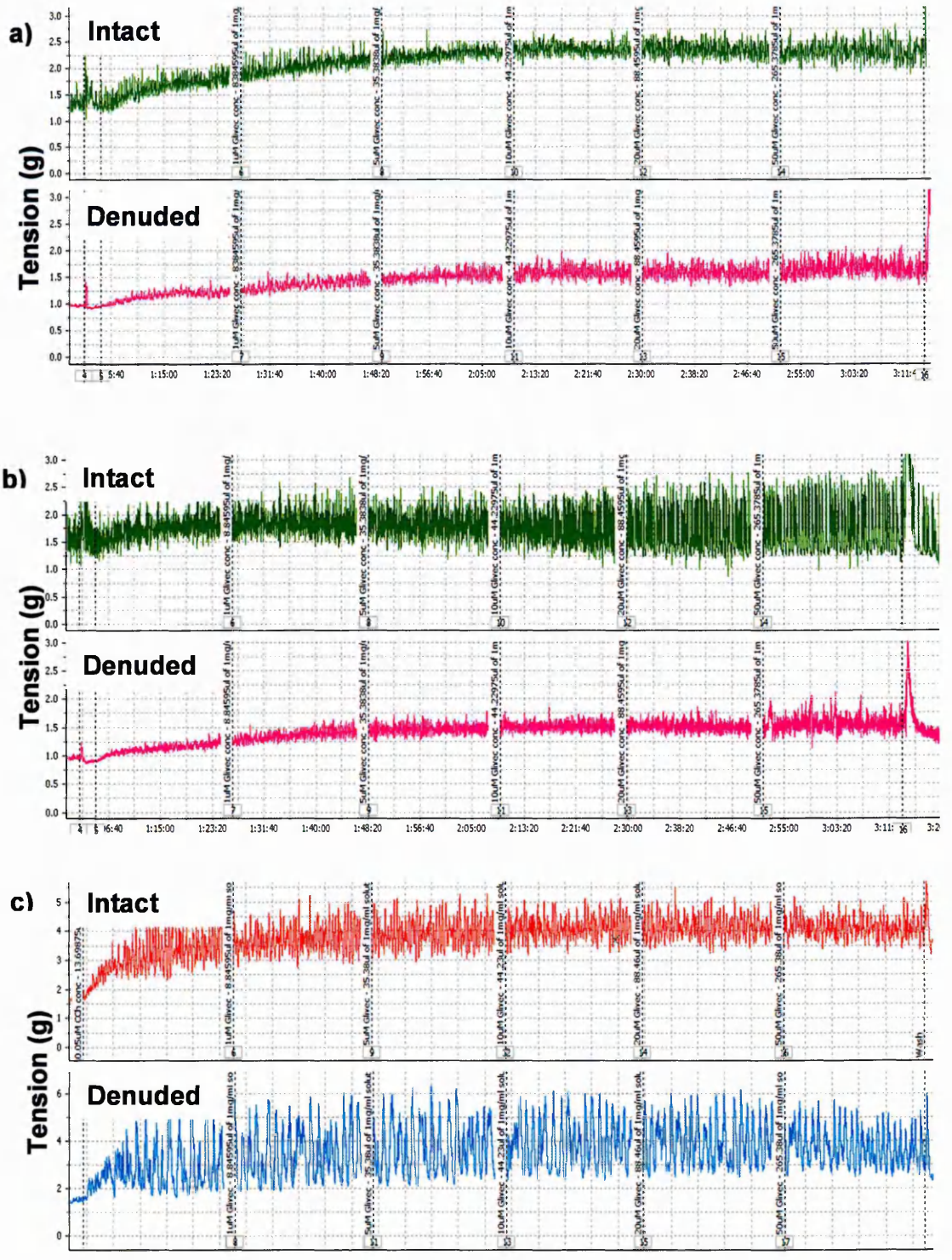
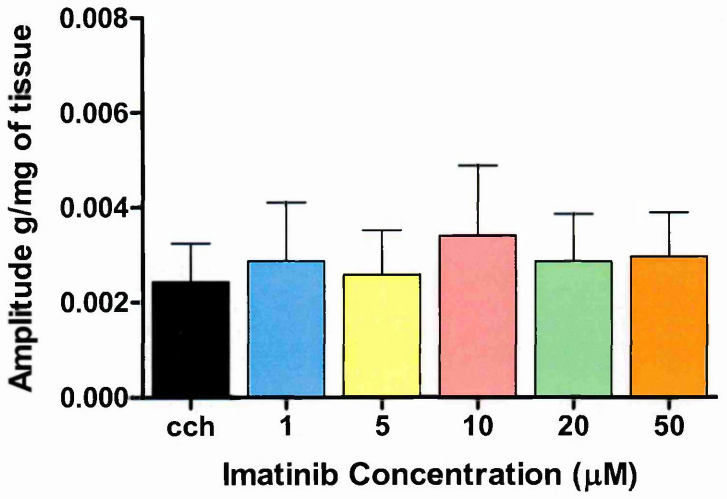
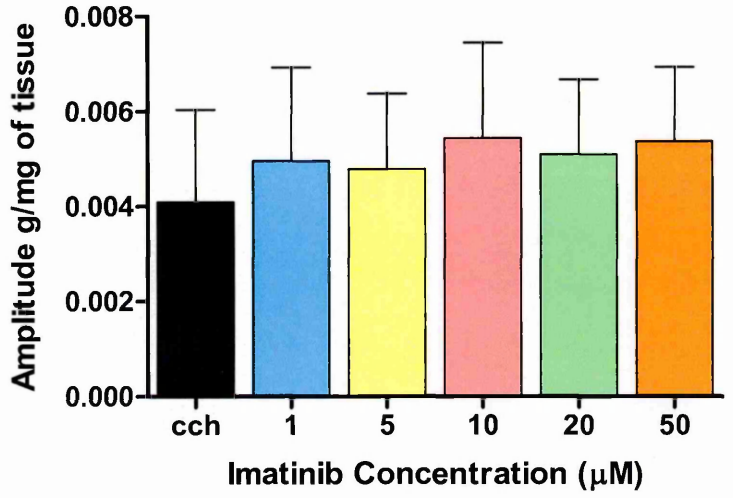


Figure 4.12 - Representative traces showing the effect of imatinib on CCh modulated phasic contractions from intact and denuded a) dome, b) body and c) trigone bladder regions.

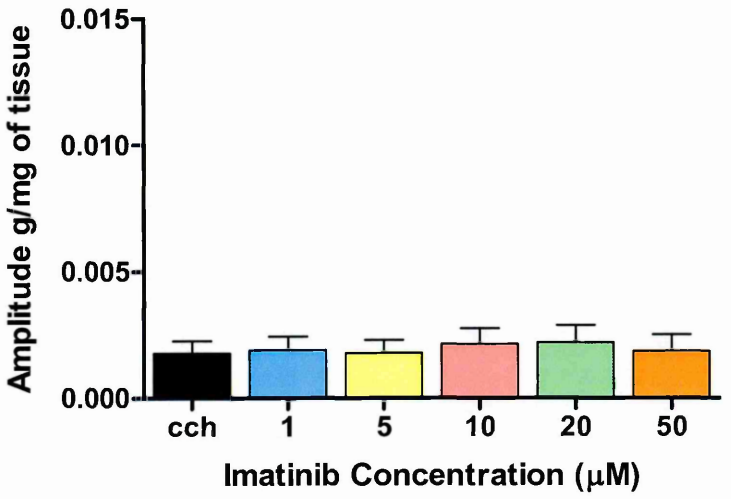


a)

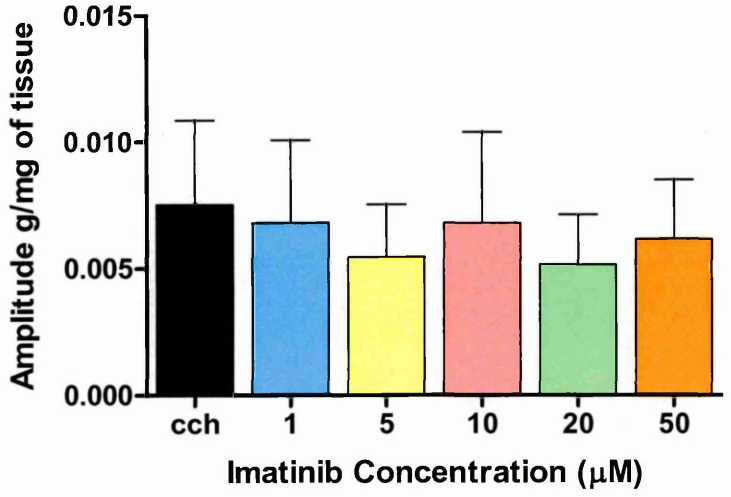


b)

Figure 4.13 - Effect of imatinib on the amplitude of CCh modulated PCs (normalised for tissue weight) in a) intact and b) denuded strips of pig bladder dome. All data are presented as mean ± SEM, n=4.

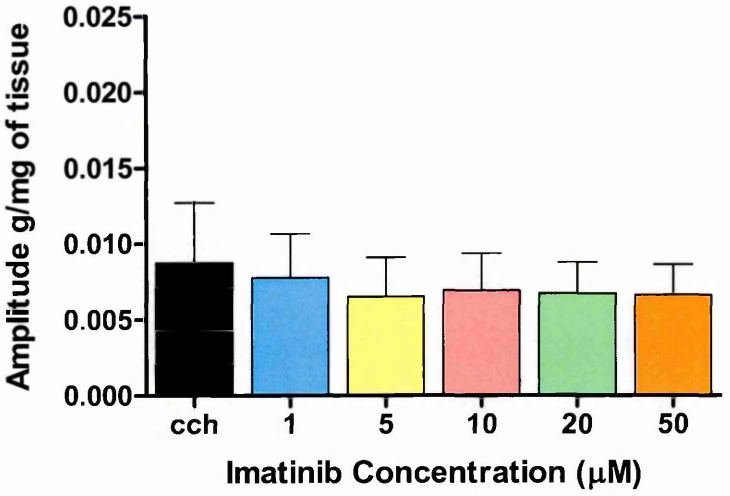


a)

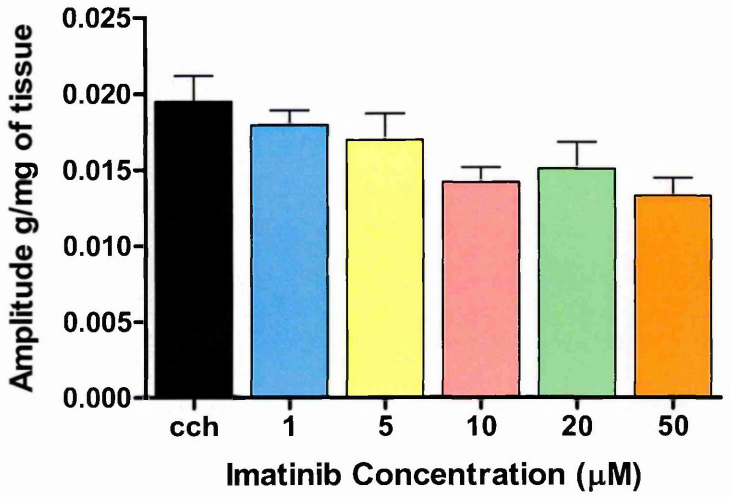


b)

Figure 4.14 - Effect of imatinib on the amplitude of CCh modulated PCs (normalised for tissue weight) in a) intact and b) denuded strips of pig bladder body. All data are presented as mean ± SEM, n=4.

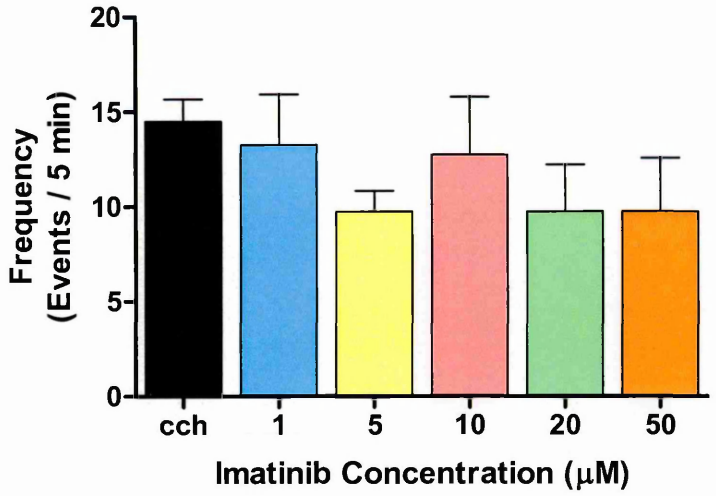


a)

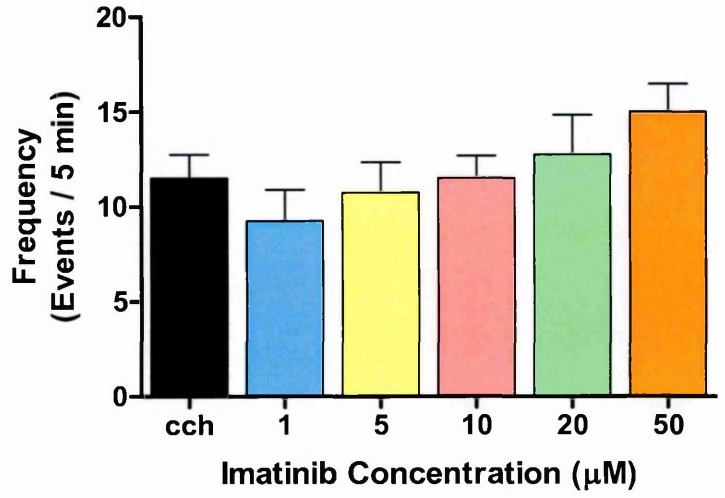


b)

Figure 4.15 - Effect of imatinib on the amplitude of CCh modulated PCs (normalised for tissue weight) in a) intact and b) denuded strips of pig bladder trigone. All data are presented as mean ± SEM, n=3.

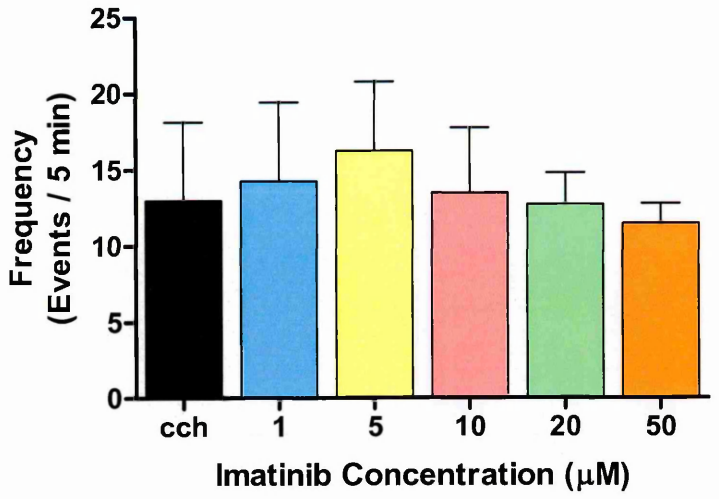


a)

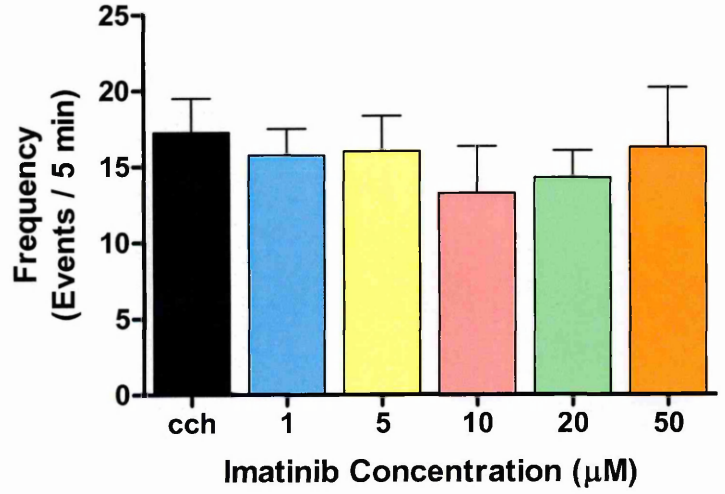


b)

Figure 4.16 - Effect of imatinib on the frequency of CCh modulated PCs (normalised for tissue weight) in a) intact and b) denuded strips of pig bladder dome. All data are presented as mean ± SEM, n=4.

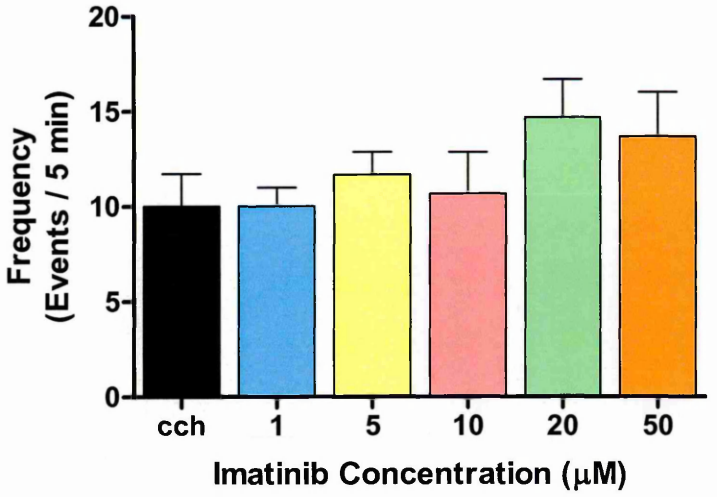


a)

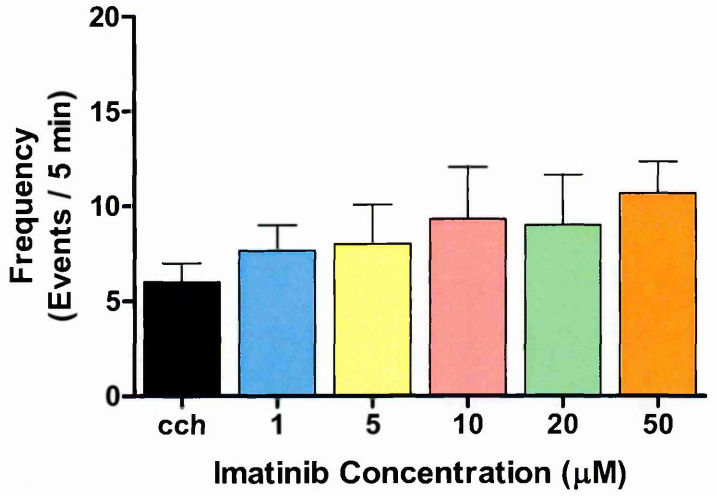


b)

Figure 4.17- Effect of imatinib on the frequency of CCh modulated PCs (normalised for tissue weight) in a) intact and b) denuded strips of pig bladder body. All data are presented as mean ± SEM, n=4.



a)



b)

Figure 4.18- Effect of imatinib on the frequency of CCh modulated PCs (normalised for tissue weight) in a) intact and b) denuded strips of pig bladder trigone. All data are presented as mean ± SEM, n=3.

4.5.2 IMMUNO-HISTOCHEMISTRY OF THE PIG BLADDER

Results of the optimisation experiments showed that for each bladder region, the most effective concentration of the SMA antibody was 1:200, whereas for vimentin 1:300 was the ideal concentration. Antibody concentration of 1:300 was used for c-Kit experiments because using a higher concentration was only increasing background autofluorescence. Optimised antibody concentrations were used to stain 12 micron thick sections that were fixed using the second fixative method, with overnight primary antibody incubation at 4°C. All secondary antibodies were used at a 1:500 dilution.

Using dual immunohistochemistry, it was possible to identify smooth muscle bundles that stained positive for α-smooth muscle actin and also for the vimentin filament. This was observed in all 3 regions of the pig bladder dome, body and trigone and is shown in the images presented in Figures 4.19, 4.20 and 4.21 below. The different components of the bladder wall; urothelium, sub-urothelium and the detrusor smooth muscle bundles, can be seen in Figure 4.19, which is tissue from the dome region, whilst figures 4.20 depict positively stained smooth muscle bundles from the dome. Detection of ICC and c-Kit positive cells was attempted by use of antibodies directed against c-Kit and vimentin. Figure 4.22, 4.23 and 4.24 show the resulting immunofluorescent staining obtained with these antibodies in the dome, body and trigone regions respectively. In all of these figures, it is possible to see positive staining for vimentin. In contrast, no specific immunofluorescent staining for c-Kit could be observed (Figure 4.22a, Figure 4.23b and Figure 4.24b). Figure 4.25 shows images from the negative control where the primary antibody was omitted.

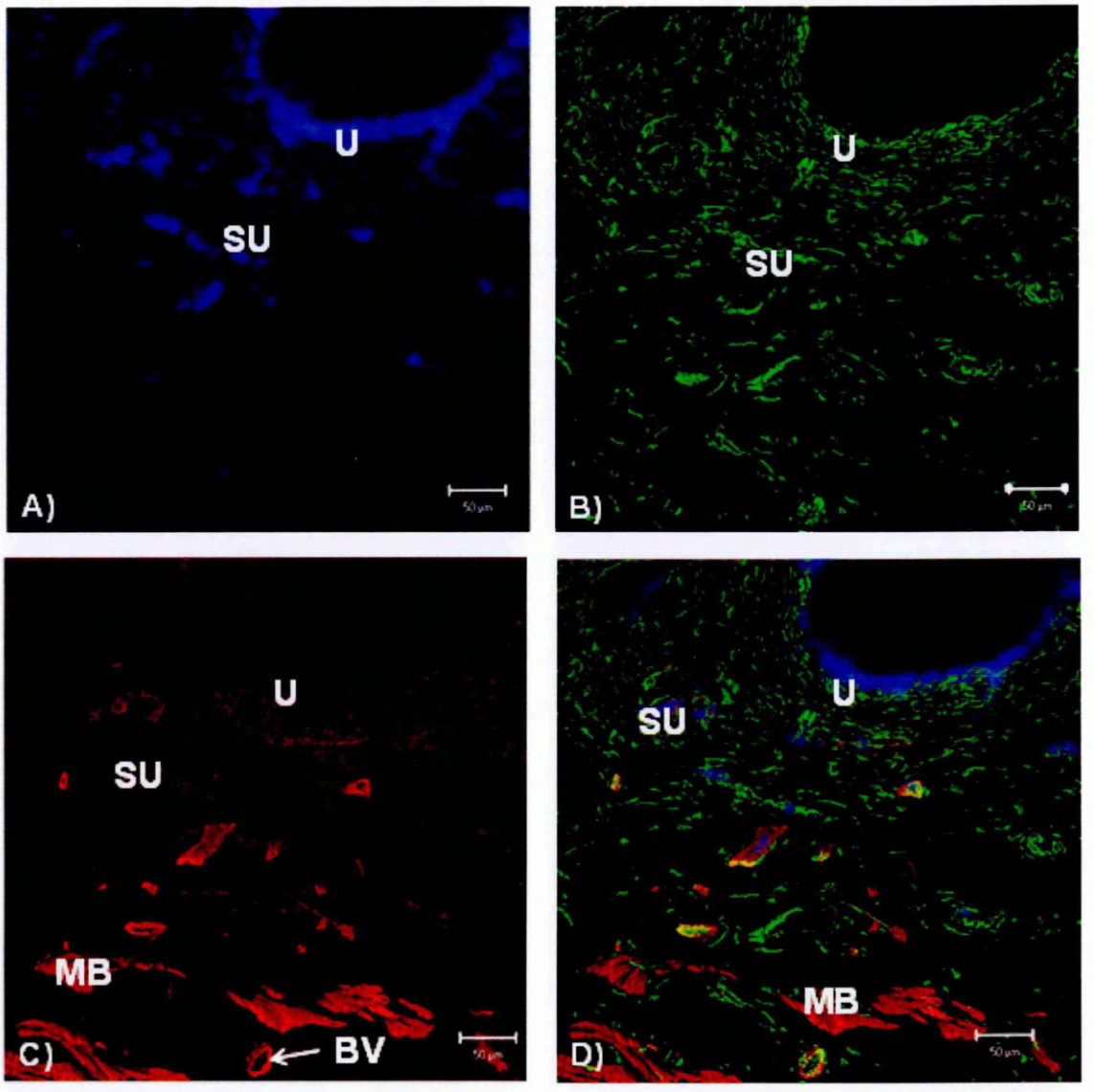


Figure 4.19 - Immunofluorescent dual staining performed on 12 micron sections of pig bladder dome showing a) Dapi b) vimentin (green), c) α-smooth muscle actin (red) and d) combined overlaid stain image showing the urothelium (U), sub-urothelium (SU), muscle bundle (MB) and blood vessels (BV); magnification 20X. The vimentin stain is localised within the urothelium, suburothelium, in and around smooth muscle bundles (green stain).

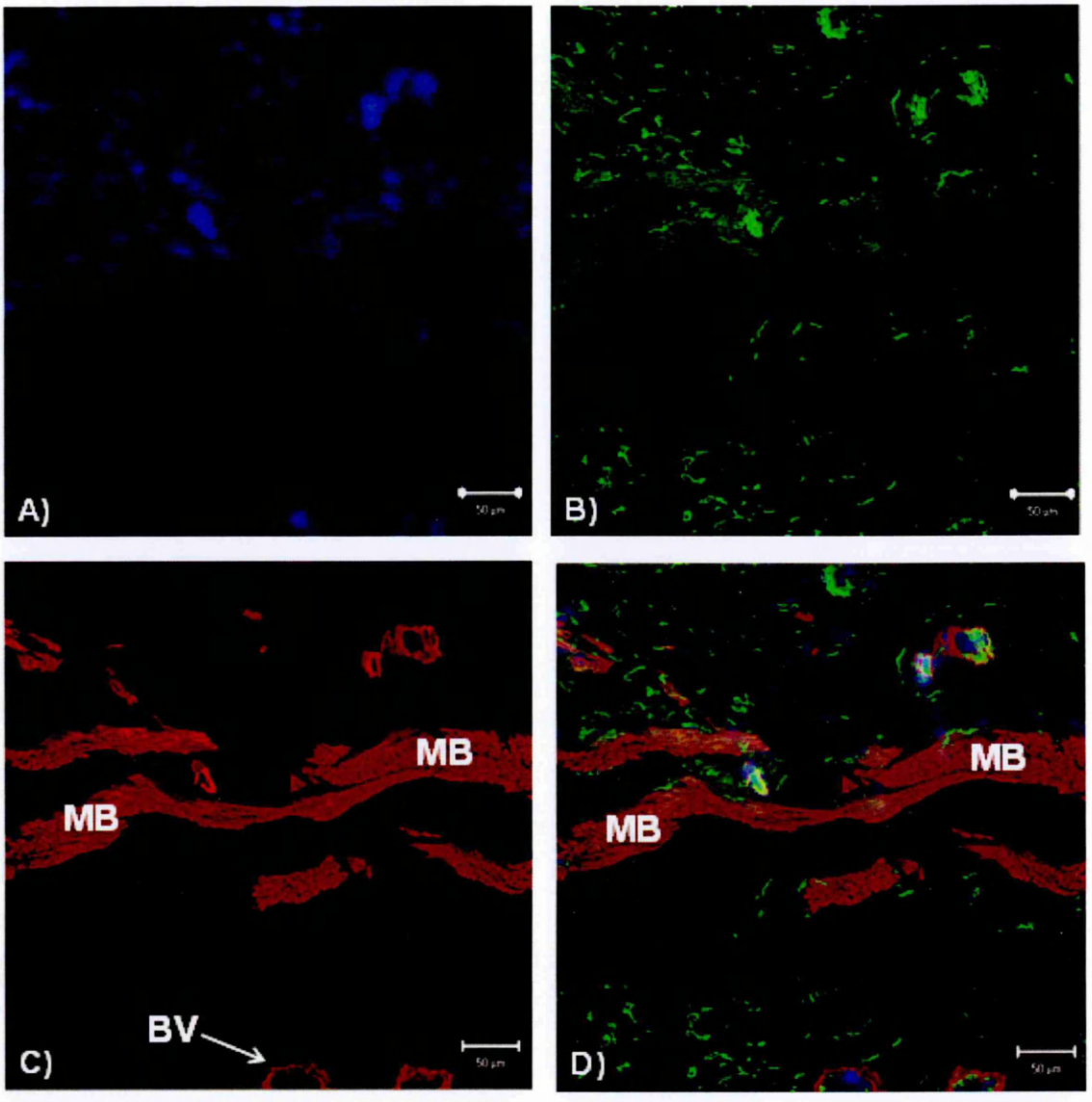


Figure 4.20 - Immunofluorescent dual staining performed on 12 micron sections of pig bladder dome showing a) Dapi (blue), b) vimentin (green), c) α-smooth muscle actin (red) and d) combined overlaid stain image showing the smooth muscle bundles (MB) and blood vessels (BV); magnification 20X. The vimentin stain is localised within the urothelium, suburothelium, in and around smooth muscle bundles (green stain).

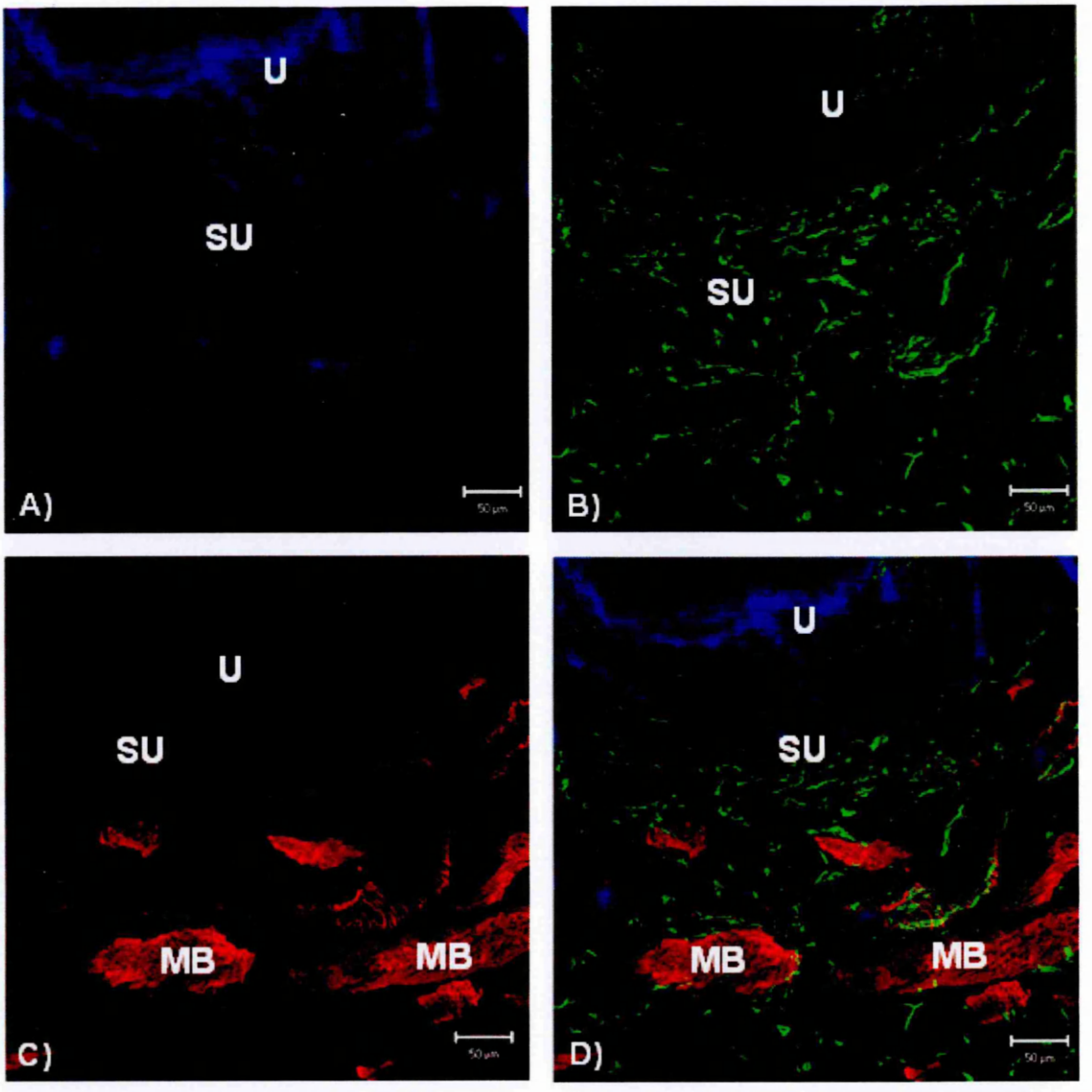


Figure 4.21- Immunofluorescent dual staining performed on 12 micron sections of pig bladder body; a) Dapi b) vimentin (green), c) α-smooth muscle actin (red) and d) combined overlaid stain image showing the urothelium (U), sub-urothelium (SU), muscle bundle (MB) and blood vessels (BV); magnification 20X. The vimentin stain is localised within the urothelium, suburothelium, in and around smooth muscle bundles (green stain).

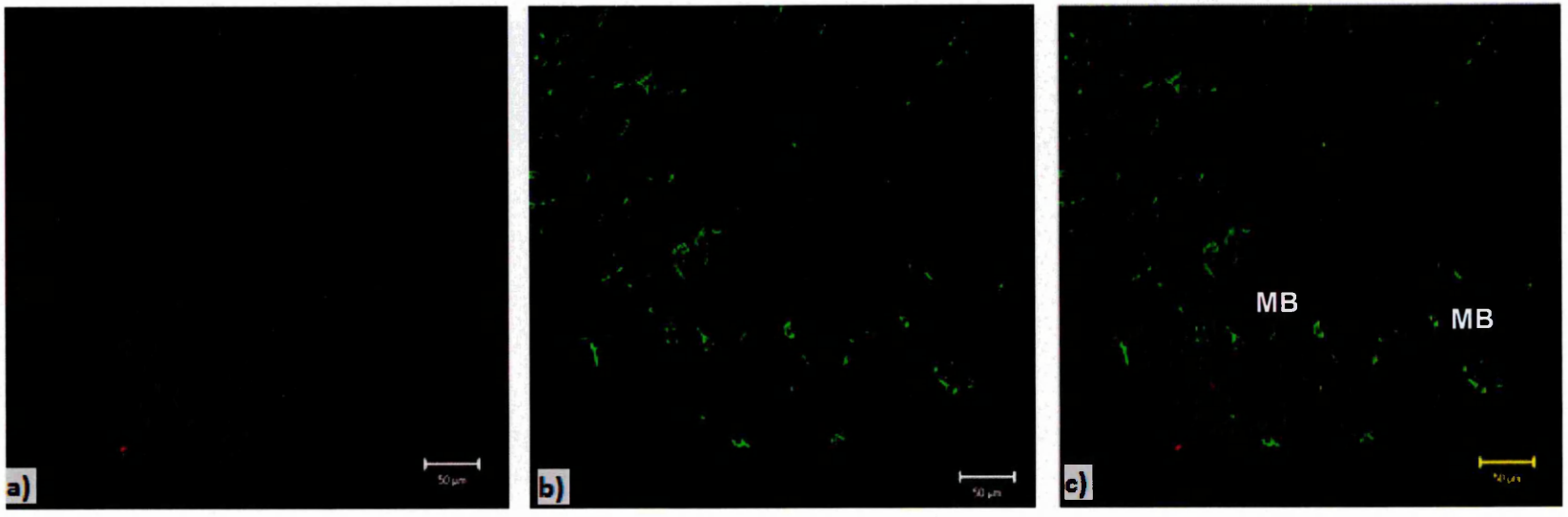


Figure 4.22 - Dual positive immunofluorescent staining performed on 12 micron thick pig bladder dome sections using a) c-Kit (red) and b) Vimentin (green) and c) combined dual stain image showing muscle bundles (MB), magnification 20X. c-Kit antibody produced only non-specific background staining.

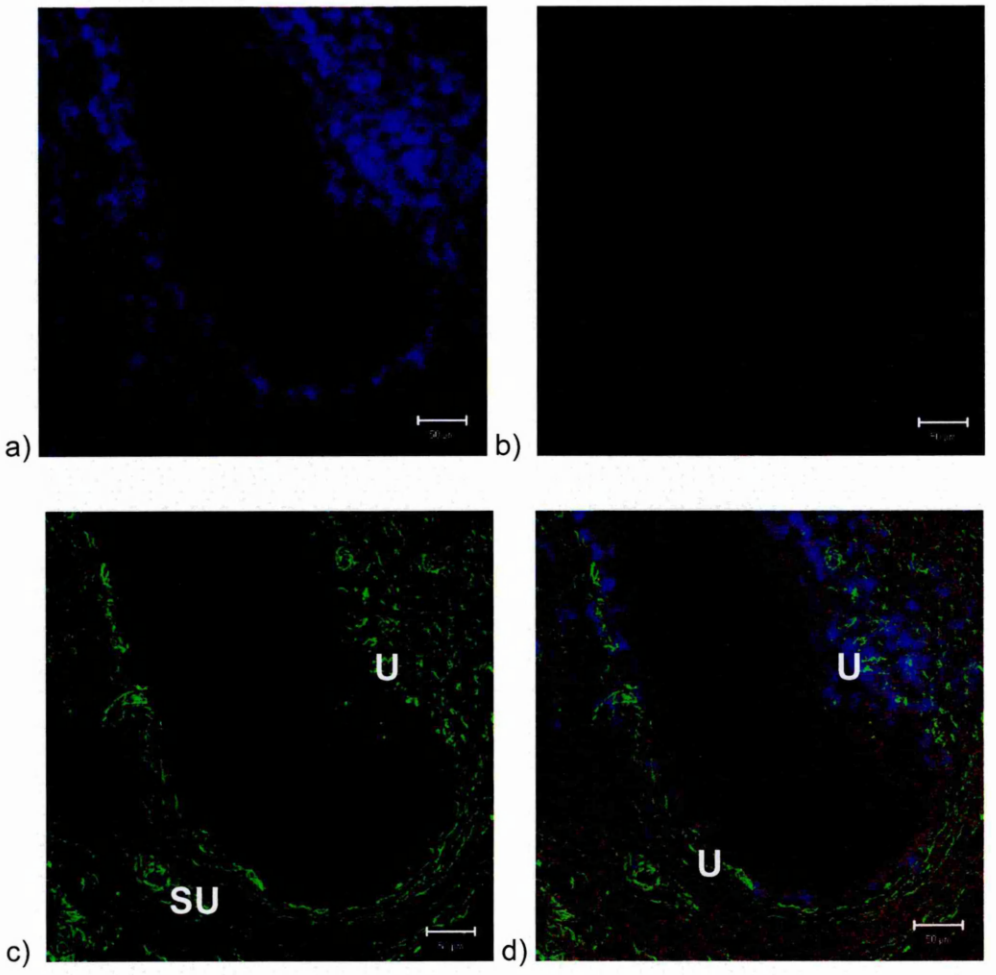


Figure 4.23 - Dual immunofluorescent staining performed on 12 micron thick pig bladder body sections using a) dapi (blue) b) c-Kit (red), c) Vimentin (green) and d) combined dual stain image showing urothelium (U), magnification 20X. c-Kit antibody produced only non-specific background staining.

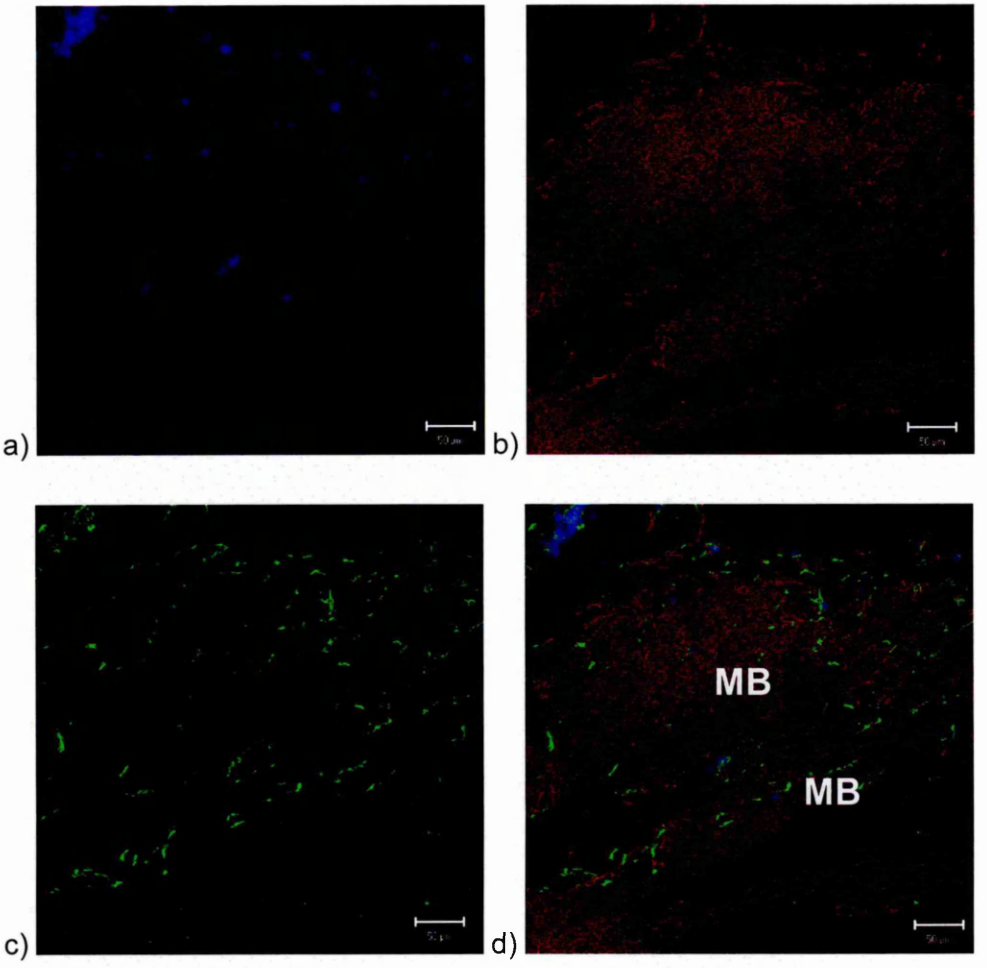


Figure 4.24- Dual immunofluorescent staining performed on 12 micron thick pig bladder trigone sections using a) dapi (blue) b) c-Kit (red), c) Vimentin (green) and d) combined dual stain image showing muscle bundle (MB), magnification 20X. c-Kit antibody produced only non-specific background staining.

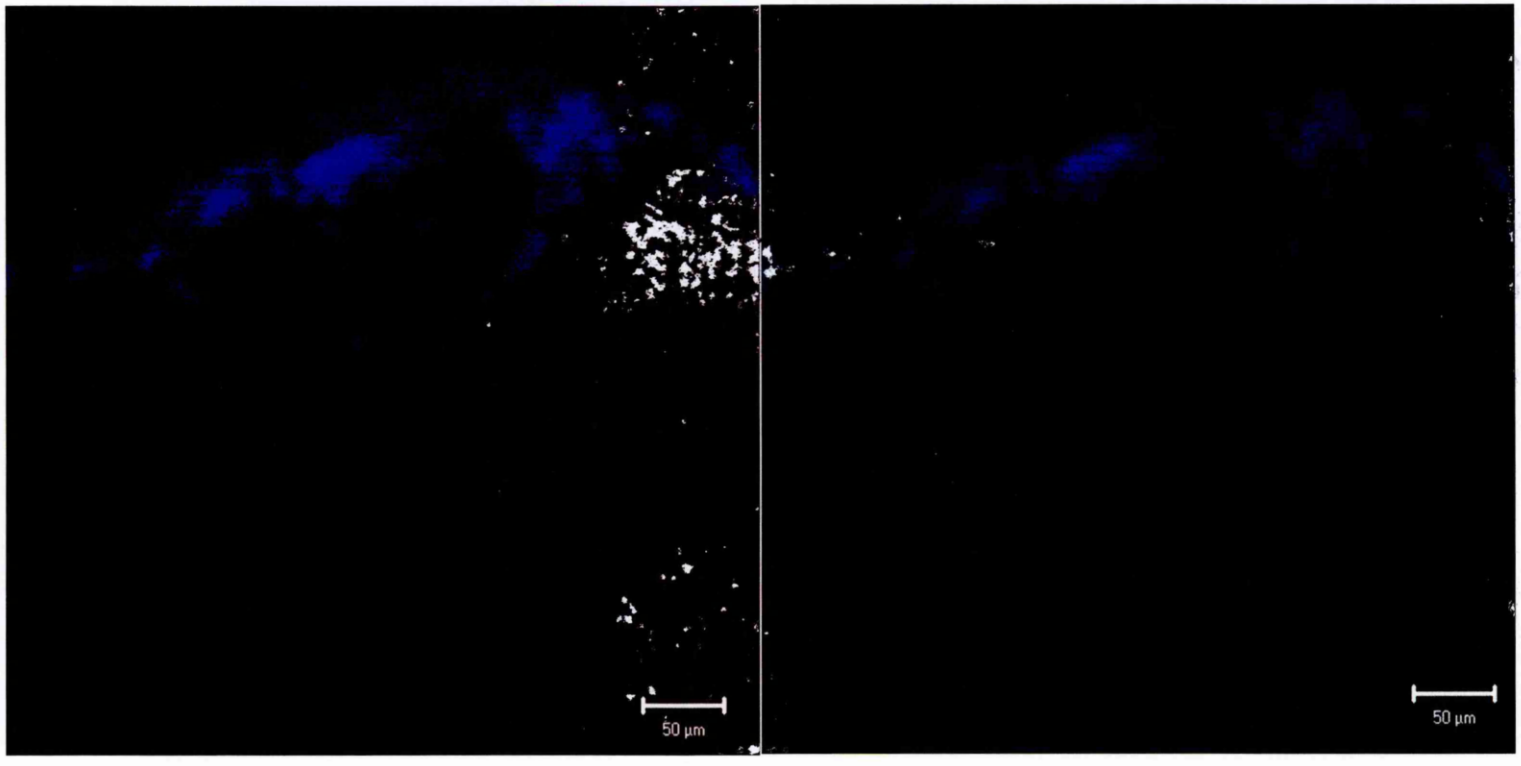


Figure 4.25 - Negative control staining performed on pig body tissue sections showing a) FITC b) TXRED.

4.6 Discussion

Interstitial cells of Cajal discovered by Ramon y Cajal are a group of specialized cells in the gastrointestinal tract that drive the peristaltic activity of the gut. They have been shown to play a role in signal transmission from nerves to smooth muscle in the GI tract (Sanders 1996; Horowitz *et al.,* 1999). Since then, research of non-GI ICCs in other body systems has become a focus of interest. ICCs in the urinary tract have been the most widely studied non-GI ICCs (McCloskey 2011). Smet *et al.* (1996) was the first to identify cells reminiscent of gut ICCs in the guinea pig and human bladder. These authors described cells with lateral processes or branches that stained positive for vimentin, a specific marker which is found in cells of mesenchymal origin including ICCs but not in SMCs leading to the speculation that the urinary bladder may contain ICC-like cells. This finding was further corroborated by Gillespie *et al.* (2004). ICCs have also been shown to display specific immunoreactivity for antibodies directed at c-Kit, a tyrosine kinase receptor (Hashitani 2006; Lang *et al.,* 2006).

The functional role of ICC in the bladder is not clear, however they may function as a sensory network, acting as intermediates between the urothelium and detrusor and afferent innervation (McCloskey 2010; McCloskey 2011). They can generate spontaneous action potentials (Metzger *et al.,* 2008) and may modulate phasic activity (Vahabi *et al.,* 2011c). In chapters 2 and 3, spontaneous phasic contractions were observed in isolated pig bladder strips from the different bladder regions and it was logical to investigate whether ICC play a role in these PCs in the pig bladder as seen in previous studies by Vahabi *et al.* (2011) in rat bladder and in human bladder (Johnston *et al.,* 2010). Although c-Kit positive cells have been found in the pig lower urinary tract (Metzger *et al.,* 2008), the precise location in the pig bladder has not been investigated.

4.6.1 Functional Investigation of the Effect of Imatinib in Pig Bladder Dome, Body and Trigone

To investigate the role of c-Kit positive cells in the PCs observed in isolated strips of pig bladder, imatinib, a specific inhibitor of the tyrosine kinase receptor KIT was used. Imatinib has been used previously as a pharmacological tool to investigate ICC and their role in mediating bladder phasic contractions. Imatinib has been shown to suppress contractile smooth muscle activity in bladders from several species including human and rat (Biers *et al.,* 2006; Vahabi *et al.,* 2011c), also in guinea pig stomach (Hashitani *et al.,* 2008), guinea pig bladders (Kubota *et al.,* 2004; Kubota *et al.,* 2006), human gall bladder (Lavoie *et al.,* 2007) and mouse small intestine (Shimojima *et al.,* 2005). In the present study, increasing cumulative concentrations of imatinib had no

effect on the amplitude of PCs in either intact or denuded strips from the pig bladder dome, body and trigone regions. This is in contrast to several published data that have shown an inhibitory effect of imatinib in the bladder. Biers *et al.* (2006) demonstrated that in human bladder from patients with OAB, imatinib had a greater effect in inhibiting detrusor contraction in comparison to strips from normal human bladders. Another study on guinea pig bladder also showed a dose dependent inhibitory effect of imatinib on the amplitude of spontaneous activity (Kubota *et al.,* 2004; Kubota *et al.,* 2006). Using rat bladders, Vahabi *et al.* (2011) showed that imatinib decreased the amplitude of CCh-induced PCs in both control and streptozotocin-induced diabetic bladder strips in a dose dependent manner although in the latter, the inhibitory effect was only evident at the higher imatinib concentrations (10 μM and 50 μM).

Imatinib also had no effect on the frequency of PCs in all three bladder regions in both intact and denuded tissues. These results are in line with Min *et al.* (2011) and Kubota *et al.* (2004, 2006) who showed that cumulative increasing concentrations of imatinib had no effect on the frequency of whole rat bladder preparations. In contrast, imatinib has also been shown to decrease the frequency of CCh-induced PCs in bladder strips of control and diabetic rats (Vahabi *et al.,* 2011c).

Effect of Imatinib on Carbachol Modulated Phasic Contractions in the Pig Bladder Dome, Body and Trigone

Various studies using animal models have shown increased PCs in bladders afflicted with different bladder dysfunctions including DO and OAB (Mills *et al.,* 2000; Drake *et al.,* 2006). In addition, immunohistochemical evidence has shown an increased number of ICCs in bladders of patients with OAB compared to normal bladders (Biers *et al.,* 2006). Using a guinea pig BOO model Kubota *et al.* (2008) also demonstrated that BOO bladder tissue exhibited a widely distributed population of ICCs in the sub-urothelial layer compared to normal guinea pig tissue (Kubota *et al.,* 2008).

In this study, carbachol (CCh), an analogue of acetylcholine (ACh) was used to modulate the phasic contractions that developed in the pig bladder tissue strips, to mimic the increased activity seen in OAB and then the effect of imatinib on these PCs was investigated. Comparison of amplitude of PCs between control strips and imatinib treated strips showed that imatinib had no effect on the amplitude in both intact and denuded tissue strips from all three bladder regions. This supports published data on rat bladders showing imatinib to have no effect on the amplitude of PCs when ACh was included in the experiments (Min *et al.,* 2011).

Imatinib also did not have any effect on the frequency of the CCh modulated PCs in intact and denuded tissue strips from the dome, body or trigone regions, again in line with Min *et al.* (2011) who showed that even in the presence of ACh, imatinib did not have any effect on the frequency of PCs (Min *et al.,* 2011).

The reason for the lack of effect of imatinib on PCs of the pig bladder in the present study is not clear. One reason could be species differences. Previous studies that have shown an effect of imatinib on PCs have been performed on rat (Vahabi *et al.,* 2011c), guinea pig (Kubota *et al.,* 2004; Kubota *et al.,* 2006) and human bladder (Biers *et al.,* 2006; Popescu *et al.,* 2006). Another possible explanation for the lack of effect of imatinib on PCs could be the age of the tissue. Unpublished data by Vahabi *et al.* has shown imatinib to have a significant effect in juvenile pig bladders (6 week old). The experiments presented in this chapter were performed on tissue from adult pigs (at least 6 months old) and thus it is possible to deduce that age may play a role. It is also possible that although ICC in the pig bladder might be involved in PCs, this particular class of cells may not necessarily express the c-Kit receptor and this could explain the lack of effect of imatinib on pig bladder PCs. Although c-Kit positive cells have been shown previously in the upper and lower urinary tract of the pig, the study did not precisely identify them in the bladder (Metzger *et al.,* 2008). Thus, this was a key aim of the present study, to identify c-Kit positive cells in the pig bladder.

4.6.2 Identification of c-Kit Positive Cells in the Pig Urinary Bladder by Immunohistochemistry

ICCs express c-Kit, a proto-oncogene that encodes for the tyrosine kinase receptor KIT. These cells also have immunoreactivity for vimentin, connexin-43 and cyclic guanine monophosphate (cGMP) (Smet *et al.,* 1996; Sui *et al.,* 2002; Wiseman *et al.,* 2003), although c-Kit is exclusively expressed by these cells and not in SMCs or fibroblasts. In the present study to investigate the presence of ICC in the pig urinary bladder, antibodies directed against c-Kit, vimentin and SMA were used. Histological studies of bladders from different species have shown ICC to be distributed in the sub-urothelium layer, between and along smooth muscle bundles and perivascularly (Johnston *et al.,* 2010). In the present study, the results show positive immunofluorescent staining for SMA and vimentin in tissues from all three of the pig bladder regions but not for c-Kit. Vimentin, a type III intermediate filament, is one of the major cytoskeletal components of cells. It is expressed in cells of mesenchymal origin, of which ICC are included (Johnston *et al.,* 2010). In this study, the results presented were also able to show that vimentin is located in between the muscle bundles and along the boundary of SMCs in the bladder wall with the highest concentration located within the suburothelium layer.

This distribution is similar to that described in human bladders (Sui *et al.,* 2002; Johnston *et al.,* 2010) and guinea pigs (McCloskey and Gurney 2002; Davidson and McCloskey 2005).

With regards to the identification of c-Kit, there was a high level of non-specific binding with the c-Kit antibody used in the present study. The antibody was raised against the human anti-c-Kit protein, although predicted to have cross species reactivity with pig tissue. This made it difficult to definitively identify c-kit positive cells in the pig bladder, as has been shown in previous studies (McCloskey and Gurney 2002; Biers *et al.,* 2006; Johnston *et al.,* 2010). The fact that the tissues did stain positively for vimentin filaments implies that there is a population of ICC, but without c-Kit specific staining it is not possible to make conclusions whether c-kit positive cells are present.

The lack of available antibodies specific for pig antigens was a major limitation in the present study. Both the c-kit antibodies used were reactive to human and mouse but with predicted reactivity to pig, and since there is substantial similarity between the pig and human bladder these particular antibodies were selected. The lack of a definite identification of c-Kit via immunohistochemistry may therefore not be an achievable aim until more specific porcine-directed antibodies become commercially available. To conclusively identify c-Kit positive ICC in the pig bladder, time and expense allowing, the use of non-fluorescent methods of detection and/or PCR could have been utilised.

4.7 SUMMARY/CONCLUSION

- Imatinib mesylate had no effect on the amplitude or frequency of PCs of both intact and denuded tissue strips from the dome, body or trigone regions of the pig bladder. This applied to both the inherent phasic contractions and CCh modulated phasic contractions and suggests that c-Kit positive cells are not involved in these PCs in the pig bladder.
- Although a population of ICC was identified in the pig bladder, using antibodies directed at vimentin, without specific c-Kit staining, c-kit positive cells could not be conclusively identified.

CHAPTER 5

INVESTIGATION OF ATP RELEASE FROM PIG URINARY BLADDER

5.1 INTRODUCTION

Adenosine triphosphate (ATP) is one of the most important biomolecules and has a vital role as the source of energy for all living cells. It is primarily composed of a nitrogenous base (adenine), a sugar backbone (ribose) and a chain of 3 ionised groups (phosphates) attached to the ribose (Bodin and Burnstock 2001). In addition to its function as an energy source, ATP has also been revealed to act as a neurotransmitter. Although acetylcholine (ACh) is the key excitatory transmitter in the bladder, neurotransmission due to ATP is also important. In the parasympathetic nerves innervating the bladder, ATP is co-released with ACh to mediate detrusor contraction (Burnstock 2009). The purinergic system constantly relays information on the state of the bladder to the CNS and initiates detrusor contraction, while the cholinergic system maintains the detrusor contraction to enable micturition (Abbracchio *et al.,* 2009). In addition, Ferguson *et al.* (1997) highlighted the important sensory role of ATP in the bladder when they showed ATP release from rabbit bladder urothelial cells in response to pressure changes/stretch. This ATP acts on purinergic receptors located in cells of the sub-urothelial plexus and subsequently modulates bladder afferent signalling. Release of ATP in the bladder has been shown in various species including guinea pig (Burnstock *et al.,* 1978), rabbit (Ferguson *et al.,* 1997), rat (Sadananda *et al.,* 2009), human (Kumar *et al.,* 2010) and pig bladder (Sadananda *et al.,* 2012).

5.2 PURINERGIC SIGNALLING IN THE BLADDER

There are two major sources of ATP required for signalling in the bladder - specific release of ATP from nerves, as evidenced by the presence of vesicles in both sympathetic and parasympathetic nerves (Burnstock *et al.,* 1978), and non-neuronal release in response to sheer stress, chemical stimuli and pressure changes (Pearson and Gordon 1979; Coutts *et al.,* 1981).

5.2.1 NEURONAL ATP

In most animal species, detrusor contraction is initiated by the parasympathetic nervous system via dual muscarinic-purinergic activation by ATP co-released with ACh (Burnstock *et al.,* 1978; Hoyle 1996). This is in contrast to healthy human bladders whose contractions are predominantly mediated by ACh which binds to detrusor muscarinic receptors thereby enabling contraction (Bayliss *et al.,* 1999; Yoshida *et al.,* 2001). ATP makes up part of the NANC (atropine-resistant) component of detrusor contraction and is thought to be especially important in pathological conditions.

5.2.2 NON-NEURONAL ATP

Bladder urothelial cells have been labelled "sensor-transducers" to describe their ability to release and respond to various neurotransmitters (Sun and Chai 2010). It is now well established that the urothelium can release ATP due to mechanical stimulation (Ferguson *et al.,* 1997), low frequency field stimulation, by cholinergic and purinergic agonists (Calvert *et al.,* 2001) and acid, but not tachykinins or capsaicin (Sadananda *et al.,* 2012).

Release of ATP has been shown in various species including guinea pig (Burnstock *et al.,* 1978), rabbit (Ferguson *et al.,* 1997), rat (Sadananda *et al.,* 2009), human (Kumar *et al.,* 2010) and pig bladder (Sadananda *et al.,* 2012). ATP may be released not only from urothelial cells but also to a lesser extent from myofibroblast/ICC and detrusor (Cheng *et al.,* 2011).

5.2.3 PURINERGIC RECEPTORS

Purinergic receptors belong to a family of transmembrane molecules thought to play a vital role in various physiological functions such as neuronal transmission, smooth muscle contraction and inflammation (Gever *et al.,* 2006). There are several members in this class of receptors. P1 purinergic receptors are G-protein coupled and are activated by adenosine (Burnstock and Kennedy 1985). P2 purinergic receptors are sub-divided into ionotropic (P2X) ligand gated ion channels and metabotropic (P2Y) G-protein coupled receptors (Fredholm *et al.,* 1994; Ralevic and Burnstock 1998). P2Y receptors are mainly activated by adenosine triphosphate (ATP), adenosine diphosphate (ADP), uridine diphosphate (UDP), uridine triphosphate (UTP) and uridine diphosphate glucose (UDP-glucose) nucleotides whereas P2X receptors are activated mainly by ATP. To date, seven P2X receptor subtypes have been identified and they all share a similar structural topology consisting of two transmembrane molecules with an extended extracellular loop where ATP binds and an intracellular carboxyl and amino termini of various lengths as shown in figure 5.1 (Brake *et al.,* 1994; Ennion *et al.,* 2000; Jiang *et al.,* 2000). The functional receptor is composed of two or three of these subtypes creating a transmembrane pore.

Various experimental techniques including molecular, functional and X-ray crystallography studies have been employed to elucidate the composition of functional P2X receptors and the current consensus is that P2X channels exist as homomeric or heteromeric units. Of the seven subtypes of P2X channels, there are four types that have a role in urinary bladder function - homomeric $P2X_1$, homomeric $P2X_2$, homomeric $P2X_3$ and heteromeric $P2X_{2/3}$ (Gever *et al.,* 2006).

5.2.3.1 P2X channels in the bladder

Homomeric $P2X_1$ channels have been shown to be densely distributed within the detrusor of the urinary bladder (Valera *et al.,* 1995; Burnstock and Knight 2004). The role of $P2X_1$ in smooth muscle contractility emerged from experiments that showed ATP as the main neurotransmitter involved in non-adrenergic non-cholinergic (NANC) atropine resistant detrusor contractions in the guinea pig bladder (Burnstock 1972). It is now well established that these channels mediate the purinergic component of nerve mediated smooth muscle contraction in sympathetically and parasympathetically controlled systems (Hoyle *et al.,* 1989; Valera *et al.,* 1995) by co-releasing ATP with ACh to initiate contraction

$P2X_2$ channels located on sensory and autonomic neurons of the peripheral nervous system suggest a role in afferent and efferent signalling pathways (Cockayne *et al.,* 2005). In the bladder they have possible pacemaker activity and a role in smooth muscle contraction as they are expressed on ICC and smooth muscle cells (Burton *et al.,* 2000; Burnstock and Lavin 2002). Homomeric $P2X_3$ and heteromeric $P2X_{2/3}$ channels are expressed on unmyelinated C-fibres and myelinated Aδ sensory afferents. $P2X_2$/$P2X_3$ deficient mice studies have confirmed that these channels play a role in mediating the primary sensory effects of ATP (Burnstock 1999; Burnstock 2001; Burnstock 2003) are found in epithelial tissues such as the urothelium, which has led to the concept that they may modulate mechanosensory and chemosensory responses in the bladder since ATP released from the urothelium can activate these channels on adjacent primary sensory afferents (Burnstock 2003; Burnstock 2009).

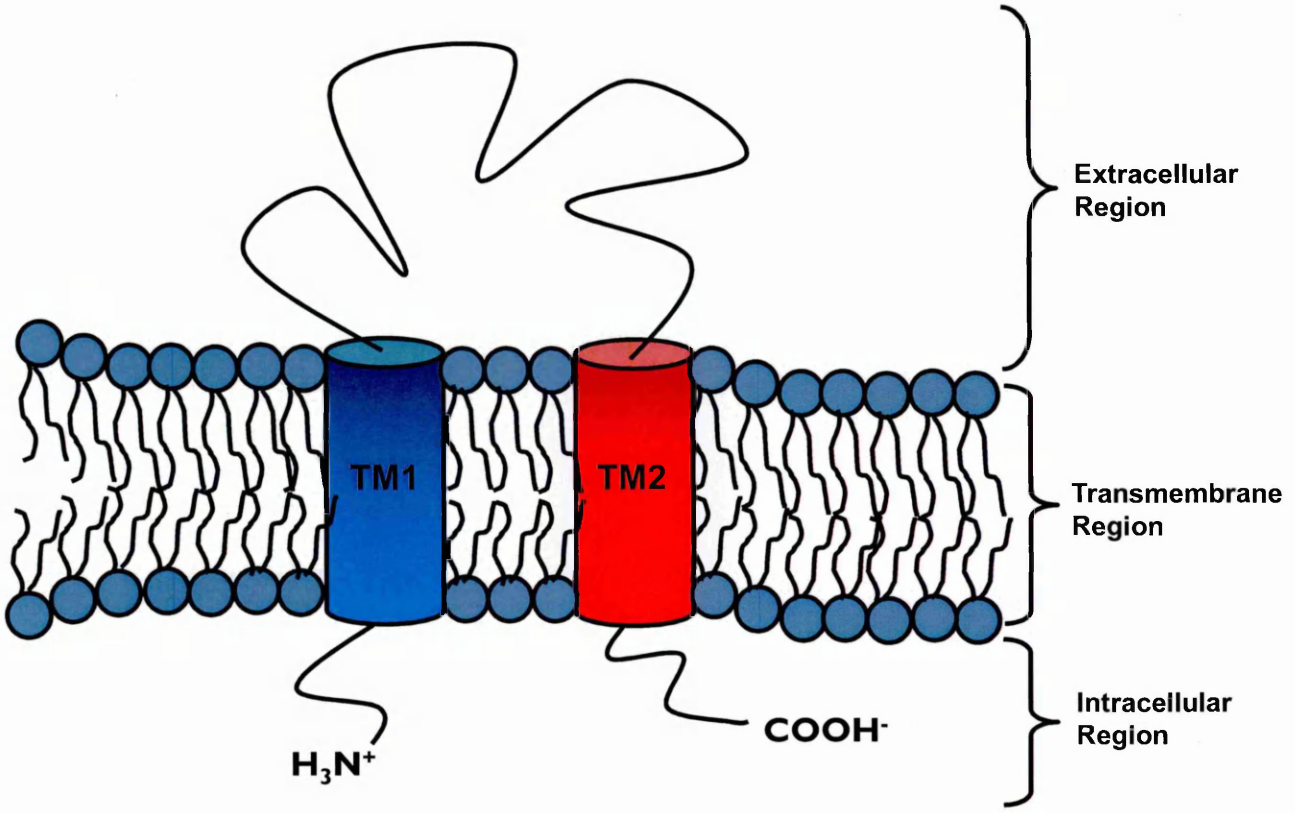


Figure 5.1 - Structure of a single P2X channel subunit showing the extracellular domain where ATP binds, transmembrane region showing TM1 & TM2 and the intracellular domain with carboxy and amino termini. TM1 is involved with channel gating while TM2 lines the ion pore. The functional receptor is composed of 2 or 3 of these subunits. Adapted from (Burnstock 2007)

Receptor Subtype	Localization	Function	Agonist	Antagonist
$P2X_1$	Within smooth muscle lining hollow organs including bladder, arteries and intestines	Mediates the purinergic component of sympathetic and parasympathetic nerve mediated smooth muscle contraction	ATP α,β-MeATP B_zATP	Suramin PPADS
$P2X_2$	Peripheral and central nervous system, non-neuronal cell types such as SM, ICC's and lymphocytes	Afferent and efferent signalling pathways ATP mediated fast synaptic transmission at both nerve terminals and interneuronal synapses	ATP ATP-γ-S 2-MeSATP	PPADS TNP-ATP Reactive blue-2
$P2X_3$ $P2X_{2/3}$	Small to medium C-fibers and Aδ sensory neurons Bladder urothelium, airway epithelial and pulmonary neuroepithelial bodies	Nociceptive transmission and mechanosensory transduction within visceral hollow organs Sensory neurotransmission	B_zATP α,β-MeATP ATP 2-MeSATP	TNP-ATP Suramin PPADS

Table 5.1 - Characteristics of ionotropic (P2X) receptors. Abbreviations - adenosine triphosphate (ATP), alpha,beta-methylene ATP(α,β-MeATP), 3'-O-(4-benzoyl)benzoyl ATP (BzATP), pyridoxal-5'-phosphate-6-azo-phenyl-2,4-disulfonate (PPADS), 2',3'-O-(2,4,6-trinitrophenyl) ATP (TNP-ATP), 2-(methylthio)ATP (2-MeSATP), adenosine 5'-O-(3-thiotriphosphate) (ATP-γ-S).

5.2.4 ROLE OF ATP & PURINERGIC SIGNALLING IN PATHOLOGY OF THE BLADDER

Purinergic signalling has been implicated in a number of pathological conditions such as bladder outlet obstruction (OBB), spinal cord injury (SCI), interstitial cystitis/painful bladder syndrome (IC/PBS), diabetes and also in ageing (Sun and Chai 2010). This is due to the important role of purinoceptor signalling functions in both sensory transduction and maintenance of the normal bladder. The relationship of cholinergic and purinergic modulation in pathological conditions of the bladder is inverse, in that the former is diminished while the latter is enhanced. The mechanisms underlying increased urothelial release of ATP are not fully understood but it seems to be due to intrinsic changes in various urothelial signalling pathways.

Various animal models have been utilized to investigate the release of ATP and its role in disease states of the bladder. IC/PBS is a chronic symptom condition characterised by urinary urgency, frequency, nocturia and bladder pain (Sun and Chai 2010). Up-regulation of purinergic signalling in the bladder urothelium has been suggested as the underlying aetiology for IC/PBS symptoms. Studies of bladder outlet obstruction (BOO) and overactive bladder (OAB) using a rabbit model showed that in response to electric field stimulation (EFS), there was an increased purinergic component in rabbits with early stage BOO and this was predominant in comparison to the cholinergic component (Calvert *et al.,* 2001).

Birder *et al.* (2003), using a feline interstitial cystitis (FIC), model investigated the mechanisms involved in ATP release evoked by applying a hypotonic stimulus to urothelial cells from normal versus FIC cells. The results showed enhanced ATP release in FIC cells compared to the normal cells. Experiments performed on rats with bladder inflammation due to cyclophosphamide-induced bladder injury also exhibited similar increased ATP release (Dang *et al.,* 2008). Urothelial cells from patients with benign prostatic hyperplasia (BPH) have been shown to release significantly more ATP than urothelial cells from control patients (Sun *et al.,* 2002). Kumar *et al.* (2004) compared ATP release in bladders from pig and normal human tissue. This study found increased ATP release due to mechanical and electrical stimulation in both species. The mean ATP release from the pig bladder was comparable to ATP measured in the human samples, and in both, the main source of ATP was the urothelium and not the detrusor (Kumar *et al.,* 2004). The ATP released was not sensitive to tetrodotoxin suggesting its source is non-neuronal. Subsequent studies investigating ATP release from the urothelium of patients with idiopathic and neurogenic detrusor overactivity similarly showed that mechanical stretch and electric

field stimulation resulted in increased ATP release in both NDO and IDO. The increased ATP release was more significant in IDO than in NDO tissue strips (Kumar *et al.,* 2010)

The heteromeric $P2X_3/P2X_2$ receptor is classed as a nociceptive receptor and thus increased expression in the bladder could mediate IC/PBS symptoms. The increased ATP levels due to cyclophosphamide-induced bladder injury were attributed to alterations in purinergic signalling in bladder afferent pathways whereby there was increased P2X receptor expression in pelvic and lumbar neural pathways contributing to bladder hypersensitivity associated with the inflammation (Dang *et al.,* 2008). Another study by Kim *et al.* also investigated purinergic signalling in the urothelium from rat bladders with BOO and concluded that there was increased $P2X_3$ expression in the urothelium of BOO group in comparison to the control (Kim *et al.,* 2008). With regards to ageing, structural and functional changes inevitable with an increase in age are accountable for the high prevalence of voiding dysfunction in older people. Studies performed on detrusor strips from three different age groups - group 1 <50 years, group 2 51-70 years and group 3 >70 years - indicated a positive association between age and purinergic neurotransmission and a negative correlation between age and cholinergic neurotransmission (Yoshida *et al.,* 2001). With increasing age, there is a decreased cholinergic component in neurogenic contraction, whereas the reverse is true for the purinergic component (Yoshida *et al.,* 2001).

All of the above studies highlight that changes in purinergic signalling may be important in bladder dysfunction and therefore there is a need for further understanding of purinergic signalling in the bladder, which may open new avenues for novel pharmacological therapies to treat LUTS. It is with this in mind that the experiments in this chapter were performed using pig bladders to investigate ATP release in the dome, body and trigone in response to stretch.

5.3 CHAPTER AIMS

The aims of this chapter were:-

- To measure ATP released in response to stretch in isolated strips of pig bladder dome, body and trigone
- To compare the amount of ATP released by tissue strips from the different bladder regions.

5.4 MATERIALS AND METHODS

5.4.1 *ATP MEASUREMENT AND DETERMINATION*

A bioluminescent assay kit for ATP measurement can be used to determine levels of ATP in various samples ranging from 2 x 10^{-12} to 8 x 10^{-5}moles/L. The reaction involves consumption of ATP resulting in emission of light due to the oxidation of D-luciferin by the firefly luciferase enzyme as shown below.

$$ATP + Luciferin \xrightarrow[Mg^{++}]{\text{Firefly Luciferase}} Adenyl - luciferin + PP_i \quad (1)$$

$$Adenyl - luciferin + O_2 \rightarrow Oxyluciferin + AMP + CO_2 + \text{light} \quad (2)$$

The amount of light emitted is proportional to the amount of ATP present in the sample (Strehler 1974; Leach 1981). The ATP assay kit used in the present study was obtained from Sigma Aldrich (U.K.). Each vial of ATP lyophilized assay mix (containing luciferase, luciferin, $MgSO_4$, DTT, EDTA, BSA and tricine buffer salts) was dissolved in 5ml of sterile water and allowed to stand on ice for 1 hour to ensure complete dissolution. The assay was performed by adding 0.1ml of the ATP assay mix to each well of a 96-well plate and this was allowed to stand for approximately 3 minutes to ensure any endogenous ATP was hydrolysed. The samples were then rapidly added to the assay mix in the wells, swirled briskly and the amount of luminescence produced was measured using a luminometer (Victor^{TM} X4 2030, Perkin Elmer, U.K).

For each assay performed a standard curve was prepared, against which the amount of ATP in the samples was determined. An ATP standard stock solution of 1x10^{-6} moles/ml was prepared using sterile H_2O. Using serial dilution, ATP standards ranging from 1x10^{-7} - 1x10^{-14}moles/ml and 3x10^{-7} to 3x10^{-14}moles/ml were prepared in order to create a calibration curve as shown in Figure 3.1. The amount of ATP in the samples was determined using the equation obtained from the standard curve as shown. Samples were measured in triplicate.

5.4.2 OPTIMISATION OF PARAMETERS FOR ATP DETERMINATION

In order to ascertain the optimal settings for recording luminescence and to optimize the sensitivity of the ATP assay, a number of parameters were investigated. These included the effect of microplate type, height of the sensor, the size of aperture and the stability of the reaction.

5.4.2.1 Effect of plate type

Several different microfluor 96-well plates are available for use in luminometry based on their reflective properties. For plate type optimisation, three different plate types - clear, black and white (Thermo Scientific, U.K.) were investigated to determine the most suitable plate type for the luminometer used. Effects of height, aperture and reaction stability in the three plates were measured.

5.4.2.2 Effect of height

The luminometer height controls the distance between the sample and the instrument sensor. If the sensor is too close to the sample, it will not be able obtain accurate readings of dispersed light and conversely if it is too far from the sample it may inaccurately incorporate overspill light from neighbouring wells. It is therefore imperative to adjust the sensor height to a suitable distance that would maximize luminescent light detection and minimize light contamination from neighbouring wells. The effect of sensor height on luminescence readings was determined using ATP standards and three different distances between the sensor and sample, being 3mm, 5mm and 7mm. The samples were measured for 1 second using 4mm aperture width.

5.4.2.3 Effect of aperture

Sensor aperture determines the amount of light reaching the sample by varying the sensor diameter. The effect of the aperture size on luminescence readings was investigated by varying the sensor diameter - 1mm (small), 4mm (normal) and 5mm (large) - with measurements taken for 1 second at 7mm luminometer height setting.

5.4.2.4 Stability of light emission

Finally, determination of the stability of the ATP-luciferin-luciferase reaction was performed on all three plate types using the optimised parameter settings for luminometer height and aperture size. Readings of the ATP standards were taken at 0, 20, 50 and 90 minutes.

5.4.3 Optimisation of sample collection

To determine the optimum method for collecting samples from bladder strips for ATP determination, a number of sample collection methods were trialled.

5.4.3.1 Microdialysis method for collection of samples

Microdialysis is a technique that allows for sampling from within an extracellular space of the tissue of interest. It was mainly developed for brain research but in recent years has been adapted to be utilised in various tissues including bladder for measurement of

ACh and ATP release (Inadome *et al.,* 1998; Yoshida *et al.,* 2004; Yoshida *et al.,* 2006). This technique involves inserting a probe into a region of interest within the tissue and slowly perfusing buffer or medium through the probe. Analytes cross the probe membrane from the extracellular space with the perfusate and are collected over time for analysis (Figure 5.2).

The 0.22mm x 10mm dialysis probe used in the present study (OP-100-10, Eicom Corp., Dublin U.K.) has a regenerated cellulose membrane that is sustained by a 0.04mm platinum wire. The probe was inserted through intact pig bladder strips into the sub-urothelial space using a microscope with the probe membrane maintained within the tissue. The inlet cannula of the probe was connected to a microdialysis syringe pump (ESP-64, Eicom, Dublin U.K.). Krebs' buffer was continuously perfused at a rate of 4µl/min. The tissue strips were suspended in a 10ml tissue bath as described in Chapter 2 and shown in Figure 5.2. Tissues were allowed to equilibrate for 60 minutes with the Krebs' buffer replaced every 15 minutes. Each strip was then stretched to 50% of its length and the microdialysis pump started to pump the Krebs' buffer through the tissue. The dialysate was collected on ice and frozen at -20°C prior to measurement of ATP.

5.4.3.2 Determination of optimal sample collection for measurement of basal ATP release and stretch-evoked ATP release

Isolated strips of bladder were mounted in tissue baths (as described in chapter 2 section 2.3.4) and allowed to equilibrate under a load of 1g for 60 minutes with tissue bath content replaced with fresh Krebs' buffer every 15 minutes. After the equilibration period, the contents of the tissue bath were collected every 60 seconds for a period of 10 minutes to determine whether basal ATP changed over time. As described above, tissue was allowed 15 minutes and another wash performed. The tissue was then stretched to 50% or 100% of its length and left for 60 second before the tissue bath content was collected to measure ATP release due to stretch.

5.4.3.2 Tissue bath collection of samples

These experiments utilized the tissue bath set up described in chapter 2 section 2.3.4. Pig bladder tissue taken from the different bladder regions was dissected into strips of approximately 1cm length, with the mucosa intact. The strips were mounted in 5ml tissue baths as described in chapter 2 section 2.3.4. The strips were allowed to equilibrate for 60 minutes under a load of 1g with tissue bath content replaced every 15 minutes. After the final wash of the equilibration period, the tissue was left for 30 seconds and the organ bath content was collected for basal ATP measurement.

Tissue was then allowed 15 minutes and another wash performed. The tissue was subsequently stretched to 50% of its length, left for 60 seconds and the tissue bath content collected. 1ml of the tissue bath content was immediately frozen at -20°C awaiting ATP measurement.

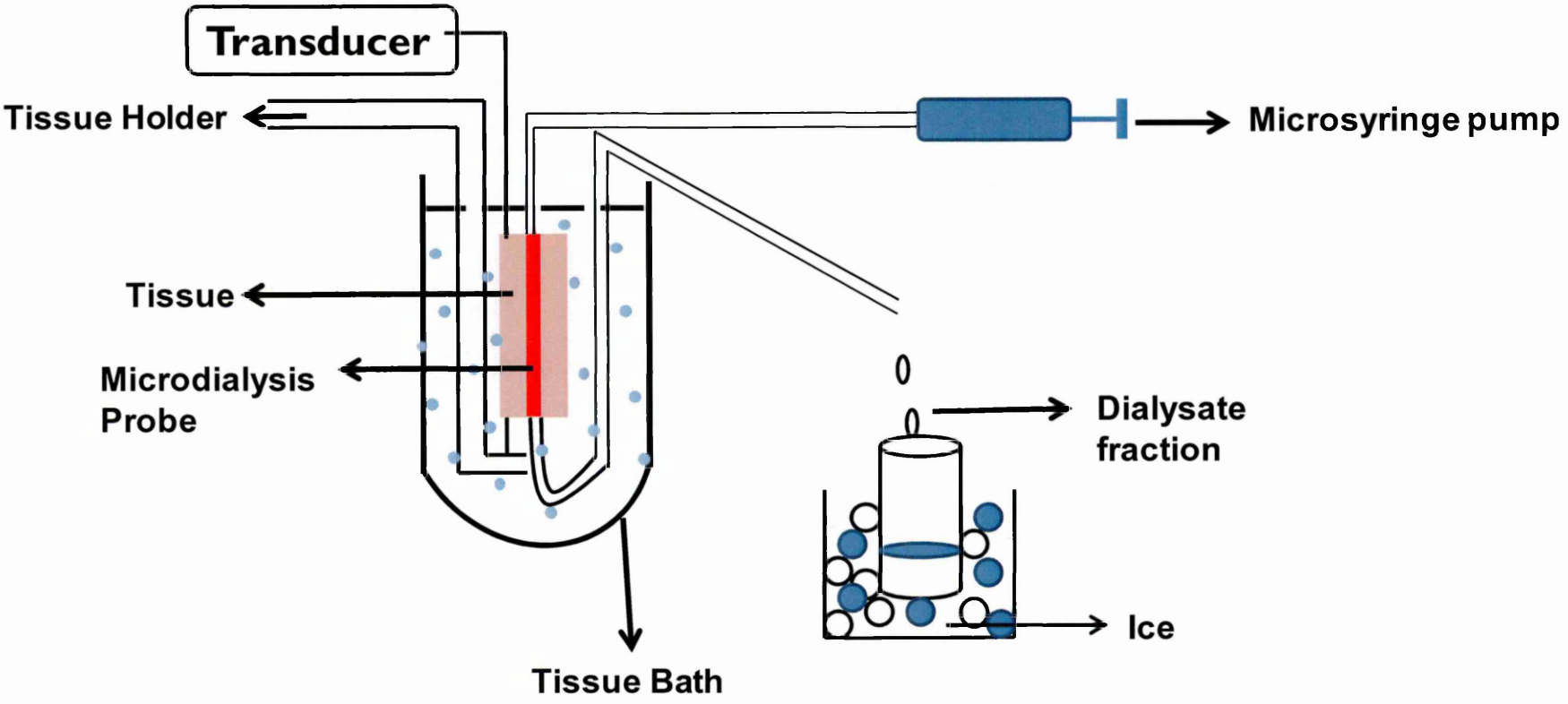


Figure 5.2- Experimental setup for dialysate collection for ATP measurement. The microsyringe pump perfused the tissue at a rate of 4µl/min and dialysate collected for 25 minutes.

5.5 Data and Statistical Analysis

The light luminescence measured as counts per second (CPS) from the analysed samples was converted into log luminescence values for both the ATP standards and the samples. An ATP standard curve was prepared with each assay of samples. ATP concentration in the sample was determined from the standard curve. Data was normalised for tissue weight and expressed at Mols/mg of tissue. All data are presented as mean ± SEM.

5.6 RESULTS

5.6.1 ATP STANDARD CURVE

An example of the ATP standard curve used for the calculation of the amount of ATP in collected samples is shown in figure 5.3.

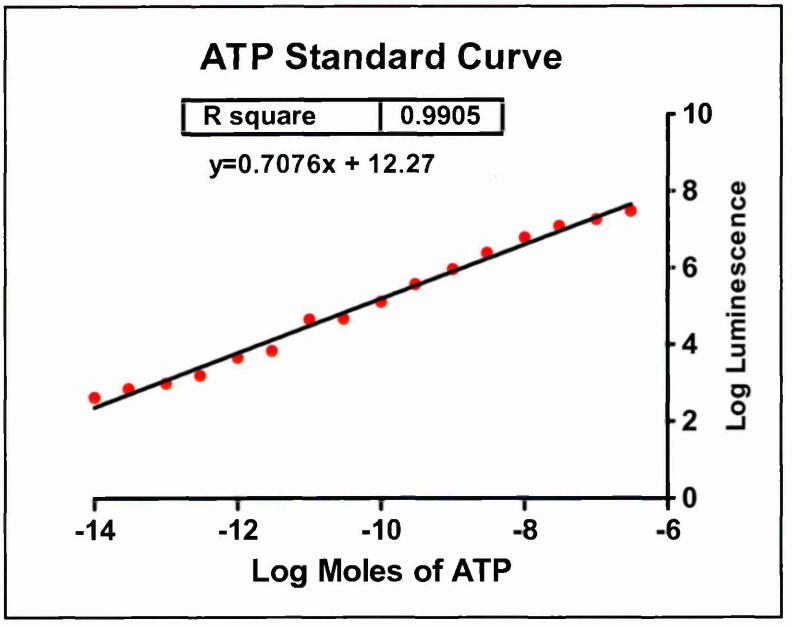


Figure 5.3 - Representative standard curve used to determine the amount of ATP in experimental samples. ATP standards were used and a linear curve fit performed to produce the equation y=0.7076x + 12.27 in this case, which is rearranged to determine the value of x which corresponds to the amount of ATP in the sample.

5.6.2 Optimisation of parameters

5.6.2.1 Effect of plate type

Figure 5.4 shows the results obtained for ATP standard curves measured on the 3 different plate types. The black plate (Figure 5.4a) provided the best linear relationship and sensitivity over the range of ATP standards in comparison to the white and clear plates (Figure 5.4b & c). The white plates produced a higher luminescence reading but this seemed to plateau resulting in the loss of sensitivity and linearity. This was also the case for the clear plates.

5.6.2.1 Height optimisation

Comparison of sensor height across the 3 plate types did not reveal any great differences within each plate type. The graphs shown on Figure 5.5 reveal that at 3, 5 and 7mm sensor height, no significant differences were observed. However, comparing the plate types against each other, the black plates maintained a better linear response (Figure 5.5a) over the range of standards in comparison to the white (Figure 5.5b) and clear plates (Figure 5.5c).

5.6.2.3 Aperture optimisation

Analysis of the effect of aperture size on luminescence readings across the 3 types of plates revealed that the 1mm aperture resulted in lower luminescence in comparison to the 4 and 5 mm aperture sizes as shown in Figure 5.6. The white and clear plates (Figure 5.6b & c) showed a trend whereby a linear response was observed with decreasing concentration of ATP standards and this plateaued off, whereas for the black plates (Figure 5.6a), the linear response was maintained over a larger range of ATP standards.

5.6.2.4 Stability of reaction

All 3 plate types showed a gradual decrease in luminescence measured with increasing time interval between the assay and the plate being read. This is shown in Figure 5.7. The white plates showed the highest luminescence reading (Figure 5.7b) in comparison to the black (Figure 5.7c) and clear plates (Figure 5.7b). Linearity of luminescence in the black plates was maintained over a larger range than in the clear and white plates.

Each optimisation parameter experiment was performed 3 times to ensure the consistent results for each plate type. These optimisation experiments resulted in the

decision to use black plates measured at 7mm height using normal (4mm) aperture size for 1 second for determining ATP amounts in collected samples.

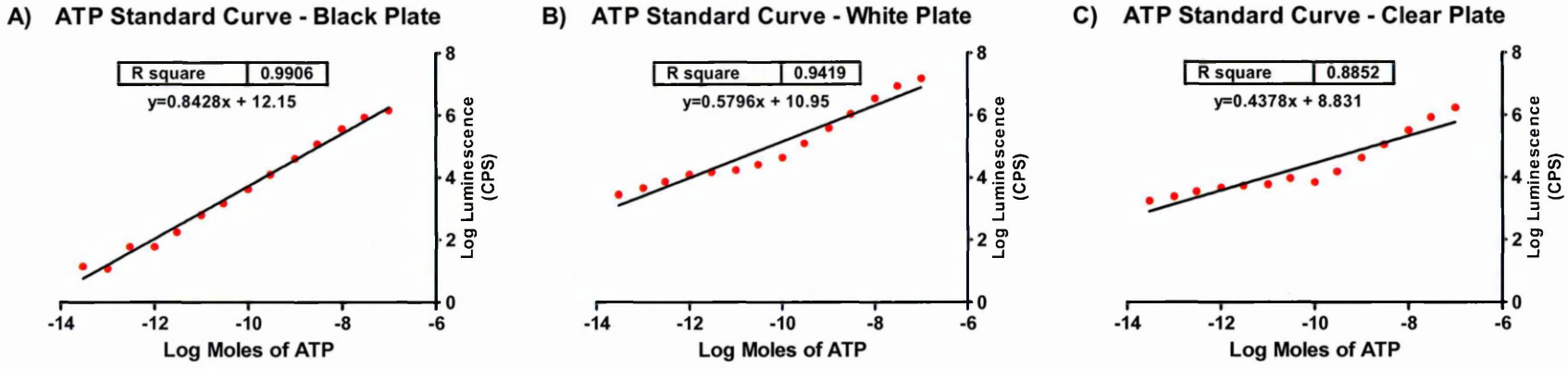


Figure 5.4 – Effect of microplate type on ATP standard curves measured on black (A), white (B) and clear (C) plates; n=3.

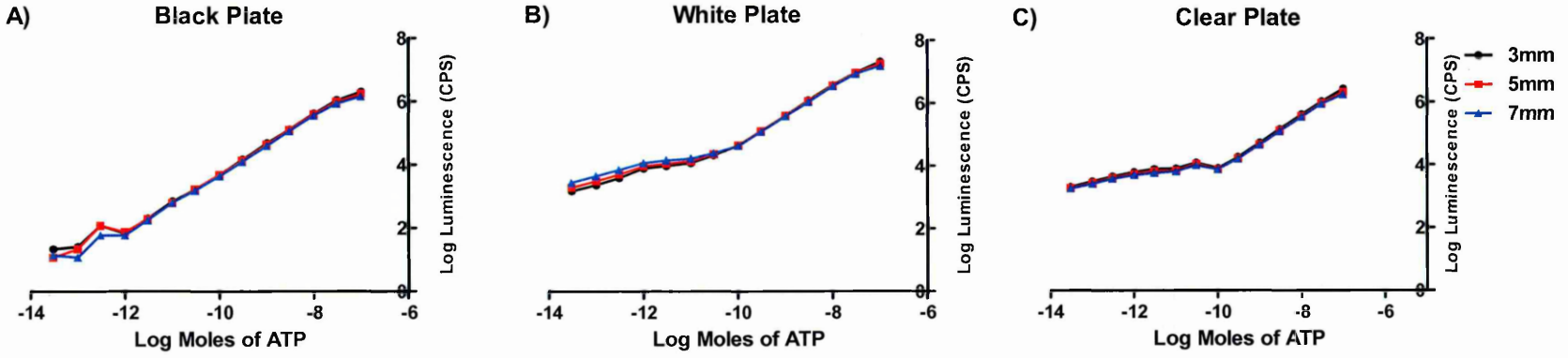


Figure 5.5 - Effect of microplate type and sensor height on ATP - standard curves measured on black (A), white (B), and clear (C) plates with sensor heights of 3, 5 and 7mm; n=3.

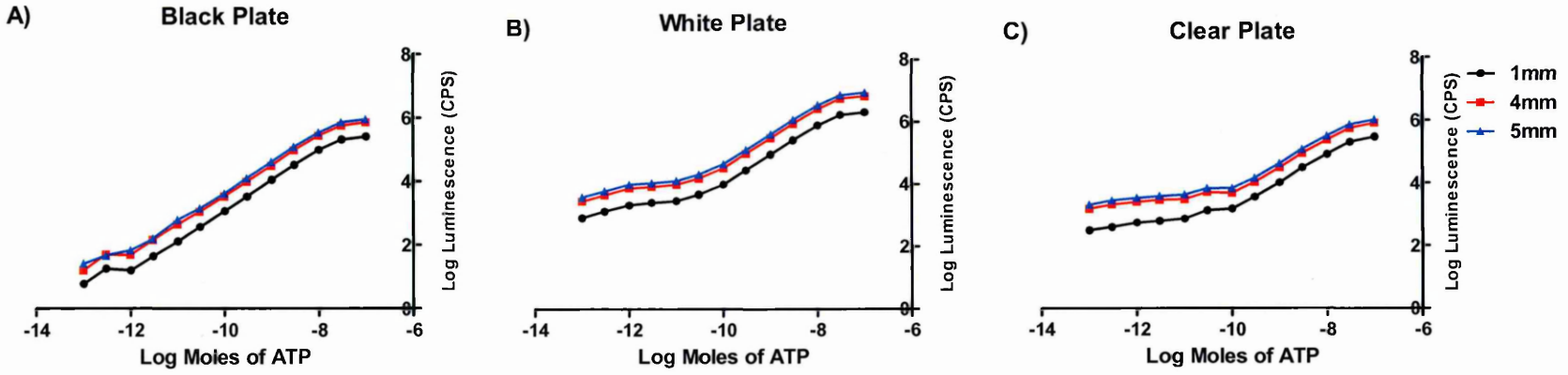
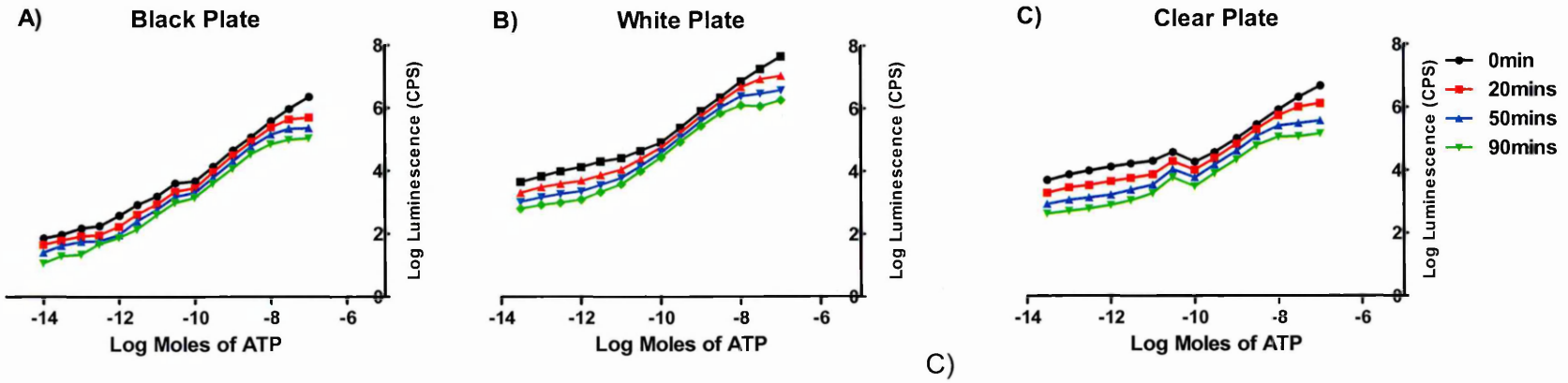


Figure 5.6- Effect of microplate type and aperture size on ATP standard curves measured on black (A), white (B) and clear (C) plates with sensor aperture sizes of small (1mm), normal (4mm) and large (5mm); n=3.



C)

Figure 5.7- Effect of microplate type and time ATP standard curves measured on black (A), white (B) and clear (C) plates at 0, 20, 50, and 90 minutes following assay; n=3.

5.6.3 Optimisation of sample collection for basal ATP release

Sample collection using the microdialysis method was not very successful. The amount of sample collected, the time it took to collect the sample and the low levels of ATP measured led to the investigation of alternative sample collection method. Collection of whole tissue bath content was tried and this method provided consistent results hence, subsequent experimental sample collection was performed this way.

Following 60 minutes equilibration of bladder strips, basal ATP release was determined over an 8 minute period with samples collected every 60 seconds. In strips of both dome and body (Figure 5.8) from the pig bladder, performing these multiple washes reduced basal ATP levels but the amount of basal ATP released was not significantly reduced over 8 minutes ($p = 0.2998$ and $p = 0.6767$ for dome and body respectively). In light of these results, sample collection for basal ATP was performed immediately after equilibration following 3 consecutive washes of the tissue.

5.6.4 Effect of Stretch on ATP Released

5.6.4.1 ATP Release evoked by 50% stretch

Isolated strips of pig bladder dome (n=8) stretched to 50% of the tissue length showed no significant increase in ATP levels following stretch (Figure 5.9A). Strips from the bladder body (n=8) showed increased levels of ATP release upon stretch but this increase was not statistically significant (Figure 5.10A). The same results presented as scatter plots showing the median and range of data for ATP release from the dome and body (Figure 5.9B and Figure 5.10B) mirrored the previous results.

5.6.4.2 ATP Release evoked by 100% stretch

Intact strips of dome (n=12) stretched to 100% of the tissue length showed increased ATP release vs. basal release, which was statistically significant (**$p<0.01$), as shown in Figure 5.11A, and this is further supported by the scatter plot (Figure 5.11B) which shows increased levels of ATP release at 100% stretch compared to basal levels. Intact strips of body (n=12) showed increased levels of ATP release following 100% stretch (Figure 5.12A & B) when expressed as mean data, though not significantly so.

Upon expressing stretch-evoked ATP release data as % increase relative to basal ATP release (taken as 100%), analysis revealed that when the tissue was stretched by 50% of its length, ATP release was significantly greater than. basal ATP release in the body ($p<0.05$) but not in the dome region – Figure 5.13A. On the other hand, the % increase of ATP release following 100% stretch was significantly greater than basal ATP release

in both the dome and the body regions ($p<0.01$ and $p<0.05$ respectively) as shown in Figure 5.13B. Figure 5.14A & B compare stretch-evoked % increase in ATP release in the dome and body regions respectively. The increase in ATP release was not statistically significant vs. basal ATP release in both regions when the tissue was stretched by 50% and 100%.

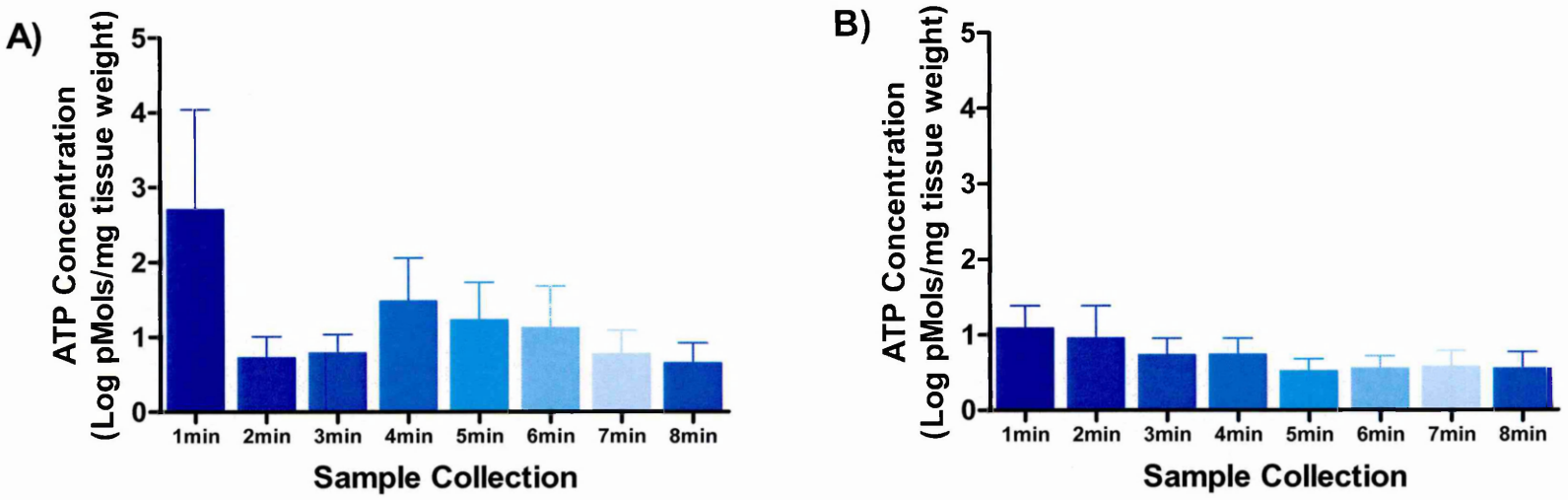


Figure 5.8– Optimisation of sample collection for measurement of basal ATP in A) dome and B) body. ATP concentration is expressed as pMols/mg of tissue weight; Data are presented as mean (n=5) ± SEM.

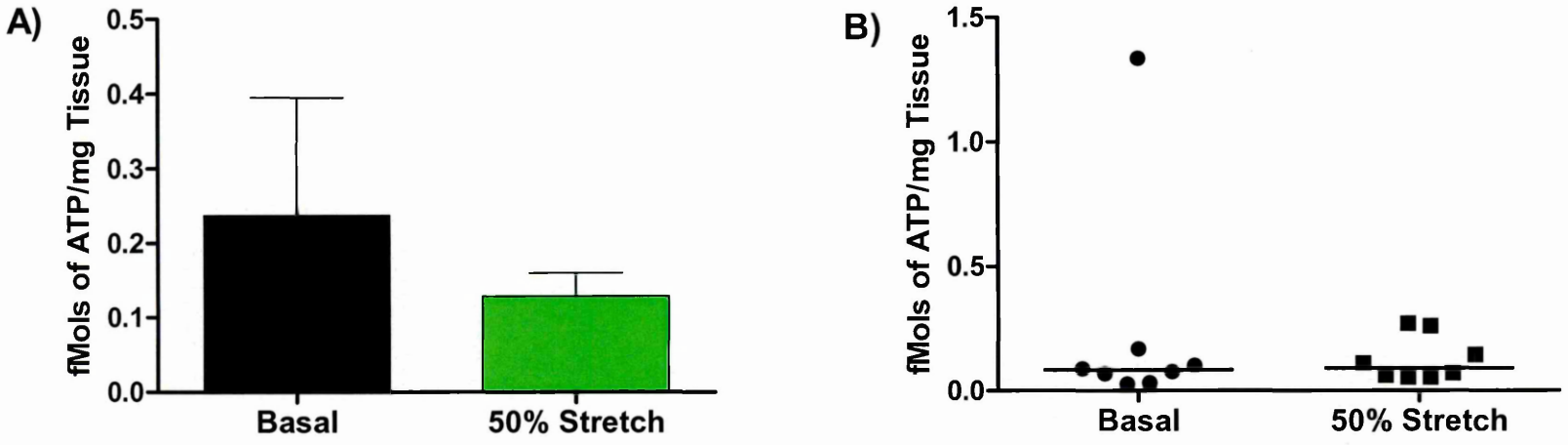


Figure 5.9- Stretch-evoked (50% of original tissue strip length) ATP release in the dome region. A) Mean ± SEM ATP released for basal vs. stretch. ATP concentration is expressed as fMols/mg of tissue weight. B) Scatter plot showing the range of ATP amounts measured and the median within the dome region; n=8.

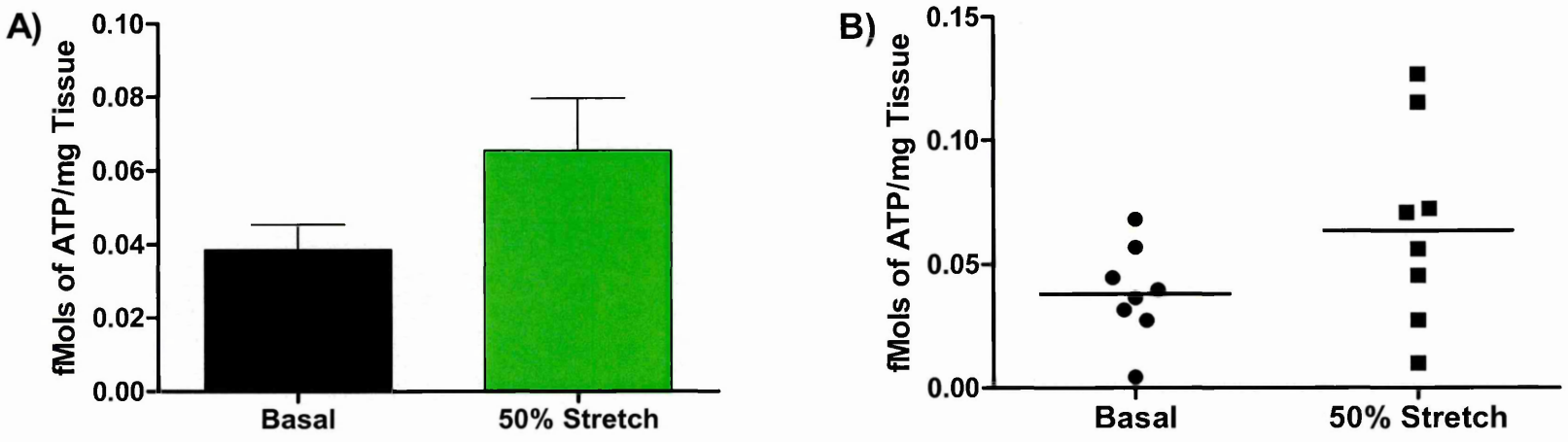


Figure 5.10 - Stretch-evoked (50% of original tissue strip length) ATP release in the body region. A) Mean ± SEM ATP release basal vs. stretch. ATP concentration is expressed as fMols/mg of tissue weight. B) Scatter plot showing the range of ATP amounts measured and the median within the body region; n=8.

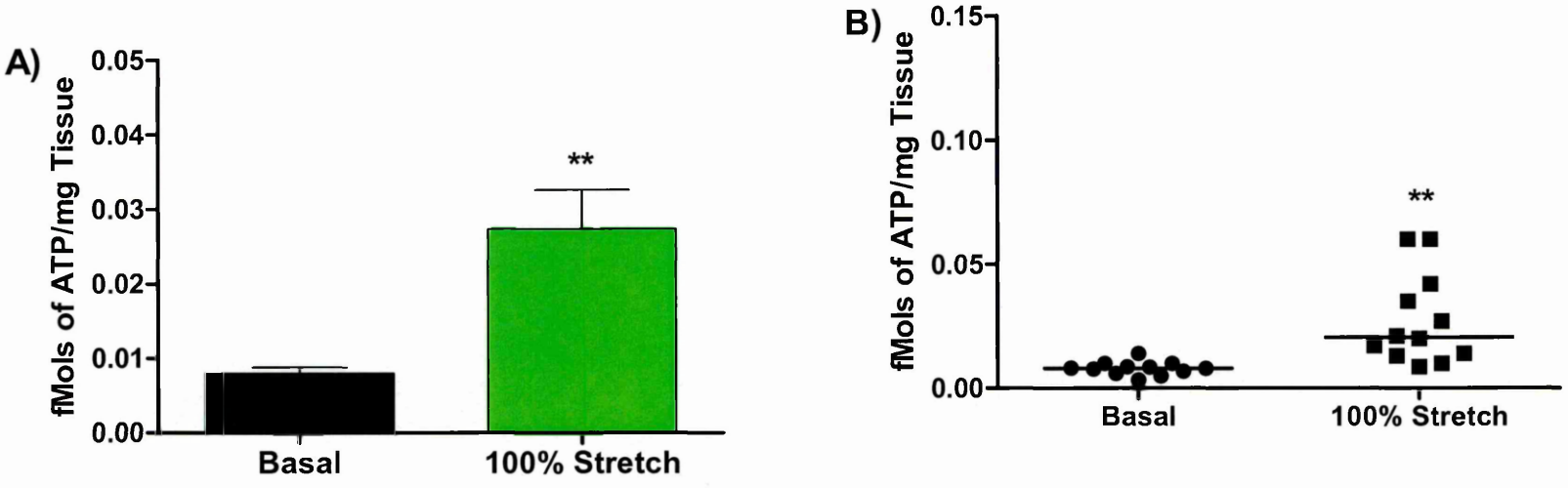


Figure 5.11- Stretch-evoked (100% of original tissue strip length) ATP release in the dome region. A) Mean ± SEM ATP release basal vs. stretch. ATP concentration is expressed as fMols/mg of tissue weight. B) Scatter plot showing the range of ATP amounts measured and the median within the dome region; n=12, **p<0.01 vs. basal.

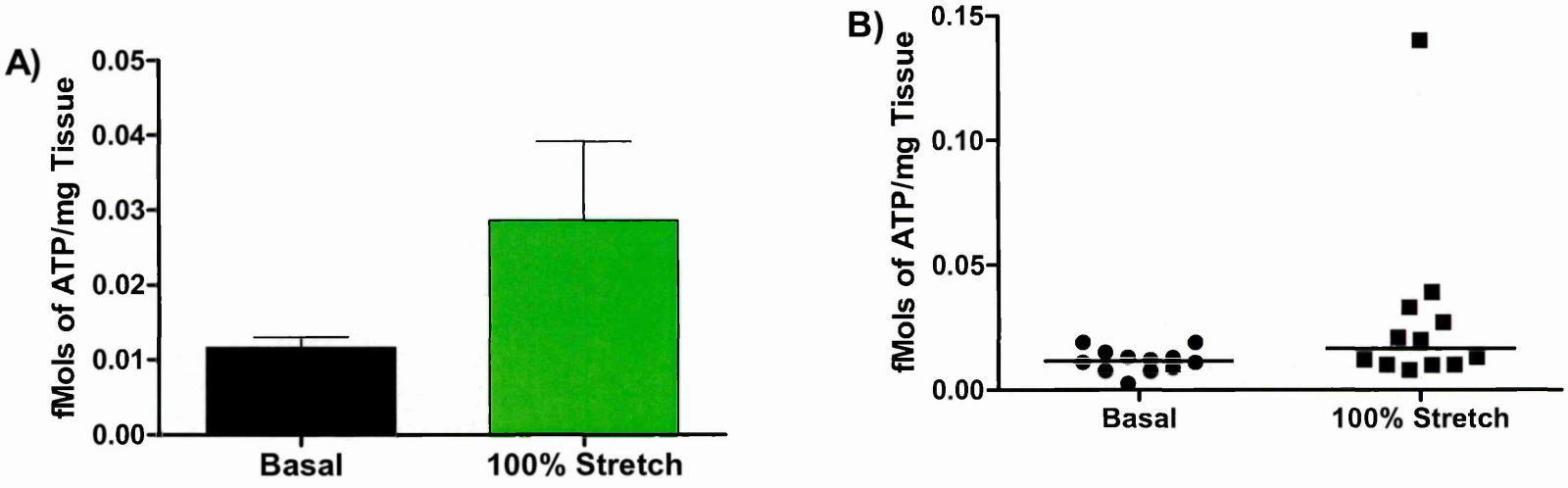


Figure 5.12- Stretch-evoked (100% of original tissue strip length) ATP release in the body region. A) Mean ± SEM ATP release basal vs. stretch. ATP concentration is expressed as fMols/mg of tissue weight. B) Scatter plot showing the range of ATP amounts rneasured and the median within the body region; n=12.

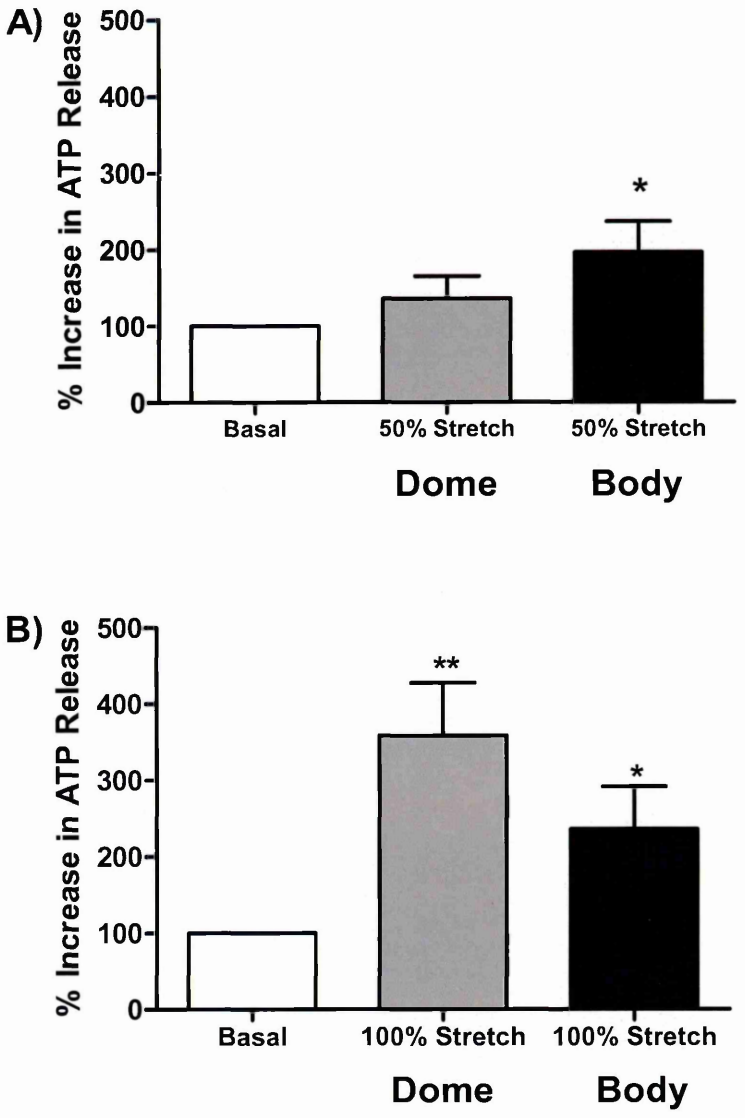


Figure 5.13 - Stretch-evoked ATP release shown as a % increase vs. basal ATP levels. A) 50% stretch (n=8) and B) 100% stretch (n=12) in the dome and body regions. Data are presented as mean ± SEM, *p<0.05, **p<0.01 vs. basal ATP.

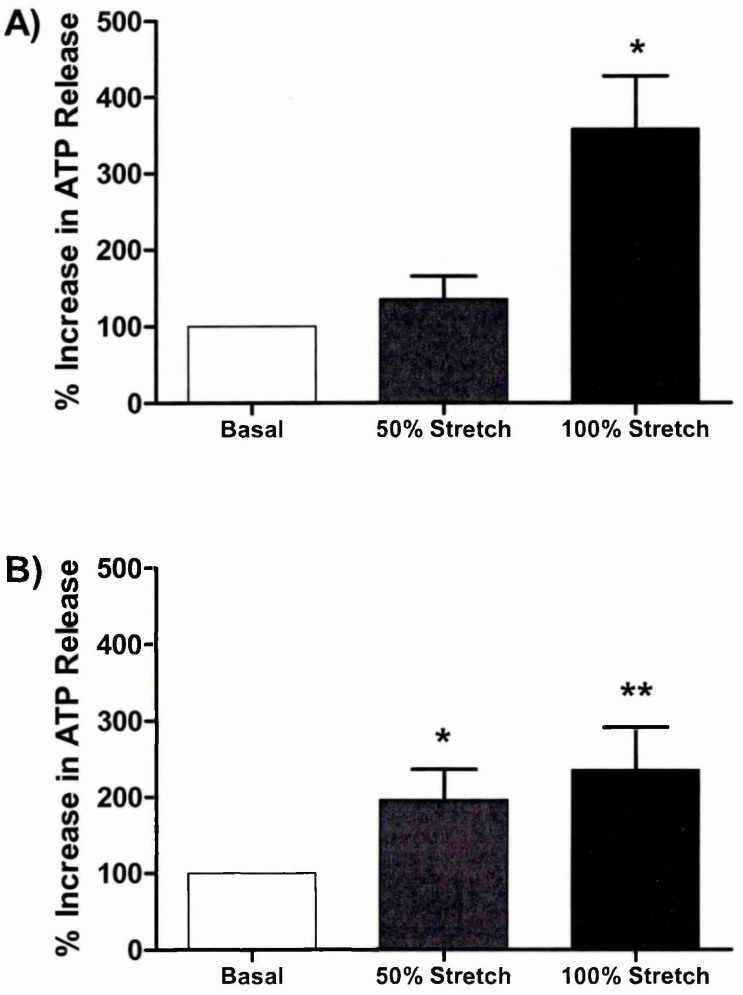


Figure 5.14 - Stretch-evoked ATP release - comparison of 50% (n=8) vs. 100% (n=12) stretch shown as a % increase against basal ATP levels in A) dome and B) body regions. Data are presented as mean ± SEM.

5.7 Discussion

ATP release is thought to play an important role in bladder signalling by acting on sub-urothelial afferent nerves and the activation of purinergic receptors located in the bladder wall (Ferguson *et al.,* 1997). ATP is released in response to various stimuli in the bladder. In this chapter, the effect of stretch of bladder tissues on ATP release was studies in the different bladder regions. The results confirm that stretch is an effective stimulus for ATP release in pig bladder tissue especially when tissues were stretched to 100% of original tissue length.

5.7.1 Optimisation of sample collection

Sample collection using the microdialysis method was not ideal for various reasons. The amount of time it took to collect a sample sufficient enough for the measurement of ATP was too long. It took 25 minutes to collect 100µl of sample perfusate at a rate of 4µl/min. ATP extraction efficiency is dependent on the flow rate. At a high flow rate, ATP diffusing from sampling site into dialysate per unit time is smaller than at a lower rate (Lonnroth *et al.,* 1987), hence the slower flow rate that was used for optimisation experiments. Measurement of the perfusate showed variable low levels of ATP in the samples, and this was not consistent. This could be due to the process of implantation of the probe as it has been reported this can alter the morphology resulting in disturbed microcirculation, rate of metabolism and the integrity of physiological barriers (Ungerstedt 2009). It is also possible that the ATP was rapidly broken down by enzymes and not representative of what was being released due to the stretch.

Initial preliminary experiments showed elevated levels of basal ATP in comparison to ATP release in response to stretch, but this was not consistent across all tissues. It was therefore important to determine the optimum method to obtain samples for measurement of basal ATP. In order to do this, the contents of the organ bath were analysed for ATP content at 60 second intervals following the 60 minute equilibration period - in an attempt to reduce any ATP that had accumulated over the equilibration period. However, in strips of both dome and body, there were no significant differences in the amount of basal ATP measured in the samples over the different time points. It was therefore decided to perform just a single basal ATP measurement immediately following the equilibration period.

5.7.2 Optimisation of ATP assay

Determination of the optimum parameters for the measurement of ATP release using the ATP bioluminescence assay was the first vital step in performing the ATP

experiments. Three different plates - black, white and clear - were tested. In addition to this, height, aperture size and stability of the ATP reaction required optimisation. The distance between the plate reader sensor and the sample describes the sensor height and the diameter of the sensor refers to the aperture. The black plates absorb light and therefore decrease the amount of background light from the reaction. The white plates on the other hand reflect the light and produce higher background readings, whereas the clear plates disburse the light and thus may not give accurate readings of luminescence in each well. The combination of the sensor aperture size and its height is therefore important in giving an accurate reading. It is known that light travels in a straight line and if the aperture is too small and too close, the readings obtained may not be accurate as some of the reflected light may not be registered by the sensor. On the other hand, if the sensor is too high with a small aperture, then overspill of light over the top of the plate is not captured and hence inaccurate luminescence readings. The balance of the right size aperture and height is therefore crucial in the measurement of accurate ATP measurement from the samples. With regards to the height, the optimum was found to be 5-7mm with the black plates giving the best linearity for the standard curve. The ideal aperture size was found to be 4 or 5mm diameter and again the black plate gave higher sensitivity and stability of the reaction in comparison to the clear or white plates.

Assessing the stability of the assay reaction was important as it informs us on how quickly the luciferase reaction occurs and the decline in light intensity over time. The optimisation experiments showed the reaction to be quite stable, with a gradual decline in luminescence over time in all 3 plate types. The highest luminescence readings were those taken at time zero - immediately upon addition of the sample - and this declined over the 90 minute time frame.

From these optimisation experiments, the optimal settings for the assay measurement were the use of black plates measured at a sensor height of 7mm, with an aperture size of 4mm, measured at time zero immediately upon addition of the sample into the assay mix.

5.7.3 ATP RELEASE IN PIG BLADDER STRIPS

It is well known that ATP plays an important role in bladder function. It is co-released with ACh from parasympathetic nerves and is essential for the initiation of the normal micturition reflex (Cockayne *et al.,* 2000). It has been shown to be released non-neuronally and act on $P2X_3$ receptors subsequently increasing afferent activity (Burnstock 2007) and eliciting sensations of bladder fullness via low threshold nerves

and pain via high threshold nerves (Ferguson *et al.,* 1997). However, our understanding of the role of non-neuronally released ATP is still not complete, and it is possible that it acts on the underlying ICC and detrusor muscle, and may have a role in the modulation of PCs in the bladder, especially in pathological conditions where there is increased expression of ICC and ATP release (Birder *et al.,* 2003; Birder *et al.,* 2004; Kumar *et al.,* 2010).

Previous studies on the release of ATP have been performed on cells in culture (Ferguson *et al.,* 1997; Birder *et al.,* 2003), animal models (Birder *et al.,* 2004; Lu *et al.,* 2007), whole bladder strips and mucosal strips (Sadananda *et al.,* 2009). Birder *et al.* used urothelial cells cultured from cat bladders to demonstrate release of ATP in response to hypotonic stimulation and this response was enhanced in cells taken from animals with feline interstitial cystitis. Urothelially released ATP, due to changes in hydrostatic pressure, has been shown to act on afferent nerves in the sub-urothelium to initiate afferent signalling mechanisms in the rabbit bladder (Ferguson *et al.,* 1997). Recently, Kumar *et al.* demonstrated increased ATP release from human bladder strips taken from patients with idiopathic detrusor overactivity (IDO) and neurogenic detrusor overactivity (NDO). In that study, stretch was used as a stimulus for ATP release (Kumar *et al.,* 2010). Previously, O'Reilly *et al.* (2002) had shown increased expression of $P2X_2$ receptors in patients with IDO and this highlighted the importance of urothelial purinergic signalling function in pathological bladder conditions (O'reilly *et al.,* 2002).

Limited studies have investigated ATP release from the pig bladder, in spite of it being shown to be a good model for human bladder (Hashitani and Brading 2003; Templeman *et al.,* 2003; Kumar *et al.,* 2004; Akino *et al.,* 2008; Sadananda *et al.,* 2008; Hernandez *et al.,* 2009). Of those that have, all have shown increased ATP release due to various stimuli. Kumar *et al.* (2004) showed ATP release due to mechanical and electrical stimuli in the pig and human bladder to be relatively similar. Using cell cultures of the different cell types present in the bladder - urothelial, myofibroblast and detrusor muscle cells - Cheng *et al.* (2011) showed consistent increase in ATP release in the respective cell cultures in response to exposure to hypotonic Krebs' buffer solution. Mucosal strip studies by Sadananda *et al.* (2009) also showed increased ATP release in response to 50% stretch of the tissue and these strips contained a mixed population of cells. In the present study, intact pig bladder strips were used to investigate stretch-evoked ATP release. These intact strips contained all the bladder cell types and thus it was not possible to attribute ATP release to a specific cell type, as can be performed on isolated cells, and as previously performed by Cheng *et al.* (2011).

However, given the evidence available it is highly likely that a significant amount of ATP was released from the urothelium.

The exact mechanism by which ATP is released from the urothelium is not entirely clear and several hypotheses have been postulated to explain this. Electron microscopy ultra-structural studies were first to provide preliminary evidence of vesicle involvement in the release of neurotransmitter release (Bodin and Burnstock 2001). Analysis of pure populations of synaptic vesicles via sub-cellular fractionation techniques conclusively identified neurotransmitter localization in these vesicles (Dowdall *et al.,* 1974) and it now widely accepted that exocytosis is a possible release mechanism of neurotransmitters (Bodin and Burnstock 2001). The trigger for the fusion of these vesicles and release of their contents is mainly the presence of Ca^{2+}. The arrival of an action potential causes membrane depolarization producing increased permeability of Ca^{2+} which causes an increase in the influx of Ca^{2+} via voltage gated channels and this subsequently triggers vesicle exocytosis (Bodin and Burnstock 2001). The ATP from these vesicles can ultimately stimulate the purinergic receptors in the urothelium, sub-urothelium and possibly some unmyelinated C-fibres and these in turn activate the afferent arm of the micturition reflex (Kumar *et al.,* 2010). Increased ATP release may therefore contribute to the detrusor overactivity in both IDO and NDO.

In this chapter, ATP release due to stretch was measured in the dome and body regions only. Protocol optimisation for these experiments took significantly longer than anticipated and preliminary results showed variability between the tissues from the different regions, hence the decision to just optimise experiments on the dome and the body regions. The amount of basal ATP released in the dome and body regions showed great variability (note differences in scales). Cheng *et al.* (2011), using cultured urothelial, myofibroblast and detrusor cells, and Sadananda *et al.* (2009), using rat bladder mucosal strips also both showed variability of ATP release due to stretch. The amounts of ATP release presented in the aforementioned studies were significantly higher in comparison to that measured in the present study using pig bladder. This may be attributed to the fact that the size of organ/tissue baths used was very different – 0.2ml for the rat bladder mucosal strips vs. 5ml for the pig bladder strips. It is possible that the volume of the tissue bath greatly diluted the amount of ATP, hence the lower measurements of fMols of ATP compared to nMols in the smaller baths. In addition, in the present study the data was obtained using whole bladder thickness strips from dome and body regions, and this may account for the differences observed in the amounts of ATP levels measured - i.e. ATP produced in the whole tissue strips may not adequately diffuse into the Krebs' buffer and may remain in the

tissue, therefore it may be that there is not an accurate representative measure of ATP release in the samples. In addition, although all of the tissue used was obtained fresh from the abattoir and handled in a consistent manner, it is possible that the variability of the data in the present study may be due to the inherent variation in the different animals sacrificed. These animals were not kept in controlled environments, where routine and uniformity in the care and handling can be kept constant.

The increase in ATP release in pig bladder strips from the body and dome regions was stretch dependent i.e. there were increased ATP levels with an increase in stretch, although this was not significant versus basal ATP release. This data is similar to several published works that show significant increased levels of ATP in response to stretch (Ferguson *et al.,* 1997; Birder *et al.,* 2003; Birder *et al.,* 2004; Smith *et al.,* 2005; Sadananda *et al.,* 2009; Kumar *et al.,* 2010; Sadananda *et al.,* 2012). Even though no significance was noted in the levels of ATP release, it is possible that due to the varied nature of the tissue, an increase in the number of experiments performed may reveal a significant difference. The variability may be due to various experimental factors, as mentioned above, and may also include rapid ATP breakdown by endogenous ectonucleotidase enzymes. Experiments by Ferguson *et al.* (1997) using isolated rabbit bladder strips to investigate the effect of calcium on EFS-evoked ATP release showed that absence of calcium significantly potentiated the release of ATP in comparison to data collected in the presence of calcium. This is supported by a study that established that there is increased breakdown of ATP by ectonucleotidases in the presence of calcium (Seguchi *et al.,* 1982) in rabbit transitional bladder epithelium. The Krebs' buffer used for the experiments contains physiological levels of Ca^{2+} and therefore could possibly be a reason for the variability observed in the data presented in this chapter. ATP breakdown could have been prevented by freezing the collected samples immediately in liquid nitrogen prior to measurement.

Another possible explanation for the differences observed in the present study in comparison to published data maybe the use of different stimuli to evoke stretch. The use of a hypotonic solution to induce stretch results in an osmotic effect whereby there is an uptake of fluid into the cell thereby simulating stretch. This may actually cause a more profound effect than physically stretching the tissue.

When examining mean data and comparing stretch-evoked ATP release at 50%, no significant differences were observed in both dome and body regions. When the stretch was increased to 100% of the tissue length, ATP release due to stretch was significantly increased in the dome but not in the body region. Calculation of

percentage change in response to stretch versus basal release values altered the findings. At 50% stretch, there was an increase in ATP release in both the dome and body regions vs. basal ATP, but this was only significant in the body region. On the other hand, at 100% stretch ATP release was significantly increased in both dome and body regions in comparison to the basal levels. This is in line with published data. Performing the % change calculation removes the effect of comparing mean values for all tissue strips as a representative for basal and stretch respectively. Single calculations of the percentage change, taking into account individual basal values and ATP due to stretch, provide a more accurate depiction of the ATP levels. Intra-regional comparisons of 50% vs. 100% stretch for both dome and body revealed no significant differences of increasing the amount of stretch performed on the tissue. Moro *et al.* (2011) recently suggested a more physiological approach to stretch is to gradually stretch the tissue over time rather than subjecting the tissue to sudden stretch for short periods of time. Using the phased stretch approach of a few mm every 30 seconds over a few minutes may be more physiological (Moro *et al.,* 2011a) and might account for the lack of significance in the data obtained in the present study.

The physiological relevance of stretching to 50% or 100% of tissue strip length is difficult to determine, and different protocols have been used in previous studies examining ATP release from bladder cells and tissues. In light of this, a more physiological approach may be to measure ATP release in response to filling, by distending whole bladders and collecting the bladder luminal content afterwards to measure ATP release. In this way particular consideration can be paid to pressure and volume. This is something that could be pursued in the future in light of the recent published finding validating a functional isolated pig whole bladder model for physiological experimentation (Parsons *et al.,* 2012). This study confirmed that large animal bladders are viable *in vitro* and can provide valuable insight into the physiology of large animal bladders, as they have the potential of being more informative in comparison to cell culture studies, tissue strip studies and rodent animal models (Parsons *et al.,* 2012).

5.8 CONCLUSION

The present study has shown ATP release in response to stretch in isolated whole bladder thickness strips of pig bladder dome and body. However, there was a level of variability in the tissues and the response to stretch was not consistent across the experiments.

- The source of ATP release is not clear, and urothelial, myofibroblast and detrusor smooth muscle are all a possibility, although given the evidence for the literature urothelial cells may be the dominant source

CHAPTER 6

GENERAL DISCUSSION

Overactive bladder is a common and distressing condition of the LUT which affects millions of people worldwide. Our understanding of OAB and its underlying causes is limited, and although there have been many advances in our knowledge of normal bladder function in recent years, particularly in the role of the urothelium, much still remains unclear. Thus, a greater understanding of bladder function, both normal and pathological, is needed in order to lead to more effective treatments. The aim of this thesis was therefore to investigate and better understand bladder function, with particular focus on the inherent phasic activity of the bladder and components of the bladder wall thought to be involved in mechanosensation. In addition, a secondary aim was to study these aspects in the pig bladder, which has been used widely as a good model for human bladder with respect to pharmacology and physiology, but used less for studies characterising mechanosensation.

OAB is characterised by involuntary contractions of the bladder during the filling phase, the cause of which is not understood. It has been suggested that these involuntary contractions may be linked with increased phasic activity, and a mechanosensory basis for OAB has emerged, which suggests changes in transmitter release from the bladder urothelium, the functioning of ICC, increased afferent nerve activity and altered detrusor excitability.

In chapter 1 it was demonstrated that *in vitro*, in the absence of electrical or chemical stimulation, isolated bladder strips develop rhythmic phasic contractions, and that this occurs in both intact strips and denuded strips. The time for development of these PCs was however significantly increased in the denuded strips, in which the mucosa (urothelium and sub-urothelium) had been removed. The absence of the mucosa therefore resulted in a delay in the time for PCs to develop. This implies a clear role for the urothelium/sub-urothelium in regulation/modulation of PCs, but that these layers are not responsible for generating these PCs, since they are still able to develop in denuded strips. Within these layers are a number of cell types that may play a role in this regulation of PCs. The release of neurotransmitters from the urothelium, such as ATP, ACh, PGs and NO, may be important as they may act on the underlying ICC, afferent nerves and/or the detrusor. The hypothesis that the mediators released from the urothelium underlie the increase in PCs seen in various LUT conditions was investigated by Fry *et al.* (2012). These authors proposed that there is a syncytium within the sub-urothelial space where PCs are generated and went on to provide evidence of this using a rat spinal cord transected rodent model, showing calcium and membrane potential (E_m) waves that originated in the sub-urothelial space and propagated to the detrusor and urothelium (Fry *et al.,* 2012). Addition of various

purines increased the conduction velocities of the Ca^{2+}/E_m waves. These findings support the idea of the presence of a syncytium whereby neurotransmitters are released from the urothelium and act on ICC and afferent nerves to initiate PCs in the bladder. The findings presented in this study suggest a similar mechanism may be at play in the pig bladder, but it appears the activity does not initiate in the mucosa. It may originate in the detrusor or from ICC around the detrusor, and the cells in the mucosa may play a role in modulating the PCs via transmitter release. Non-neuronal tetrodotoxin resistant bladder contractions have been named autonomous activity, micromotions, intrinsic activity, non-micturition contractions, rhythmic and phasic activity. Whether these phenomena are one and the same activity is not yet clear, and the origin of these PCs has been debated upon and several theories have been proposed. It has been established that this 'autonomous activity' is not generated by post-ganglionic parasympathetic nerves, or by the activation of the neuromuscular junction, and that the underlying mechanism is different to that of detrusor activation during micturition (Drake *et al.,* 2003; Gillespie 2004). Instead it seems that there exists a complex system capable of generating coordinated contractions. Bladder PCs have therefore been suggested to be due to ICC or myofibroblasts in the sub-urothelium, and indeed reports suggest that this layer can contract in response to mediators such as ATP, NO and ACh (Sadananda *et al.,* 2008; Moro *et al.,* 2011a). Sub-urothelial myofibroblasts have been shown to possess muscarinic M3 receptors whereupon mediators may act to induce PCs. In addition, a novel muscularis mucosae layer of cells, located between the urothelium and the detrusor has been described, and although its properties and role in bladder function is unclear, guinea pig mucosal tissue strips have been shown to exhibit spontaneous phasic contractions that appear to originate in the muscularis mucosae (Heppner *et al.,* 2011). Thus this layer of SM that is physiologically and pharmacologically different to the detrusor, may contribute to the phasic contractions of the bladder

An interesting finding in the present study was that PCs in the bladder trigone region were not affected by removal of the urothelium, indicating a functional heterogeneity. This supports the suggestion from previous immunohistochemical studies that showed morphological differences in the bladder regions (Gillespie *et al.,* 2004; Gillespie *et al.,* 2005). In addition differences exist in the receptor density and distribution in these regions (Sánchez *et al.,* 2011). This is most likely associated with the different physiological roles of the regions - a contractile function for the dome and body versus. a funnelling function for the trigone. During bladder filling, the trigone is fixed in shape and helps to anchor the ureteric orifices to prevent backward flow of urine into the

ureters and, more importantly, contributes to bladder neck closure, for which it is vital that the trigone generates its own muscle tone (Akino *et al.,* 2008). In subsequent chapters these PCs were investigated further and some of these ideas.

Firstly, was a study to determine whether PCs in the pig bladder could be modulated cholinergically by low concentrations of carbachol, since there have been suggestions that an increased release of ACh might underlie increased phasic activity seen in OAB (Kanai *et al.,* 2007). Also, anticholinergics are now thought to act at sites other than just the detrusor muscle, and therefore block actions of ACh at multiple cell types in the bladder wall (Chapple 2000; Andersson *et al.,* 2009). Indeed in the present study, PCs were increased by low concentrations of carbachol, and thus it appears that a muscarinic receptor mediated system is important in PCs, as well as the characterised role for muscarinic receptor pathways in detrusor contraction that leads to micturition. The target of the carbachol could have been multi-site, since muscarinic receptors are widespread in the bladder wall and are found on nerves (Matsumoto *et al.,* 2010; Nandigama *et al.,* 2010; Sellers and Chess-Williams 2012), detrusor muscle (Hegde *et al.,* 1997; Sellers *et al.,* 2000; Chess-Williams 2002; Mansfield *et al.,* 2005; Creed *et al.,* 2010; Sellers and Chess-Williams 2012), urothelium (Chess-Williams 2002; Bschleipfer *et al.,* 2007; Kim *et al.,* 2008; Moro *et al.,* 2011b; Moro *et al.,* 2011b; Yoshida *et al.,* 2011; Sellers and Chess-Williams 2012) and ICC (Grol *et al.,* 2009). However, it was not possible to determine the precise target in the present study. Relatively selective antagonists for the muscarinic receptor subtypes were used to determine which receptor subtype(s) may be involved. Muscarinic receptor antagonists are the first line of therapy for OAB symptoms and urinary incontinence and the study of muscarinic receptors using the available agonists and antagonists has been important in establishing treatment for OAB. Although there is much information with regards to muscarinic receptors in the bladder, studies have been hampered significantly due to the lack of receptor subtype selective agents. In the present study, it was not possible to obtain conclusive results as to the muscarinic receptor subtype responsible for the cholinergic modulation of the PCs. The lack of muscarinic antagonist selectivity for a single receptor subtype compounded the complexity of determining the particular subtype responsible for the PCs in pig bladder. The fact that the bladder wall co-expresses more than one receptor subtype makes the determination of the function a single subtype more difficult. The presence of a heterogeneous population of cells within the bladder wall expressing various receptors and lack of selective antagonists contributed to the inability of identification of the responsible receptor subtype. From

these studies there remains the potential that non-neuronal acetylcholine released from the urothelium in the pig bladder may act on underlying cells to modulate bladder PCs.

Since it is possible that this ACh acts on ICC in the pig bladder wall, as this class of cells has been implicated in PCs, this led into the next study, which investigated whether ICCs were involved in PCs in the pig bladder, as has been shown in other species (Hashitani *et al.,* 2004; Hashitani *et al.,* 2008; Vahabi *et al.,* 2011c). The bladder wall appears to have a heterogeneous population of ICC, expressing different markers and found in different locations (Smet *et al.,* 1996; Davidson and McCloskey 2005; Lagou *et al.,* 2006; Lavoie *et al.,* 2007; Johnston *et al.,* 2010; McCloskey 2010). Since c-Kit is expressed only in ICC and not SMCs, an inhibitor of this tyrosine kinase receptor - imatinib - was used to investigate the role of ICC, with particular emphasis specifically on c-Kit positive cells in PCs of the pig bladder. The results showed that imatinib did not affect PCs in the pig bladder, and indeed using an antibody directed against c-kit, it was not possible to detect c-Kit positive cells in the pig bladder. Thus, it might be concluded that c-Kit positive cells are not functionally involved in PCs in the pig bladder. However, due to the lack of specificity of the available antibodies for pig tissues, it was difficult to draw definite conclusions as to whether these cells are present. It is possible that they may be present, but that they are not involved functionally in the PCs. These studies also did not rule out the possibility that PCs of the pig bladder are mediated by other ICC, since not all ICC express c-Kit, and potentially PCs may be mediated by other types of ICC, myofibroblasts or muscularis mucosae and detrusor. In response to the questions raised in chapters 2 and 3, it is possible that more elaborate technical approaches are required, such as electrophysiological recording from whole bladder tissues or cells and molecular studies to investigate c-Kit expression of the various cell types and receptors in the pig bladder in the different regions.

Given the emerging role of transmitter release from the urothelium in bladder function and PCs, the next stage was to study ATP release from the pig bladder. The rationale for this was the evidence that ATP is important in normal bladder function and has been shown to have an increased role in bladder pathology (Birder *et al.,* 2003; Khera *et al.,* 2004; Smith *et al.,* 2005; Salas *et al.,* 2007). One of the most successful, yet little understood treatments for OAB is injection of botulinum toxin into the bladder, which has been suggested to act via alteration of ATP signalling in the bladder wall. It was with this in mind that these experiments were undertaken. The original aims of the study were to optimise the measurement of ATP release from bladder tissues and then

progress to study the effects of botulinum toxin on this and investigate these mechanisms in a pathological model of bladder dysfunction.

A significant amount of time was spent on optimisation of the protocol for measuring ATP release in the pig bladder. To begin with, microdialysis probes were used in the collection of perfusate to measure ATP release. This method produced inconsistent and variable results, which may be attributed to the fact that the method of collection was not efficient (4µl/min) and the delay in collecting enough perfusate ample enough for the assay may have resulted in ATP breakdown by ectonucleotidase enzymes hence the inconsistent results. There is also the possibility that the process of inserting the microdialysis needle and probe may have caused substantial tissue damage that could have altered ATP release. Collection of whole tissue bath content was more successful and provided consistent measurement of ATP release. The outcome from the optimisation process meant that it was only able to investigate ATP release from the dome and body regions of the pig bladder. The results presented in this study showed ATP release from whole bladder strips and this increased upon stretching of the tissue. The amount of ATP measured in comparison to other published data was relatively low, and this may be due to the experimental protocol used. The size of the tissue baths (5ml vs. 0.2ml) may have significantly altered the concentration of ATP hence the lower values measured, and in hindsight a better approach may have been to freeze the samples immediately upon collection on dry ice or liquid nitrogen, to minimize ATP breakdown. Preliminary data obtained on the effect of botulinum toxin on ATP release showed no effect on ATP release (n=3, data not included) thereby suggesting that botulinum toxin did not affect ATP release from the three bladder regions, regardless of whether it had been injected into the tissue or incubated with the bladder tissue overnight.

SUMMARY AND CONCLUSIONS

The study highlights a need for further investigation and understanding of not only pathways that mediate normal bladder function but also the complex interaction of the various emerging mechanosensory components of the bladder including the urothelium, the ICCs, muscularis mucosae, the mediators they release and how they interact with underlying afferent nerves and muscle to enable bladder function.

The physiological investigation of pig bladder tissue showed that in vitro, isolated strips from the three bladder regions - dome, body and trigone - could develop PCs and that the urothelium plays a role in the time it took for these PCs to develop. Further analysis of these PCs revealed significant functional heterogeneity between the different bladder regions. These PCs could be modulated cholinergically, although it was not possible to identify the exact receptor subtype(s) responsible for this. Identification of ICCs in bladders of other species naturally led to the question of whether they were present in the pig bladder and if so, did they have a role in the PCs that developed in the pig tissue? Functional experiments using imatinib, a tyrosine kinase inhibitor, showed that the c-Kit ICC population, if present in the pig bladder, did not have an effect on the PCs. This led to the investigation of these cells using antibodies to try and identify if and where they are located. Immunohistochemical results showed a group of cells staining positive for vimentin in similar areas of the bladder as shown in other species, but the lack of specific antibodies could not definitively identify-Kit positive cells.

Data investigating transmitter release showed that ATP was released from the pig bladder in response to stretch. This ATP may exert its effects by acting on the receptors present in the underlying nerves, muscle and ICC.

The results presented in this thesis using pig bladder tissues have provided some important information with regards to bladder function that can be applied to future experiments on human bladder samples. The burden of OAB and the lack of compliance with the available pharmacological tools highlights a need for new drug therapies to tackle this condition that grossly affects quality of life for millions of people, and this will only be achieved with further understanding of the normal and pathological function of the bladder.

FUTURE WORK

Although the bulk of data presented in this study was obtained using tissue bath techniques, it seems that more elaborate approaches are needed in order to fully answer some of the questions raised. Molecular biology techniques such as PCR could be used to identify the expression of the muscarinic subtypes in the different regions of the bladder. This could also be used to confirm the presence of ICC in the bladder. Further investigation of ICC using antibodies specific to the pig could also be performed to firmly establish the localization of these cells in the different regions. The lack of an effect with imatinib suggests that c-Kit positive ICCs are not involved in PCs. Confirmation of their location and their expression with PCR would lay to rest whether the pig bladder expressed c-Kit or not..

The recent validation of a method to use large animal whole bladders could be exploited to investigate further ATP and ACh release, and maybe even other transmitters, from whole organs rather than using tissue strips. ATP experiments may need to include an ectoATPase inhibitor to ensure accurate measurement of ATP levels. Future research areas of interest would be completion of studies investigating the mechanisms underlying botulinum toxin and investigating potential changes in these mechanosensory pathways in pathological models/human tissue samples.

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