Muscarinic and Purinergic Signalling within the bladder

Thesis submitted to University College London for the Degree of Doctor of Medicine

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Acknowledgements

Declaration

The work contained in this thesis was carried out at University College London NHS Trust, The Royal Free NHS Trust and the Whittington NHS Trust between August 2006 and March 2009.

All of the work is my own. None of the data form part of any other thesis. This study was approved by the Whittington and Moorfields research Ethics Committee. All patients gave informed consent prior to their involvement in this study.

Abstract

The aim of this thesis is to improve our understanding of muscarinic and purinergic neurotransmission within the urinary bladder both within the detrusor muscle and the urothelium as both sites are therapeutic targets.

The M_2 receptor is the most populous muscarinic receptor in the detrusor muscle however its role is unclear, as detrusor contractility has been demonstrated to be mediated principally by the M_3 receptor. The role of the M_2 muscarinic receptor in guinea pig and human detrusor contractility was examined through organ bath experiments. Significant M_2 modulation of contractility in patients with neuropathic overactivity and overactive bladder symptoms was demonstrated through inhibition of agonist dose response curves and electrical field stimulation with the selective M_2 inhibitor methoctramine. Furthermore cAMP elevation through the adenylate cyclase activator forskolin produced an identical and non-additive inhibition to that achieved through methoctramine suggesting that cAMP inhibition is an important mechanism of M_2 activation in the detrusor.

Detrusor contractility was further assessed through an isolated cell technique and this demonstrated further evidence of M_2 mediated contraction of the detrusor indicating that the site of action of M_2 agonism is directly within the detrusor cells.

Ussing chamber experiments to examine the effect of the exogenous addition of neurotransmitters on the electrical properties of the urothelium were carried

out. These demonstrated that cholinergic agonists had no effect but ATP resulted in an increased negativity of the basolateral surface of the urothelium only when added to the luminal but not the basolateral surface. As ATP release from the urothelium has been found to be associated with inflammation and the sensory nerves are adjacent to the basolateral surface, we believe this represents a sensory mechanism whereby a luminal inflammatory signal is transduced electrically across the urothelium to activate the sensory nerves.

Chapter 1

Introduction

1.1 Urinary Bladder Physiology

Anatomy

The bladder is a pelvic organ, which serves to store urine produced by the kidneys until it is convenient for it to be expelled. It is a hollow bodied organ, that's fills with urine, which enters the bladder via the ureters (Fig 1a). The roof of the bladder is termed the dome and the base of the bladder the trigone, which extends from the two ureters to the urethra. The urethra remains contracted during the storage phase through the tonic contraction of the internal and external urethral sphincter preventing any expulsion of urine from the bladder into the urethra. Anatomically the bladder walls has a number of distinct layers, from inside to out (Fig 1b):

1)   Urothelium, which consist of layers of transitional epithelial cells.

2)   Suburothelium, which is in close proximity to afferent nerves.

3)   The detrusor smooth muscle, which is innervated by efferent nerves of the autonomic nervous system. It consists of 3 layers; an inner and outer longitudinal layer and in the middle a circular layer (Wakabayashi et al. 1994).

4)   The serosal layer which is the outer lining, which is in contact with the peritoneum superiorly.

Filling is not passive but a process of active relaxation of the detrusor smooth muscle. The high degree of compliance allows for increase in urine volume to near maximal levels with no increase in vesical pressure (Harris et al. 1996).

Neurological Control

The bladder and urethral sphincter are innervated by a combination of

Fig 1a Anatomy of the bladder

Urinary Bladder

Ureter

Peritoneum

Detrusor muscle

Submucosa

Mucosa

Rugae

Fibrous connective tissue

Ureteral opening

Trigone

Internal urethral orifice

Prostate gland

External urethral orifice
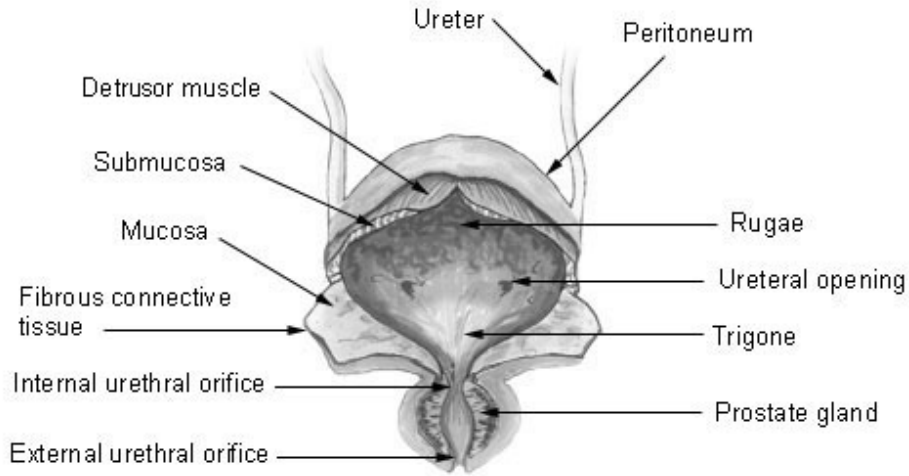
Fig 1b The lining of the bladder

Urothelium

Smooth Muscle

Urine

Afferent nerve

Efferent nerve

Na^+

ATP

P2X

P2X

$P2X_1$

ATP ACh

P2X

ATP

M3

M2

Depolarisation

Interstitial Cells
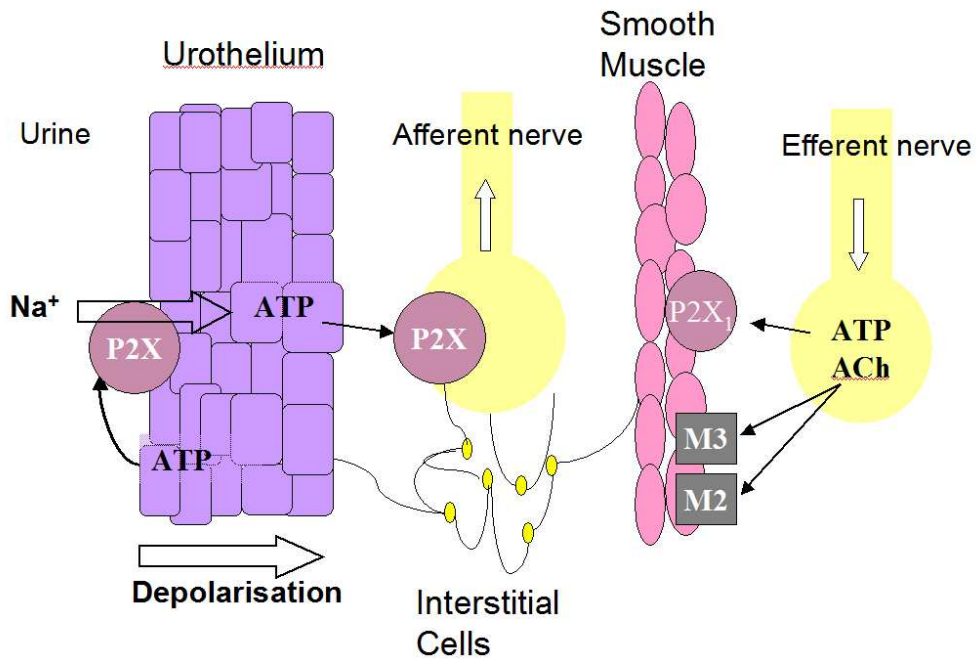
Fig 1c Neurological control of voiding. PMC –pontine micturition centre, SMC –sacral micturition centre
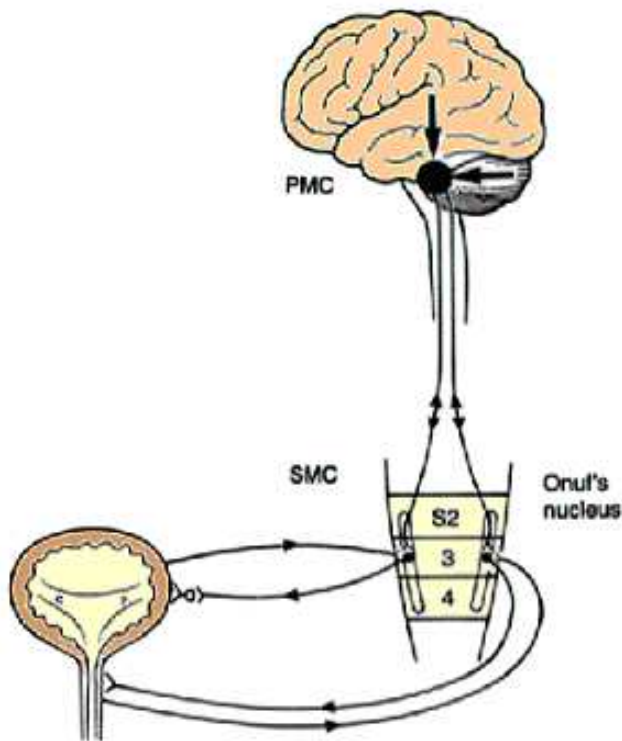
somatic, sympathetic and parasympathetic nerves. Control of voiding function

involves the full length of the spinal cord, the brainstem and higher centres.

This complexity accounts for the diversity of disease patterns seen in brain

and spinal cord injuries. The bladder receives parasympathetic innervation via

the pelvic nerve, which receives nerve fibres from the S2-S4 spinal roots (Fig

1c) . The principal neurotransmitter that initiates muscle contraction is

acetylcholine. The bladder is also innervated by the sympathetic nervous

system via the hypogastric nerve, which receives fibres from the T10-L2

region of the spinal column. It opposes the actions by promoting storage and

contributes to the relaxation of the bladder during the storage phase. The

internal urethral sphincter receives inhibitory stimulation via the sympathetic

nervous fibres and stimulatory fibres of the parasympathetic nervous system

carried via the pudendal nerve. The external urethral sphincter has part

somatic innervation via the pudendal nerve. Much of bladder function is locally

controlled at the spinal cord by two spinal reflexes. The sacral micturition

centre (S2-S4 of the spinal column) controls receptive bladder relaxation, as

the bladder is filling and reflex bladder contraction, which is triggered when

the bladder has been sufficiently filled for vesical pressure to start to increase.

Onuf nucleus (S2-S4) contributes to the guarding reflex, which is a continence

mechanism that insures that urethral sphincter tone is increased when

abdominal pressure increases so that stress incontinence does not occur. The

combination of these two mechanisms in isolation produces a reflex bladder,

which fills until the pressure starts to rise and voiding commences

immediately. Onufs nucleus and the sacral micturition centre are under control

of the Pontine micturition centre unless spinal cord injury has occurred. This

coordinates bladder contraction with urethral sphincter relaxation and assists in maintaining sustained bladder contractions during voiding (de Groat 2006). The pontine micturition centre is influenced by information from higher centres within the brain. These connections do no fully function until after 1-2 years of age and when active allow for the deferment of voiding until it is socially convenient to do so.

Neurotransmitters at the smooth muscle neuromuscular junction (Motor function)

At the neuromuscular junction a number of neurotransmitters are released from vesicles, in response to electrical excitation of the nerve axon. These neurotransmitters mediate contractility of the detrusor muscle. The principal neurotransmitter released is acetylcholine (Bayliss et al. 1999), which acts on transmembrane muscarinic receptors located on the detrusor cells. MRNA for all five muscarinic receptor subtypes has been detected in the human detrusor, however the M_2 and M_3 receptor subtypes are predominantly expressed (Mansfield et al. 2005;Wang, Luthin, & Ruggieri 1995).

M_2 receptor numbers within the detrusor, exceed that of the M_3 receptor across a range of mammalian species, including humans where they exceed M_3 numbers by a ratio of 3:1 (Mansfield et al. 2005;Wang, Luthin, & Ruggieri 1995). However, the findings from animal knockout models and the use of selective muscarinic agonists points to contractility largely being mediated by the M_3 receptor (Mansfield et al. 2005).The M_2 receptor is believed to have a regulatory role, though this has yet to be clearly established.

M_3 receptor activation has been demonstrated to activate the $G_{q/11}$ G protein. This was demonstrated through the inhibition of acetylcholine mediated contractions with specific $G_{q/11}$ antibodies (An et al. 2002). Activated $G_{q/11}$ activates phospholipase C that leads to the production of IP_3 and diacylglycerol. IP_3 activates the IP_3 receptor on the sarcoplasmic reticulum, which controls the release of Ca^{2+} from the sarcoplasmic reticulum (An, Yun, Lee, Yang, Shim, Jeong, Shin, Kim, Kim, & Sohn 2002). A rise in intracellular Ca^{2+} leads to a contraction by binding to calmodulin, which in turn activates myosin light chain kinase (MLCK). MLCK phosphorylates myosin filaments, and this allows crossbridge formation with actin and therefore shortening and the generation of force. However, the work of Schneider in human detrusor muscle points to an alternative mechanism for M_3 activity as the phospoholipase inhibitor U73122 did not significantly inhibit detrusor contractility, whereas nifedipine the Ca^{2+} channel blocker and the rho kinase inhibitor Y27632 did so (Schneider et al 2004).

M_2 receptor activation leads to the activation of $G_{i/0}$ G protein. Activation of which leads to inhibition of adenylyl cyclase, which results in a reduction in cAMP concentration. A reduced cAMP level may potentiate detrusor contraction by a reduction in protein kinase A activity or increased rho-kinase activity (Somlyo & Somlyo 2000), both of which may result in reduced phosphorylation of myosin light chain phosphatase and reduced dephosphorylation of myosin, which constitute a calcium independent mechanism of potentiating the force of contractions.

Atropine resistance of electrically mediated detrusor contraction has led to the search for alternative neurotransmitters. ATP has been demonstrated to

mediate detrusor contractions. ATP potentially mediates its effect through P2X receptors, which function as a cation channel and may promote extracellular Ca^{2+} entry; either directly through the channel or indirectly through depolarisation of the cell membrane and activation of voltage gated Ca^{2+} channels. ATP may also mediate contraction through activation of G protein coupled P2Y receptors. In the normal human detrusor the most commonly expressed receptor at the mRNA level is the $P2X_1$ receptor (O'Reilly et al. 2001). This also appears to be the case in outflow obstruction (O'Reilly et al. 2002), however in idiopathic detrusor overactivity $P2X_2$ receptor expression is increased (O'Reilly, Kosaka, Knight, Chang, Ford, Rymer, Popert, Burnstock, & McMahon 2002).

Intracellular Calcium and detrusor contraction.

A rise in intracellular concentration is the key event mediating detrusor contraction (Wu, Kentish, & Fry 1995), however there is much debate regarding the relative contribution of extracellular and intracellular sources. Acetylcholine may facilitate a rise in intracellular Ca^{2+} concentrations through IP_3 activating the IP_3 receptor, which allows the egress of Ca^{2+} from the sarcoplasmic reticulum, and this is the principal mechanism mooted. However, the action of calcium channel blockers indicates that extracellular sources of Ca^{2+} are essential for contractility. Whether this is through maintenance of intracellular store levels or direct increment of intracellular Ca^{2+} concentrations prior to a contraction is a matter of contention. There are

several types of Ca^{2+} channels that may facilitate extracellular Ca^{2+} entry. Longer acting or L type voltage gated channels have been demonstrated to have a critical role in human detrusor contractility through the use of selective L type inhibitors such as nifedipine (Badawi et al. 2006). The role of the shorter T- type channel is controversial, with some authors finding the T-type inhibitor mibefradil to have little effect on human detrusor contractility while others have demonstrated the presence of functional T-type channels (Sui et al. 2007). However, Ca^{2+} homeostasis is complex and interdependent. There is evidence that extracellular Ca^{2+} entry may promote a major Ca^{2+} efflux from intracellular stores (Imaizumi et al. 1998). Likewise there is evidence that Ca^{2+} efflux from intracellular stores has influence on Ca^{2+} influx. Jiang (Jiang et al. 2005) demonstrated that the ryanodine receptor serves to decrease spontaneous contractions and concluded that the release of Ca^{2+} from intracellular stores could activate Ca^{2+} dependent K^{+} channels (Appendix Figure 5), which through their hyperpolarising effect on membrane potential may inhibit voltage gated Ca^{2+} channels. This may be compatible with the observation that muscarinic agonists have an inhibitory effect on L-type Ca^{2+} channels. Conversely Ca^{2+} efflux from intracellular stores may promote intracellular Ca^{2+} entry through the activation of store operated channels (Putney, Jr. & McKay 1999) though this mechanism has yet to be clarified. Kubota (Kubota et al. 2003) demonstrated that mitochondria play a role in buffering intracellular Ca^{2+} concentrations in the guinea pig detrusor as the addition of mitochondrial protonophore resulted in depolarisation of the detrusor membrane and a rise in intracellular Ca^{2+} concentration.

Urothelial Function

The urothelium is the epithelial lining of the bladder that is in contact with the urine. It is typically consist of 3 to 7 layers of cells. There are three types of cells; an outermost layer of large flattened hexagonal cells (termed umbrella cells) an intermediate layer of cells and a basal layer of cells which have a lower turnover rate of 3-6 months and are believed to differentiate into the other cell types (de Groat 2004). The urothelium serves as a barrier between the urine and the interstitial space as urine is a noxious substance. The umbrella cells in particular are believed to be important in this respect as they have tight gap junctions between them and are coated on their surface by a protein called uroplakin which reduces the permeability of the urothelium to small molecules such as water, urea and protons (Apodaca 2004). It has been demonstrated that this barrier function is compromised in a number of pathological conditions such as spinal cord injury (Apodaca et al. 2003) and interstitial cystitis (Truschel et al. 2002) and has been postulated that the passage of these substances through the urothelium can precipitate urinary symptoms such as urgency and incontinence.

There is growing interest in the role that the urothelium may play in relaying bladder sensation. The urothelium has some neuronal characteristics, demonstrated by the expression of numerous receptor subtypes including, muscarinic, purinergic, nicotinic and transient receptor potential receptor channels, amiloride sensitive and mechanosensitive Na^+ channels. The urothelium has been demonstrated to release a number of neurotransmitters in response to bladder stretch and inflammation; ATP (Truschel et al. 2002), acetylcholine (Yoshida et al. 2004), nitrous oxide (de Groat 2004) and

substance P. The basal surface of the urothelium is in close contact with sensory nerves and these substances may potentially modulate afferent nerves directly or via interstitial cells, which lie in close proximity. Likewise it is likely that substances released from the axons of sensory nerves modulate urothelial function. Neurotransmitters released from the urothelium may have a direct effect on detrusor contractility. It has been postulated that detrusor overactivity may be due to these substances being excessively released form the urothelium during the storage phase when the efferent parasympathetic nerve fibres are quiescent.

The direct effect of the urothelium on the detrusor has been investigated through the comparison of detrusor contractility when the urothelium is present and removed, both in human (Chaiyaprasithi et al. 2003) and guinea pig tissue (Hawthorn et al. 2000). In both cases, detrusor contractility was increased when the muscle strips were denuded of the urothelium. The authors concluded that some diffusible substance released from the urothelium was responsible for inhibiting detrusor contractions, however this substance remains unidentified as the authors concluded that it was not nitrous oxide or a catecholamine due to the lack of activity of ODQ and L-N^G – nitroarginine (inhibitors of nitrous oxide synthesis) or propanolol (B-blocker) respectively (Chaiyaprasithi, Mang, Kilbinger, & Hohenfellner 2003). Furthermore, the diffusible substance does not seem to be ATP or to mediate its actions through K^+ channels (which when activated may hyperpolarize a cell membrane and inhibit contractility), as the purinergic inhibitor suramin and the K^+ channel inhibitors apamin, TEA had no effect in inhibiting detrusor contractions (Hawthorn, Chapple, Cock, & Chess-Williams 2000).

ATP has been shown to be independently released from both the basal and apical surface of the urothelium (Lewis & Lewis 2006). The purpose of apical release is unknown given the basal location of the sensory nerves but this may serve some regulatory function. ATP release has been demonstrated to be increased in a number of inflammatory conditions, including UTI (Osterberg et al. 1991) and interstitial cystitis (Sun & Chai 2002). The expression of urothelial $P2X_2$ and $P2X_3$ receptors has been found to be increased in patients with interstitial cystitis and inflammation of the bladder. Interstitial cystitis in cats has been shown to lead to the increased excitability of sensory neurons within the dorsal root ganglion (Sculptoreanu et al. 2005). Furthermore experiments with $P2X_3$ deficient mice have shown them to have impaired voiding function (Cockayne et al. 2000). There is good evidence that purinergic signalling is an important component of urothelial sensory function.

Acetylcholine has likewise been demonstrated to be released from the urothelium in a stretch dependent fashion. Yoshida found that muscle strips released more acetylcholine when the urothelium was present when compared to denuded detrusor muscle strips. This release was demonstrated to be non neuronal as it was uninhibited by the addition of tetrodotoxin which abolishes nerve activity (Yoshida, Miyamae, Iwashita, Otani, & Inadome 2004). The authors concluded that this might be part of a bladder sensory mechanism.

Temperature-sensitive transient receptor potential vanilloid (TRPV) ion channels are believed to play a role in mediating bladder sensation (Birder et al. 2002). They are expressed within the urothelium and within c fibre sensory

nerves mediating bladder sensation. These receptors may depolarise the membrane through the opening of a cation channel and are believed to be important in nociception and perhaps serve as "environmental sensors". There are a number of subtypes expressed within the bladder. The TRPv1 receptor is activated by irritant stimuli such as capsaicin, which is a component of chilli peppers and anandamide, which is an exogenous ligand for the valinoid and canabinoid receptor. It is also activated by heat and changes in pH and osmolality. Activation of urothelial TRPV1 receptors has been demonstrated to lead to a rise in intracellular Ca^{2+} and enhanced release of the neurotransmitters ATP and Nitrous Oxide. Cultured urothelial cells from TRPV1 null mice show a decrease in hypotonic solution induced ATP release (Birder et al. 2002).

Disorder of bladder function

Overactive Bladder Symptoms (OAB)

Overactive Bladder symptoms as defined by the International Continence Society is characterised by urinary urgency; "a compelling desire to pass urine that is difficult to defer, ± urinary frequency (greater than 8 voids per day) ± nocturia (greater than one void per night), ± urge incontinence (Chapple et al. 2005). Overactive bladder symptoms are associated with detrusor overactivity, which is characterised by the presence of detrusor contractions during the filling phase on urodynamic assessment (Hashim & Abrams 2006). However detrusor overactivity and overactive bladder symptoms as they are strictly defined may exist in patients independently of each other.

OAB is a common condition that affects a large proportion of the elderly population but has a significant incidence amongst younger individuals. The prevalence of OAB has been estimated as 11% (Irwin et al. 2006; Milsom et al. 2001) and 16% (Tubaro 2004). OAB is a large social problem; a recent estimate has put the cost of OAB to the UK economy at £743 per year (Hashim & Abrams 2004). It was estimated that OAB cost five EEC countries in excess of €4.2 billion in the year 2000 (Reeves et al. 2006). OAB is independently associated with falls and fractures, urinary tract and skin infections, sleep disturbances and depression (McGrother et al. 2006; Ragins et al. 2008). The clinical impact of this syndrome is likely to increase greatly as the world's population ages (Irwin et al. 2006).

The pathophysiology of OAB is unknown though a number of theories have been proffered. Occult infection manifest by pyruria on fresh unspun urine has

been suggested as a common cause of OAB with up to 70% of patients with OAB having pyuria, yet by conventional urinary testing most of these patients would be deemed free of infection (Malone-Lee 2007). It has been demonstrated that UTI with uropathic escherichia coli is initiated by adherence of the bacteria to the outer uroplakin layer of the umbrella cells and formation of intracellular colonies termed biopods (Schilling & Hultgren 2002) and it is possible than such colonies exist in patients with OAB giving rise to chronic low grade infections that may be difficult to diagnose or treat with current therapeutic protocols.

Neuropathic disorders

Neuropathic disorders can present both with symptoms of overactivity or hyperreflexia. If the neurological lesion is superior to the sacral micturition centre and there is loss of descending inhibition from the pontine micturition centre then hyperreflexia may arise. If the motor neurones in the sacral micturition centre are damaged then the bladder will become areflexic. There is evidence of alteration in muscarinic function in patients with neuropathic bladders. Pontari (Pontari, Braverman, & Ruggieri, Sr. 2004) inferred increased M_2 muscarinic receptor signalling in four out of seven neuropathic patients. Braverman (Braverman et al. 1998) found evidence of increased M_2 muscarinic signalling in rats, which had spinal cord transection relative to control animals.

Painful Bladder Syndrome

Painful bladder syndrome is difficult to treat and to characterise pathologically but is defined by the International Continence Society as the complaint of suprapubic pain related to bladder filling, accompanied by other symptoms such as increased daytime and night time frequency, in the absence of proven urinary infection or other obvious pathology. The condition has been treated by bladder hydrodistension and various pharmacological agents. A recent review (Dimitrakov et al. 2007) found little evidence of patient benefit for any treatment other than pentosan polysulphate.

Bladder outflow obstruction

Bladder outflow obstruction is much more common in men than women who have a longer urethra and the potential for prostatic obstruction. Bladder outflow obstruction may be caused by benign prostatic hypertrophy, prostate cancer, bladder neck hypertrophy, and urethral strictures. Voiding symptoms which characterise BOO have a prevalence of 37% in males aged >60 (Irwin et al. 2006), and are most commonly due to benign enlargement of the prostate.

Voiding symptoms; poor stream, hesitancy, post micturition dribbling and incomplete emptying are associated with bladder outflow obstruction (BOO). Storage symptoms; urinary frequency, urgency, nocturia and incontinence are associated both with the overactive bladder (OAB) and BOO (Abrams et al. 2002). In BOO, the voiding symptoms are thought to be primarily due to the effect of the obstruction on urinary flow whereas the storage symptoms are

due to the effect that the obstruction has on bladder function (Morant et al. 2008).

BOO is characterised by an elevated detrusor pressure relative to the urinary flow rate in the earlier stages of the disease process. However chronic obstruction can lead to marked and irreversible changes in bladder function characterised by an increase in bladder capacity from the normal value of 500-600 mls to 1 –4 litres and a decrease in voiding pressure such that retention of urine persists after the obstruction has been surgically treated. Such patients are said to have an acontractile bladder and may be dependent on lifelong catheterisation as untreated they are prone to infections and life threatening renal impairment.

Pharmacological treatments of bladder disorder

Anticholinergic Drugs

Anticholinergic drugs are the most common form of treatment used for OAB. They act through inhibiting the function of the neurotransmitter acetylcholine. They primary effect is believed to be at the neuromuscular junction where they act to reduce contractions during the filling phase, however as much less is known about urothelial function, inhibition of sensory signalling and the influence that this has on the voiding reflex is something that cannot be discounted.

The antimuscarinic drugs used today are similar in their efficacy and side effects profile. The principal stated targets are the M_3 muscarinic receptors as these have previously been shown to mediate detrusor contractions, however all of these drugs will have activity at the other muscarinic receptor subtypes.

The most common side effects that patients complain of are due to M_3 inhibition; dry mouth, constipation and blurred vision. M_2 inhibition can potentially cause tachycardia through their action on cardiac receptors and may affect the patient's memory, if the molecule can traverse the blood brain barrier (Caulfield & Birdsall 1998).

Botulinum toxin injections

Botulinum toxin injections into the bladder have proved an efficacious treatment for patients with overactive urinary symptoms that are refractory to antimuscarinic drug treatment. This has been demonstrated in OAB (Ghei et al. 2005), neuropathic detrusor overactivity (Giannantoni et al. 2008) and painful bladder symptoms (Giannantoni et al. 2008).

Botulinum toxin acts by cleaving the subunit of SNARE1, a type of SNAP protein that is essential for the formation of presynaptic neurotransmitter vesicles (Kalandakanond & Coffield 2001). In skeletal muscle this results in abolition of presynaptic vesicular release of the neurotransmitter acetylcholine and ATP from the neuromuscular junction. By analogy it is believed to have an identical effect on the human detrusor though this needs to be demonstrated experimentally. It is possible, given that the same neurotransmitters are released from the urothelium albeit by alternative mechanisms that botulinum toxin has an effect on afferent nerve function though again this hypothesis is one that needs to be tested experimentally.

1.2 Muscarinic Receptors

Introduction

Acetylcholine is a signalling molecule, which has extensive actions within the mammalian nervous system, acting both centrally and peripherally. Unusually for a signalling molecule, acetylcholine acts on two unrelated families of receptors- the nicotinic and muscarinic receptors, distinguished by their selective response to nicotine and muscarine respectively. In fact muscarinic receptors share greater sequence homology with B adrenergic and dopaminergic receptors than the nicotinic receptors (Hosey 1992). In the periphery, nicotinic receptors mediate the synaptic action of the skeletal neuromuscular junction, whereas muscarinic receptors mediate the action of the postganglionic neurons of the parasympathetic nervous system. Nicotinic receptors are fast acting as a result of their ionotropic effects whereas muscarinic receptors generally provide a slower metabotropic response. Muscarinc receptors have been extensively studied due to their wide range of physiological actions, which includes cerebral, cardiovascular, bowel and urinary function. Whilst the complexity of the field has rapidly increased, so has the potential for therapeutic interventions for the range of disease processes which are influenced by muscarinic receptor function.

Discovery of muscarinic receptor subtypes and their genes

Five mammalian receptor subtype (M_1-M_5) have been discovered through the elucidation of the genes encoding for them (Bonner et al. 1987;Bonner et al. 1988;Hosey 1992;Kubo et al. 1986;Peralta et al. 1987)). The M_1-M_5 genes are localised to 11q12-13, 7q35-36, 1q43-44, 11p12-11.2 and 15q26 respectively within the human chromosome (Bonner 1989). There is considerable homology in amino acid sequences between the mammalian species whose receptors have been cloned thus far. For example there is 97% homology between human and porcine M_2 receptors at the amino acid level (Hulme, Kurtenbach, & Curtis 1991). However, variation of even a single amino acid can lead to greatly differing receptor pharmacology. As such transfection of the cloned human muscarinic receptor to a cell line should provide a superior model of human receptor functionality than a non human mammalian species (Caulfield & Birdsall 1998) (Table 1, page 29).

The human muscarinic receptor genes have the following number of exons; M_1 2 exons M_2 3-4 exons,M_3 5 exons,M_4 2 exons, M_5 receptor 2 exons.

In the case of the M_2 receptor gene, eight alternatively spliced gene products have been discovered. Variation is solely in the 5' UTR and the same protein (isoform a) is always produced. Alternative transcripts have not been found to be products of the other muscarinic receptor genes.

Table 1, Muscarinic receptors gene homology.
Number of amino acids (percentage of identity with human sequence).

(Caulfield & Birdsall 1998)

Species	M_1	M_2	M_3	M_4	M_5
Human	460	466	590	479	532
Pig	460 (99%)	466 (97%)	590 (96%)		
Rat	458 (98%)	466 (95%)	589 (92%)	478 (95%)	531 (89%)
Chicken		466 (92%)	639 (87%)	490 (76%)	

Structure

The three dimensional structure of the muscarinic receptor has yet to be elucidated, however muscarinic receptors are G-protein coupled receptors and as such have seven transmembrane helices. G-protein coupled receptors are the most extensive family of plasma membrane localised receptors and account for > 1% of the human genome (Breitwieser 2004). It is predicted that its three dimensional structure will be similar to that of rhodopsin (Baldwin 1993) . In addition muscarinic receptors have the following structural motifs:

The transmembrane segments are most likely alpha helical. The carboxy terminus is on the intracellular side of the membrane. As antibodies to the carboxy terminus only demonstrate immunoreactivity when the cells are permeabilised (Lu et al. 1997). There are two cysteine residues that form a disulphide bond between the first and third extracellular loops (Kurtenbach et al. 1990). All muscarinic receptors have an Aspartate residue in the distal N terminal part of the third transmembrane domain which is believed to interact with the polar head group of amine ligands eg acetylcholine (Caulfield & Birdsall 1998). The ligand-binding site is in the outer portion of the receptor. However, the sites involved in the binding of muscarinic receptor antagonists are believed to be diverse (Wess, Bonner, & Brann 1990). Muscarinic receptors possess a large third intracellular loop (i3), which becomes phosphorylated by endogenous protein kinase (Pals-Rylaarsdam & Hosey 1997). This region along with the second intracellular loop is important for the interaction between the receptor and its target G proteins. In this region there is sequence divergence between $M_1/M_3/M_5$ receptors and M_2/M_4 receptors, which determines that the former interact with q/11 G proteins and the latter

i/o G proteins. Studies with chimeras of the M_2 and M_3 muscarinic receptor have shown that the transfer of small amino acid segments of under 21 amino acids in length between these two receptors can cause an M_2 receptor to signal like an M_3 receptor and vice-versa (Wess, Bonner, & Brann 1990).

Heterodimers/ Homodimers

Protein dimerization and oligomerization is a ubiquitous process found in all living organisms and functionally important in many biological signalling systems, including G-protein coupled receptors (Milligan et al. 2003). It has been demonstrated that proteins that have the ability to self interact (dimerize) have a greater propensity to interact with other proteins than would be expected by chance (Ispolatov et al. 2005). Paralagous proteins that are involved in heterodimerization may have evolved by duplication of genes encoding proteins that are able to form homo-dimers. This property may confer proteins with several advantages including structural stability (Hattori et al. 2003), control of binding site accessibility (Marianayagam, Sunde, & Matthews 2004), increasing the range of structural complexity and phenotypes available from a genome of a particular size (Ispolatov, Yuryev, Mazo, & Maslov 2005).

There is much evidence for the existence of muscarinic receptor homodimers and higher order homo-oligomers (Park & Wells 2004) and for heterodimerization between muscarinic receptor subtypes and even other members of the G-protein coupled receptor family. Though some of the inferences need to be tempered by the possibility that the methodology

employed to study dimerization, such as the use of mutant receptors or co-immunoprecipitation may produce interactions that are not representative of those that occur between wild type receptors in vivo. These potential drawbacks can be overcome through the use of newer techniques such as bioluminescence resonance energy transfer (BRET) which studies real time interactions between proteins in vivo (Breitweiser 2004; Goin & Nathanson 2006). Ligand binding studies of muscarinic receptors have indicated the formation of dimers as complex binding pharmacology has been observed, suggesting multiple states of affinity consistent with either dual binding sites on a dimeric protein (Hirschberg & Schimerlik 1994; Potter et al. 1991) or cooperative interactions between activated monomers (Chidiac et al. 1997).

Most dimers are not connected by covalent bonds, the exception to this is disulphide bridge linkage, which has been demonstrated between M_3 homodimers and has been shown to involve the Cys 140 and Cys 220 residues. Two modes of interaction between receptors have been described; 1) contact dimerization and 2) domain "swapping" (Breitwieser 2004). Contact sites have been identified for a few GPCR homodimers such as the B-adrenergic receptor (Hebert et al. 1996) and the D2 dopaminergic receptor (Guo, Shi, & Javitch 2003). Domain swapping was first suggested as a cooperative mechanism by Maggio and his colleagues as they demonstrated functional rescue of a non functional mutated M_3 receptor via coexpression of an α_{2c} receptor (Maggio, Vogel, & Wess 1993). The question as to whether muscarinic receptors exist constitutively within the plasma membrane as dimers or whether dimerization of a monomer is promoted by agonist-induced activation of a monomer has been raised. The work of Zeng (Zeng & Wess

1999) and Goin (Goin & Nathanson 2006) suggests that it is the former rather than the latter. Relatively little is known about the functional consequences of dimerization. Of particular interest is the effect that muscarinic receptor dimerization has on the G-protein mediated interactions with the principal downstream effectors- phospholipase C and adenylyl cyclase. Novi demonstrated that activation of both components of the M_3 homodimer are necessary for the activation of Extracellular receptor kinase 1/2, but G-protein activation could occur simply by activation of a single component of the homodimer (Novi et al. 2004). Novi later demonstrated that the formation of paired agonist activated wild type M_2 dimers, M_2/M_3 heterodimers, α2 adrenergic/ M_3 muscarinic heterodimers were a prerequisite for the recruitment of the G-protein regulatory protein β-arrestin-1 to the plasma membrane (Novi et al. 2005).

Localization of muscarinic receptors and physiological function in mammalian studies

M_1 receptor; Brain (Levey et al. 1991), Memory- activation of MAP kinase, glands (Hamilton et al. 1997), sympathetic ganglia (Ramcharan & Matthews 1996).

M_2 receptor Heart (Hoover, Baisden, & Xi-Moy 1994), hindbrain (Levey et al. 1991), smooth muscle, detrusor and urothelium (Mukerji et al. 2006).

M₃ receptor; Smooth muscle (Caulfield & Birdsall 1998), detrusor and urothelium (Mukerji et al. 2006), salivary glands causing secretion, pupils – constriction (Matsui et al. 2000), brain (Levey et al. 1991).

M₄ receptor; Basal forebrain striatum (Caulfield & Birdsall 1998), central dopaminergic response regulation (Gomeza et al. 1999), adrenal chromafin cells (Endo et al. 2005).

M₅ receptor; Substantia Nigra (Reever, Ferrari-DiLeo et al. 1996), involvement in thr reward pathways of mice (Forster et al. 2002;Yeomans, Forster, & Blaha 2001), iris cilliary muscle (Ishizaka et al. 1998).

Table 2 Summary of muscarinic receptor distribution, function and mechanism of action (Caulfield & Birdsall 1998)

		M_2	M_3	M_4	M_5
Location	Brain, Glands, Sympathetic ganglia	Heart, Hindbrain, smooth muscle	Smooth muscle, Glands, brain	Basal forebrain	Substantia nigra
Preferred G protein secondary messenger	q/11 PLC IP_3/DAG Ca^{2+}/PKC	i/0 -(AC)	q/11 PLC IP_3/DAG Ca^{2+}/PKC	i/0 -(AC)	q/11 PLC IP_3/DAG Ca^{2+}/PKC
Functional response		Inhibit Ca^{2+} channels, decreased heart rate	Gland secretion, smooth muscle contraction	Inhibition of Ca^{2+} channels	

Mechanisms of action

G proteins are a family of signalling molecules, which interact with surface membrane receptors. They are heterotrimeric proteins consisting of α,β and γ subunits. There is some evidence to suggest that the normal stoichometry for this interaction is between a muscarinic receptor dimer and a G protein trimer. This is derived from analogous studies of the interaction of rhodopsin with G-proteins (Clark et al. 2001;Willardson et al. 1993). The G proteins are classified according to the α subunit, of which there are four main classes; $α_s$, $α_i$, $α_q$ and $α_{12}$. There is also considerable variability in the β and γ subunits and hence multiple permutations of G-protein complexes are possible. Ligand binding to the G-protein coupled receptor results in a conformational change which allows the α subunit to catalyse the exchange of GDP for GTP. GDP release is the rate limiting step in the cyclical function of the α subunit (Ferguson et al. 1986). The α subunits dissociate from the βγ subunits which are each independently able to activate a range of divergent downstream targets. G protein deactivation occurs by the intrinsic hydrolysis of GTP to GDP by the α subunit. It is likely that our knowledge of these pathways and their regulatory factors will increase such that we may be able to understand how a particular pathway attains physiological prominence within a specific tissue type. This in turn may lead to specific pharmacological modulation of a pathway for therapeutic purposes. The best understood signalling pathway is the $G_{q/11}$ / IP_3 pathway in smooth muscle but evidence for other mechanisms is also described below.

The M1/M3/M5 receptors activate the α subunit of the $G_{q/11}$ protein which can activate Phospholipase C and Phospholipase D. Phospholipase C activation

results in increased IP_3 and 1,2,-diacylglycerol (DAG). IP_3 activates the IP_3 receptor, which results in the release of Ca^{2+} from the sarcoplasmic reticulum. A rise in Intracellular Ca^{2+} is the critical event leading to smooth muscle contraction. Basal intracellular Ca^{2+} levels are maintained at 0.01uM to 0.1uM. Contractile proteins are sensitive to a rise in Ca^{2+} in the 0.1uM to 10uM range (Wu, Kentish, & Fry 1995). Ca^{2+} binds to calmodulin leading to the formation of Ca^{2+}-calmodulin complex, which is able to activate myosin light chain kinase (MLCK). MLCK phosphorylates myosin light chain, which triggers cycling of crossbridges with actin resulting in ATP hydrolysis and muscle contraction. Diacylglycerol activates protein kinase C, which has numerous downstream targets.

The M_2/M_4 receptor couples to the α subunit of the $G_{i/0}$ protein which inhibits adenylcyclase and results in decreased cAMP concentrations. It has been proposed that this may counteract mechanisms, which increase cAMP concentrations such as β adrenergic signalling. cAMP has numerous downstream effectors. Reduced cAMP levels could potentially modulate smooth muscle contraction via activation of rho-kinase or inhibition of protein kinase A (Somlyo & Somlyo 2004). Both of which may lead to phosphorylation and deactivation of myosin light chain phosphatase and prolonged activation of myosin. Such a mechanism could lead to increased contractility at a given intracellular Ca^{2+} concentration (Ca^{2+} sensitisation) or potentially a contraction independent to a rise in given intracellular Ca^{2+} concentration.

There are also reports of muscarinic receptors activating adenylcyclase, in a pertussis toxin sensitive manner, in the case of the rat olfactory bulbs (Onali & Olianas 1995).

Muscarinic receptors have been reported to have ionotropic function. In cardiac muscle; M_2 mediated inhibition of voltage gated Ca^{2+} channels via adenylcyclase inhibition (Mery et al. 1997) and also direct activation by M_2 receptors of inward K^+ rectifier current by $G_{\beta\gamma}$ subunits (Wickman & Clapham 1995). In the guinea pig ileum M_2 activity stimulates the opening of cation selective channels, which result in depolarisation and contraction (Zholos & Bolton 1997).

Muscarinic receptors are also located presynaptically and may function to modulate the release of acetylcholine and other neurotransmitters from parasympathetic nerve terminals. D'Agostino demonstrated that pre-junctional M_4 receptors participate in a negative feedback regulatory mechanism by inhibiting the release of acetylcholine from parasympathetic nerve terminals (D'Agostino et al. 1997).

Human genetic Polymorphism

Polymorphisms are the differences between humans or animals of the same species. The genetic variation which account for our individuality, be it physical or psychological characteristics or disease predisposition are being uncovered all the time. The human genome project has greatly facilitated our understanding of the genetic basis of disease by providing an open database, which scientists throughout the world may explore. Evolutionary pressure insures that the genome is highly conserved. According to our current knowledge there is 99.7% homology of human DNA between individuals. Thus one in every 300 bases within the genome varies and contributes to our

individuality. However, this number may rise, as less frequent genetic polymorphisms are uncovered through probing of DNA from a wider range of individuals. The most common type of genetic polymorphisms are single nucleotide polymorphism (SNP). A single DNA base is altered and thus the single subtlest change in the genome occurs. SNP will usually result in 2 alleles i.e. variation results in only 2 out of the 4 possible bases occurring at that point in the genome. Traditionally base changes are classed as SNPs if the minor allele arises with a frequency of greater than 1%. This is arbitrarily so that the changes in the genome represent variations that are conserved rather than those which arise out of spontaneous mutations. SNPs may occur in regions of DNA, which code for proteins (exon) or regulatory regions (introns) or within an intergenic region. Polymorphism arising in introns may affect the control of genes and thus the timing and quantity of protein synthesised may be altered. SNPs arising in exons may cause no change to the protein synthesised (synchronous SNP) or may alter one of the amino acids (asynchronous change) and thus may alter the function of the protein that is synthesised. Genetic polymorphism of the muscarinic receptor has been implicated in the development of asthma (Yamamoto et al. 2007), prolonged heart rate recovery from exercise (Hautala et al. 2006) and cognitive function (Gosso et al. 2006). However, in the overactive bladder, there are currently no such documented associations between muscarinic receptor polymorphism and bladder function, though an association has been found between seretonin 2A receptor genetic polymorphism and urinary incontinence in women (Noronha et al. 2010). The experience of our laboratory is that there can be a wide difference in agonist EC_{50} values (up to

30 fold) when comparing detrusor contractions from bladder biopsies from different individuals. A possible explanation for this is genetic polymorphism of the muscarinic receptor.

Known polymorphic regions within human muscarinic receptor genes

Muscarinic receptor gene polymorphism has been implicated as a predisposing factor in a number of phenotypes and disease processes, both of early and late onset. Some of these SNPs and their significance are described below. Much of the pre-eminence in this field has been gained by those studying human cognitive and behavioural function;

M_1 receptor gene, *CHRM1;*

Two SNPs (-9697C > T and -4953A > G) were associated with asthma (Yamamoto, Matsubara, Maeda, Minagawa, Takashima, Maruyama, Michikawa, & Yanagisawa 2007).

Reduced M_1 receptor cDNA expression in patients with schizophrenia (Yamamoto et al. 2007).

M_2 receptor gene, *CHRM 2;*

An SNP at intron 5 rs32460 resulting in a sequence change from T/T to C/C and an SNP at rs8191992 in the 3' – untranslated region of exon 6 resulted in significantly differing tachycardia post exercise (Hautala, Rankinen, Kiviniemi, Makikallio, Huikuri, Bouchard, & Tulppo 2006).

Two degenerate SNPS (1197T→C and 976A→ C were found not to be significantly associated with the asthma phenotype.

Association was between rs324650 and performance IQ (PIQ), where the T allele was associated with an increase of 4.6 PIQ points (Gosso, van, de Geus, Polderman, Heutink, Boomsma, & Posthuma 2006).

Three SNPs (one in intron 4 rs184240 and two in intron 5 rs324640, rs324650) showed significant association with alcoholism. Two SNPs (both in intron 4 rs184240 and rs2061174) were significantly associated with major depressive syndrome (Wang et al. 2004).

Chrm2 variability associated with agreeableness and conscientiousness (Niu, Luo, & Hao 2004).

M_3 receptor gene, *CHRM* 3;

Gene variation is associated with decreased acute insulin secretion and increased risk for early-onset type 2 diabetes in Pima Indians (Guo et al. 2006).

No association between coding region of M_3 receptors and the asthma phenotype (Donfack et al. 2003).

M_4 receptor gene, *CHRM* 4;
No published studies

M_5 receptor gene, *CHRM* 5;

A 26.8% increase in cigarette consumption in carriers of the rs7162140 T-allele. Carriers of the rs7162140 T-allele were also found to have nearly a 3-fold increased risk of developing cannabis dependence (Anney et al. 2007).

Muscarinic Agonists

Acetylcholine, the physiological agonist contains a quaternary nitrogen, which allows interaction with the anionic receptor site. This is illustrated by the fact that dimethylaminoethylacetate which is the tertiary form of acetylcholine, has an approximately 1000 fold reduced affinity for the receptor. Other agonist, such as muscarine and carbachol, share this structural feature. However, other agonists of high potency- oxotremorine and pilocarpine contain tertiary amines. Experimentally carbachol and oxotremorine have been the most utilized in functional studies. There are no highly selective agonists, though oxotremorine has some selectivity for the M_2 receptor over the M_3 receptor (Pkb =7.1 and 6.0 respectively) (Griffin et al. 2003). Carbachol and oxotremorine have the ability to activate phospholipase A2 and inhibit adenylylcyclase, albeit at different concentrations. Carbachol has been demonstrated to preferentially activate phospholipase A2 at lower concentrations whilst oxotremorine preferentially inhibits adenylylcyclase at lower concentrations (Griffin et al. 2003). Muscarinic agonist potency and hence selectivity, is greatly determined by the tissue or cell line under study and findings from a given functional study may not be generalisable. The search for functionally selective agonists have deemed to be a potential avenue for treatment of Alzheimer's disease (Fisher 2008), however there are currently no treatments that have demonstrated efficacy.

Muscarinic Antagonists

Atropine is an alkaloid originally derived from the nightshade plant- Atropa Belladonna and one of the oldest known molecules. It possesses an N-methyl group. It has a much higher pK_B than any other known antagonists and has no selectivity between muscarinic receptor subtypes. There are currently no antagonists, which are highly specific for a particular muscarinic receptor subtype, with the exception of MT7 muscarinic receptor snake venom, which is highly selective for the M_1 receptor (Bradley, Rowan, & Harvey 2003). To illustrate this problem; in attempting to distinguish between the M_2 and M_3 receptors which are highly expressed throughout the nervous system, no antagonist has a greater than 30 fold preference between these two subtypes. Gallamine is an allosteric inhibitor with preference for the M_2 receptor over the M_3 inhibitor. However its mechanism of action may produce complex interactions with other ligands. It has been demonstrated that gallamine can act as an agonist in the absence of orthosteric agonists and that this function is not inhibited by classical antagonists (Jakubik et al. 1996).

Table 3 Muscarinic antagonists and their receptor specific pK_B.

Muscarinic receptor Antagonist	M_1	M_2	M_3	M_4	M_5
Atropine	9.0-9.7	9.0-9.3	8.9-9.8	9.1-9.6	8.9-9.7
Methoctramine	71-7.8	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
Pirenzapine	7.8-8.5	6.3-6.7	6.7-7.1	7.1-8.1	6.2-7.1
Darifenacin	7.5-7.8	7.0-7.4	8.4-8.9	7.7-8.0	8.0-8.1
MT7	9.8	<6	<6	<6	<6
Gallamine		6.2	4.5		
Oxybutynin	8.5	7.8	8.7	8.2	7.6
Tolteridine	8.5	9.4	8.5	8.1	8.6

(Caulfield 1993), (Hou, Hirshman, & Emala 1998), (Eglen & Watson 1996)

Constitutive receptor activity.

It has been demonstrated that muscarinic receptors can activate G-proteins in the absence of any agonist. In a patch clamp study of atrial cells, muscarinic antagonists were able to elevate cAMP levels (Soejima & Noma 1984). Constitutive activity has also been demonstrated in studies where there was over expression of the receptor or G-proteins (Burstein, Spalding, & Brann 1997;Vogel et al. 1995). Furthermore, mutant muscarinic receptors with constitutive activity have been created in the case of M_1 (Hogger et al. 1995) and M_5 receptors (Spalding et al. 1997). In these cases the muscarinic

antagonists atropine and 4-DAMP displayed inverse agonist function and were able to revert mutant receptor activity to that of the wild type. The detection of constitutive receptor activity has led to a refinement in the model of receptor activation. The receptor has been proposed to exist in two states; an inactive and active state that is able to interact with G proteins. Agonists are able to bind to the receptor and induce a conformational change in the receptor to the active state. However an antagonist needs to be classed as either a neutral antagonist which simply binds to the receptor and has no further effect other than to prevent an agonist from binding to that site or an inverse agonist which binds to the receptor but promotes a conformational change from the active to the inactive state. Kenakin in his review suggests that up to 85% of 'antagonists' can behave as inverse agonists (Kenakin 2004). Constitutive receptor activity has recently been found to be a feature of number of pathological conditions. An example of this is Chagas disease, which is caused by the parasite Trypanosoma Cruzi. It has been suggested that infection results in the production of autoantibodies against the second extracellular loop of the M_2 acetylcholine receptor, which decrease myocardial contractility and reduce cAMP production. There is subsequent desensitisation and internalisation of the receptor. Constitutive activity has been demonstrated to occur due to genetic polymorphism; The S49G mutation in the β_1-adrenoceptor, identified in patients with heart failure, appears to result in constitutive activity of the receptor and increased desensitisation. This SNP was beneficial to the patients, as long-term survival was significantly greater than in patients who possessed the wild-type receptor (Levin et al. 2002).

The classification of antagonists as either being neutral or inverse is likely to attain greater significance to the pharmacological industry as a disease caused by a constitutively active genetic variant of the receptor may respond only to an inverse agonist but not a neutral antagonist. Furthermore, a disease caused by over stimulation of the receptor by antibodies or excessive neurotransmitter release, may in theory, be better treated by a neutral antagonist, as these have been implicated to cause less receptor upregulation (Milligan, Bond, & Lee 1995). It has also been demonstrated that there may be more than one active state of the muscarinic receptor, which may be induced by different agonists. Akam and his colleagues demonstrated in Chinese Hamster Ovary cells expressing the M_3 acetylcholine receptor that pilocarpine could induce a $G\alpha_{i/0}$ response, whereas methacholine additionally produced a $G\alpha_{q/11}$ response (Akam, Challiss, & Nahorski 2001).

Receptor regulation

Desensitisation of muscarinic receptors in response to acetylcholine is likely to occur in vivo as parasympathetic nerves exhibit tonic firing activity and this is likely to be part of a homeostatic mechanism, which regulates cellular function. The immense complexity and diversity of regulatory proteins which interact with muscarinic receptors is indicative of the wide range of physiological functions that are performed by the relatively few muscarinic receptor subtypes and tissue specific functionality is likely to be endowed by these regulatory processes. Phosphorylation of the muscarinic receptor is a key but not exclusive mechanism of receptor regulation (Willets et al. 2001). Multiple phosphorylation sites have been discovered in the third cytoplasmic

loop and the C-terminus of the muscarinic acetylcholine receptor involving serine and threonine residues (Pollok-Kopp et al. 2007) and occasionally tyrosine residues (Fan et al. 2001).

Rapid receptor phosphorylation in response to agonist stimulation is a posttranslational modification adopted by nearly all G protein–coupled receptors (GPCRs) (Pierce, Luttrell, & Lefkowitz 2001). Several classes of kinase mediate muscarinic receptor phosphorylation. Amongst them are the GPCR kinases (GRKs), protein kinase C (PKC), casein kinase 1α (CK1α) and protein kinase C (PKC).

The GRK family currently consists of seven proteins (GRK1- 7). GRKs only phosphorylate the agonist occupied receptor leading to the binding of arrestins, which suppress G protein interaction (Pierce, Luttrell, & Lefkowitz 2001). GRK 2 and GRK 3 reside in the cytosol but translocate to the cell membrane in response to agonist activation of the receptor (Haga & Haga 1992). GRK 2 and GRK 3 interact with $\beta\gamma$ subunits of the G protein via a 125 amino acid pleckstrin homology domain (Krupnick & Benovic 1998). In hippocampal neurons Willets and his colleagues concluded that endogenous GRK2 is a regulator of M_1 mACh receptor signalling and that this process involves both phosphorylation-dependent and -independent mechanisms (Willets, Nahorski, & Challiss 2005).

GRK5 and GRK 6 are ubiquitously expressed however it is not clear which receptors are their targets, though there is some evidence that M_2 and M_3 receptors are targeted (Gainetdinov et al. 1999;Willets, Nahorski, & Challiss

2005). GRK phosphorylation can promote receptor activation of G protein–independent pathways such as the MAPK cascade (Wei et al., 2003).

It has been demonstrated that M_1 and M_3 but not M_2 receptors are phosphorylated by PKC. In contrast to the actions of GRK, PKC acts in an agonist independent manner (Haga & Haga 1992).

Casein kinase 1α has been shown to phosphorylate residues within the third cytoplasmic loop of the M_3 acetylcholine receptor. However this does not seem to result in any desensitisation (Budd, McDonald, & Tobin 2000).

Casein kinase 2 has been demonstrated to phosphorylate the M_3 muscarinic receptor. This was shown to regulate the activation of Jun-kinase but not ERK1/2 and have no effect on Internalisation of the M_3-muscarinic receptor (Willets, Nahorski, & Challiss 2005).   Furthermore this phosphorylation differed between cell types.

Agonist induced activation of the muscarinic receptor leads to phosphorylation of residues within the third cytoplasmic loop of the muscarinic receptor and this facilitates internalisation of the receptor (Moro, Lameh, & Sadee 1993; ;Pals-Rylaarsdam & Hosey 1997). However, agonist activation does not seem to be the only means of inducing phosphorylation as binding of the receptor with the inverse agonist N-methylscopolamine results in significant receptor internalisation (Vogler et al. 1998). GRKs play an important role in receptor internalisation, however this is very much receptor subtype and cell type specific. Studies on GRK knockout animals have suggested that a given receptor subtype expressed in different tissues may be phosphorylated by a different set of receptor kinases (Walker et al. 1999). Receptor

phosphorylation, desensitisation and internalisation do not correlate in all studies (van Koppen & Kaiser 2003).

β-arrestin 1 and 2 can bind to the phosphorylated receptor (Goodman, Jr. et al. 1996) and also to clathrin which is a component of the endocytic system. Expression with a β-arrestin 1 mutant which is unable to interact with the phosphorylated receptor demonstrate markedly reduced receptor internalisation of M_1, M_3 and M_4 receptors in HEK293 cells (Goin & Nathanson 2006). Similarly expression of a dominant negative clathrin mutant markedly reduces receptor internalisation of M_1, M_3 and M_4 receptors (Vogler et al. 1998). Some studies have concluded that M_2 receptor internalisation may not be mediated by clathrin, as the M_2 receptor does not appear to interact with β-arrestin (Wu et al. 1997). Internalisation of M_2 muscarinic receptors via different mechanisms has been reported; Feron reported that M_2 receptors in cardiac monocytes can be internalised in caveolae which are invaginations of the plasma membrane (Feron et al. 1997). However, a recent study in cardiac atrial muscle suggests that internalisation of the M_2 receptor is mediated by β-arrestin 2 and clathrin and contributes to the desensitisation of the muscarinic K^+ current (Yamanushi et al. 2007). Muscarinic receptor internalisation involves the interaction of other molecules such as AP-2, dynamin and the cytosolic binding protein c-Src. Expression of dominant negative forms of dynamin (Claing et al. 2000) and c-src (Luttrell et al. 1999) demonstrate markedly reduced receptor internalisation. Heterodimerization has been implicated as a process that can influence receptor regulation as Goin and his colleagues have demonstrated that coexpression of M_3 mAChR with increasing amounts of the M_2 subtype receptor in JEG-3 cells resulted in

an increased agonist-induced down-regulation of M_3 receptors (Goin & Nathanson 2006).

Receptors, which have been internalised, can be resensitized. It has been proposed that the internalised receptor is dephosphorylated by a GPCR phosphatase in an endocytic compartment and subsequently recycled to the plasma membrane (Krueger et al. 1997). However internalisation of the receptor is not necessarily a prelude to resensitization (Tobin, Lambert, & Nahorski 1992) and may lead to down regulation and attenuation of signalling (Krudewig et al. 2000).

Conclusion

Over the last ten years there has been rapid growth in our understanding of muscarinic receptor function. Understanding of tissue specific regulatory mechanisms may lead to the development of specific pharmacological interventions. Likewise, an appreciation of the subtle differences in function brought about by genetic polymorphism may lead to gene specific therapies.

1.3 Purinergic Receptors

Introduction

ATP is a neurotransmitter, which acts on purinergic receptors. It has a wide range of functions within the mammalian peripheral and central nervous system. It is released often as a co-transmitter from many nerve axons including cholinergic, adrenergic, glutamergic and GABAergic nerve terminals (Zimmermann 2008) and also from non innervated tissue such as epithelial surfaces. There is evidence that altered purinergic signalling is present in a range of diseased states such as pulmonary hypertension (Sprague et al. 2001), diabetic retinopathy (Sugiyama et al. 2004) and interstitial cystitis (Osterberg, Hallander, Kallner, Lundin, & Aberg 1991;Sun & Chai 2002).

The purine receptor family (Appendix, Table 2, page 177) consists of P1 receptors, which respond to adenosine, which is the final breakdown product of ATP hydrolysis, and P2 receptors, which respond to ATP/ADP. P2 receptors are further classified as P2X receptor and P2Y receptors Currently molecular cloning has identified 4 P1, 7 P2X and 8 P2Y receptor subtypes.

P2X receptors exist primarily as trimers (Nicke et al. 1998;Nicke 2008). Each receptor has seven subunits, only two of which are transmembrane domains (Zimmermann 2008). They function as ligand gated non-selective cation channels that allow the influx of Na^+ and Ca^{2+} and efflux of K^+. The P2X receptor can bind up to three ATP molecules and binding is believed to occur

between receptor subunits. P2X receptors 1 to 5 can form homo or heterotrimers whereas the P2X$_6$ receptor forms only heterotrimers whereas the P2X$_7$ forms only homomers. The properties of purinergic receptor trimers are influenced by their subunit composition.

P2Y receptors like P1 receptors are members of the G protein coupled receptor super family. Structurally they consist of seven transmembrane α helical domains and function through activation of G proteins and intracellular secondary messengers and can be activated by a number of nucleotides and their degradation products (Appendix, Table 2). They have also been shown to activate ion channels (Hussl, Kubista, & Boehm 2007).

A diverse range of enzymes of varying specificity can break down ATP released from cells. Membrane bound ectonucleotidases which have their catalytic subunit facing the extracellular space have been classified into two subfamilies;- the triphosphate diphosphohydrolases that hydrolyse nucleotide tri and di phosphate eg ATP conversion to ADP and ADP conversion to AMP and the pyrophospatase/ phospodiesterase which have lesser specificity and are able to convert ATP to AMP and cAMP to AMP. However a range of other enzymes contribute to ATP hydrolysis such as GPI-anchored alkaline phosphatases and the GP-anchored ecto-5-nucleotidases.

Purinergic receptors are known to undergo rapid desensitisation in response to agonist stimulation occurring within seconds and taking up to fifteen minutes to recover. Differing rates of desensitisation have been noted between the receptor subtypes with the fastest rates of desensitisation occurring in P2X$_1$ and P2X$_3$ receptors (North 2002).

Detrusor Contractility

Probing of the neurotransmitter mechanism activated by electrical excitation of detrusor smooth muscles from humans and animals have demonstrated a significant proportion of the elicited contraction being resistant to atropine. This proportion is low in normal human tissue (Burnstock 2007) but has found to be increased in a number of conditions including interstitial cystitis (Palea et al. 1993), obstructive (Bayliss et al. 1999) and overactive bladder symptoms (O'Reilly et al. 2001) consistent with a concept of neuronal plasticity. O'Reilly, in their study of the P2X receptor found that $P2X_1$ was the predominant purinergic receptor subtype expressed at the mRNA level. However, all the remaining purinergic receptors were consistently expressed.

Bayliss demonstrated that atropine resistance was not present in patients with urodynamically normal bladders in contrast to those with outflow obstruction and detrusor overactivity who had the greatest atropine resistance. Furthermore this atropine resistance could be abolished through the addition of ABMA, which effectively desensitises the purinergic receptor from producing further ATP mediated responses. However when exogenous ATP was added to muscle strips there was no significant difference in the potency of exogenous ATP in eliciting responses between the three patient groups. Furthermore there was evidence that ATP and acetylcholine were cotransmitted, on the basis of the similarity between the overall electrical field stimulation frequency response curve and the atropine resistant frequency response curve in that they had the same half maximal response frequency,

suggesting that acetylcholine and ATP were released from nerves that had the same excitatory potential, if not the same nerves.

Harvey et al have found increased potency of ATP at initiating detrusor contractions in patients with detrusor overactivity may be due to decreased hydrolysis of ATP (Harvey et al. 2002).

Neuronal plasticity has been demonstrated in animal models of outflow obstruction, where increased purinergic and decreased cholinergic signalling has been demonstrated in rabbits with outflow obstruction (Calvert et al. 2001). However others have found decreased purinergic signalling in outflow obstruction (Scott, Uvelius & Arner 2004). Whilst there are undoubtedly changes in purinergic signalling as a result of pathophysiology, whether this accounts for patient's symptoms has yet to be determined.

There is evidence for an increase in purinergic signalling in the human bladder with advancing age; the atropine-resistant component of nerve-mediated contractions of the human bladder was found to be increased with age, principally as a result of increased release of ATP (Yoshida et al. 2004). The sensitivity of the bladder to the purinergic agonist $\alpha\beta$-meATP has been shown to increase with age (Wuest et al. 2005). However, there was no change in the mRNA detected for $P2X_1$ and $P2X_3$ in this study.

ATP and the Urothelium

There is growing evidence that the urothelium does not merely function as a barrier but has neuronal properties as demonstrated by its copious expression of neurotransmitter receptors and the host of chemical mediators that are released by the urothelium (Birder et al. 2002).

Stretch activated ATP release has been demonstrated from the urothelium of the bladder (Ferguson, Kennedy & Burton 1997) and also from the ureters (Burnstock 2002). The mechanism of ATP release from neuronal axons is believed to be exocytotic vesicular release however the mechanism of release form non-neuronal cells remains uncertain. A host of mechanisms accounting for this process have been proposed – vesicular release, connexin and pannexin hemi channels, ATP binding cassettes, $P2X_7$ receptors (Burnstock 2007). The basal and stretch activated (hypotonic stimulus) release of ATP from the urothelium of spinal cord injured rats is greater than controls suggesting a pathophysiological role in neuropathic detrusor overactivity. The stretch activated luminal but not serosal ATP release could be inhibited by botulinum toxin A suggesting a vesicular mechanism of release for stretch activated ATP release and an alternative mechanism for basal release (Khera et al. 2004).

There is a growing body of evidence to suggest that ATP release from the urothelium is enhanced in a number of diseases and inflammatory states. Increased ATP has been found in patients with UTI (Osterberg, Hallander, Kallner, Lundin, & Aberg 1991) and interstitial cystitis (Sun & Chai 2002) and from rats with cyclophosphamide induced cystitis (Smith et al. 2005). Studies in $P2X_3$ knockout mice have demonstrated bladder hyporeflexia and reduced response to pain (Cockayne et al. 2000). $P2X_3$ receptors are expressed within the urothelium, the suburothelium and within nociceptive sensory neurons within the dorsal root ganglion (Andersson 2002). $P2X_3$ receptors expression has been found to be upregulated in patients with interstitial cystitis (Sun & Chai 2002). ATP has been demonstrated to be independently released from

both the luminal and basolateral surface of the urothelium (Khera, Somogyi, Kiss, Boone, & Smith 2004). The question arises as to why ATP is released into the urine when the sensory nerves are located on the other side of the urothelium in proximity to the basolateral surface. One explanation is that there is increased leakiness of the urothelium and or umbrella cell death in diseased states. There are several lines of evidence however that would point to an active physiological release mechanism instead. Firstly that basal release has been demonstrated in normal as well as pathological urothelium secondly that luminal release can be specifically inhibited by botulinum toxin A (Khera et al 2004; Somogyi et al. 2005) and depletion of intracellular Ca^{2+} stores (Van der Wijk et al. 2003) and lastly the resistance of the urothelium from spinal cord injured rats is not markedly different from controls (Apodeca et al. 2003) indicating that this urothelium is no more leaky in those with spinal cord injury though there is an increase in ATP release.

1.4 Ussing Chamber

The Ussing chamber so named after it is inventor Hans Ussing, is a piece of apparatus that allows the selective perfusion of the apical and basal surfaces of an epithelial sheet, measurement of epithelial ion transport and the ensuing changes in its electrical properties. A wide range of applications have been developed for this versatile apparatus, such as measurement of drug absorption, tissue permeability and investigation of ion transport coupled /mechanosensory afferent mechanisms (Li, Sheppard, & Hug 2004). Functionally the apparatus consist of two units; a physical chamber for mounting and perfusing the tissue and an electrical circuit for recording epithelial ion transport and measuring the resistance of the tissue sample.

Ussing Chamber Types

There are two types of Ussing chambers; recirculating chamber and a continuously pefusing chamber. A recirculating chamber consist of a U tube which allows for equal column heights of solution being applied to each side of the urothelium so that the hydrostatic forces across the membrane can be balanced. Equalizing the hydrostatic forces across the membrane can reduce the risk of damaging the membrane and isolate the driving forces for transport across the membrane to electrico-chemical influences. Alternatively, adjusting the net hydrostatic forces can be used to explore mechano-sensory mechanisms and mimic hydrostatic pressures, which may exist within the lumen of visceral organs such as the bowel or bladder (Li, Sheppard, & Hug 2004). The disadvantage of a recirculating Ussing chamber is that once a

reagent is added to the perfusing solution on either side of the Ussing chamber then it remains within the chamber. This inflexibility can be overcome by using a continuously perfusing system where reagents can be continuously added and washed out from either side of the tissue. The perfusing solution is amenable to warming via water jackets around the connecting tubing.

Epithelial Electrical Properties

Epithelial surfaces being transporting membranes have an asymmetrical distribution of ion channels between their apical or luminal surface and their basal or serosal surface. This results in the presence of a negative voltage at the apical surface in reference to the basal surface. This negative voltage is present whether the epithelium is secreting or absorbing ions or substrate. In theory the overall transepithelial voltage is a sum of apical and basal voltage differences. A well-studied example of a transporting membrane is frog skin. Here the apical surface can have a polarity of +60mV, this is generated by the movement of Na^+ ions through open ion channels down their concentration gradient. At the basal membrane Na^+ is actively transported against an ionic gradient by the Na^+/ K^+ ATPase ionic exchange transporter. Intracellular K^+ concentration is maintained by the outward movement of K^+ ions down a concentration gradient and hence transmembrane potential here is near the Nernst voltage for this ion i.e. −70 mV. Thus the sum transepithelial voltage from basal to apical is −70mV −(+60mV) =130mV (Nagel 1977). In practice this voltage is never actually measured because of the resistance of the ex-vivo tissue and integrity of the membrane serves to diminish the recorded

value of measurements. Nonetheless useful measurements are possible by taking into account the direction of change of transepithelial voltage in response to selective ligands (fig 1d).

Electrical Recordings

The epithelium can be conceived of as a simple electrical circuit (fig 1e).

The Ussing chamber is designed for making accurate electrical measurements. It is crucial that the epithelium is handled and dissected very gently so as not to disrupt its continuity. The epithelium is stretched tightly over an aperture, which is typically 2 –10 mm in diameter so as to produce a watertight seal that prevents the leakage of perfusing solution around the epithelial sheet.   Different methods have been utilised for securing the epithelium such as rubber rings, pins, histoacrylic glass or air suction. Salt bridges are used to provide an interface between the electrical flux across the epithelial chamber and the measuring circuit. The AgCl /Agar KCl salt bridge is one of the most commonly used. The ideal electrode combines low resistance, reusability and minimal ionic leakage, which may affect or damage the epithelium. A KCl concentration of 0.5M to 3.0M is often used in the salt bridges. The higher the concentration of KCl used, the lower the resistance but the greater the risk from leakage of KCl to the epithelium.

In order to measure the voltage across the membrane the electrodes are typically placed as close as possible to the membrane so as to reduce the resistance R_C (Fig 1e). The electrodes measuring current can be placed further away than the voltage electrodes.

In order to calculate transmembrane resistance R_{TE}, transepithelial voltage (TEP) and short circuit current (SCC) needs to be measured. Measurements can be made using open circuit or short circuit condition using voltage or current clamping. Voltage clamping involves applying fixed voltages across the membrane and measuring the resulting current. Current clamping involves injection of a small amount of current across the epithelium and measuring the resulting voltage changes. Estimation of resistance can be made by both of these methods using Ohms Law. Of the two modes of measurement current clamping produces a lesser perturbation of the cells as the current injected only produces a temporary change to the voltage across the epithelium. The TEP can be measured directly by a voltmeter during open circuit recording, and the SCC can be estimated from Ohms Law if either voltage or current clamping is used to measure epithelial resistance. Alternatively, the SCC can be measured directly during zero voltage clamping when the TEP voltage is clamped to zero volts through the injection of small amounts of current across the epithelium, which is regulated by a feedback amplifier. The resistance of the epithelium can again be derived using Ohms law through dividing the SCC into the TEP. This is conditional on the integrirty of the urothelium and there is thereby not another alternative route for the passage of current across the urothelium (R_s being large in Fig 1e) and there being a low resistance of the ex vivo circuit (R_C being small in Fig 1e). In practice experimental conditions do not allow for accurate derivations of these values, but changes in these variables in response to specific ligands during the course of an experiment can yield useful information about epithelial transport mechanisms.

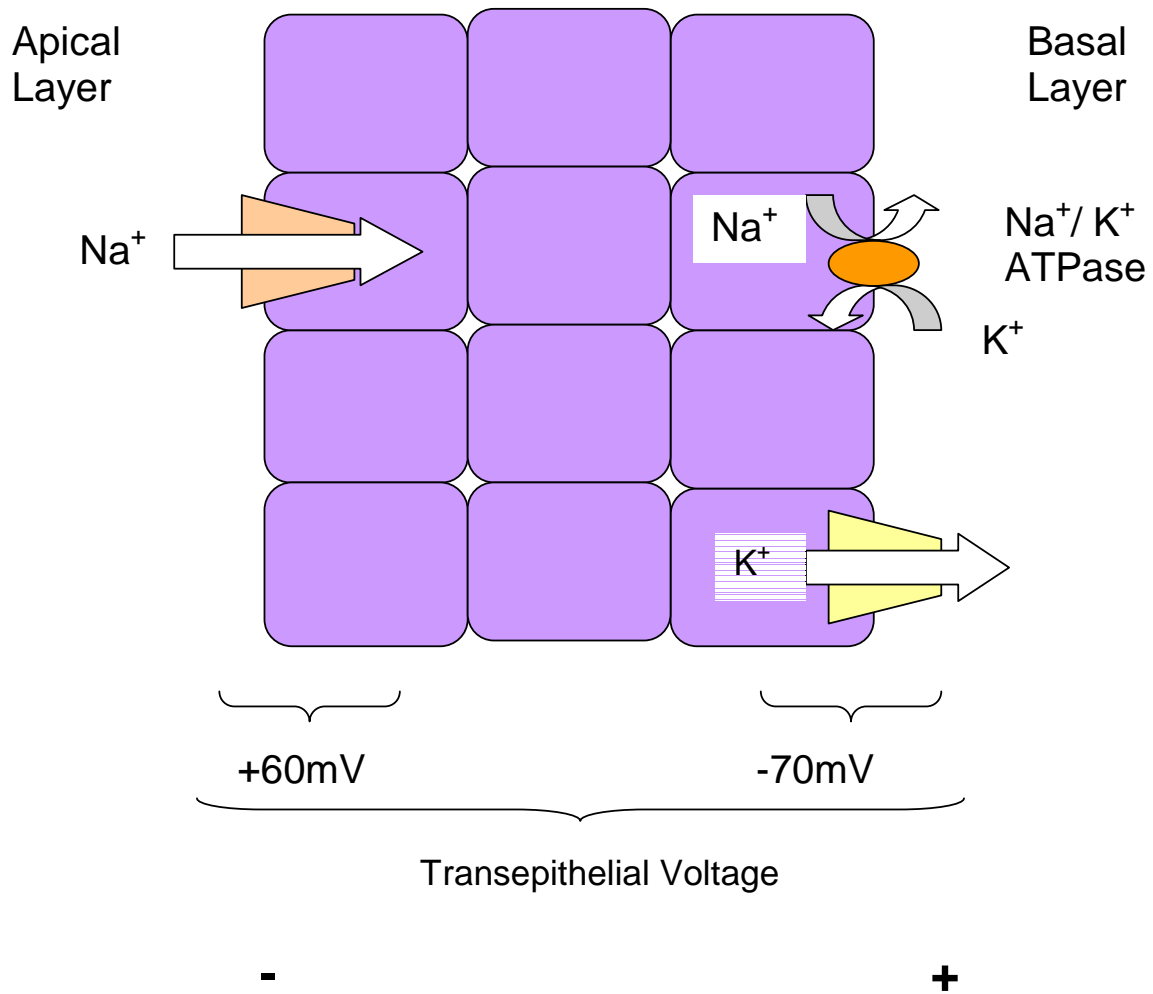Fig 1d Transepithelial voltage across an epithelial surface

Apical
Layer

Basal
Layer

Na^+

Na^+

Na^+/ K^+
ATPase

K^+

K^+

+60mV

-70mV

Transepithelial Voltage

-

+

Fig 1e; Representation of measurements of epithelial electrical properties by a circuit diagram. R_A is the apical membrane resistance and R_B the basal membrane resistance. The sum of these two values is R_{TE} the transepithelial resistance. The difficulty in measuring this absolute value and the voltage and current across the epithelium is illustrated by two further resistances. R_S is the shunt resistance, which represents an alternative route for current either through a hole in the epithelium or around the edge and ideally should be as large as possible. R_C is the resistance of the exvivo circuit consisting of the electrodes, wiring and salt bridges. If R_C is large in relation to the resistance of the voltmeter then the voltmeter underestimates the voltage across the urothelium as there will be a significant potential drop dissipated across R_C. Most voltmeters are of very high resistance nonetheless and the resistance of R_C is usually insignificant compared to the resistance of the voltmeter.
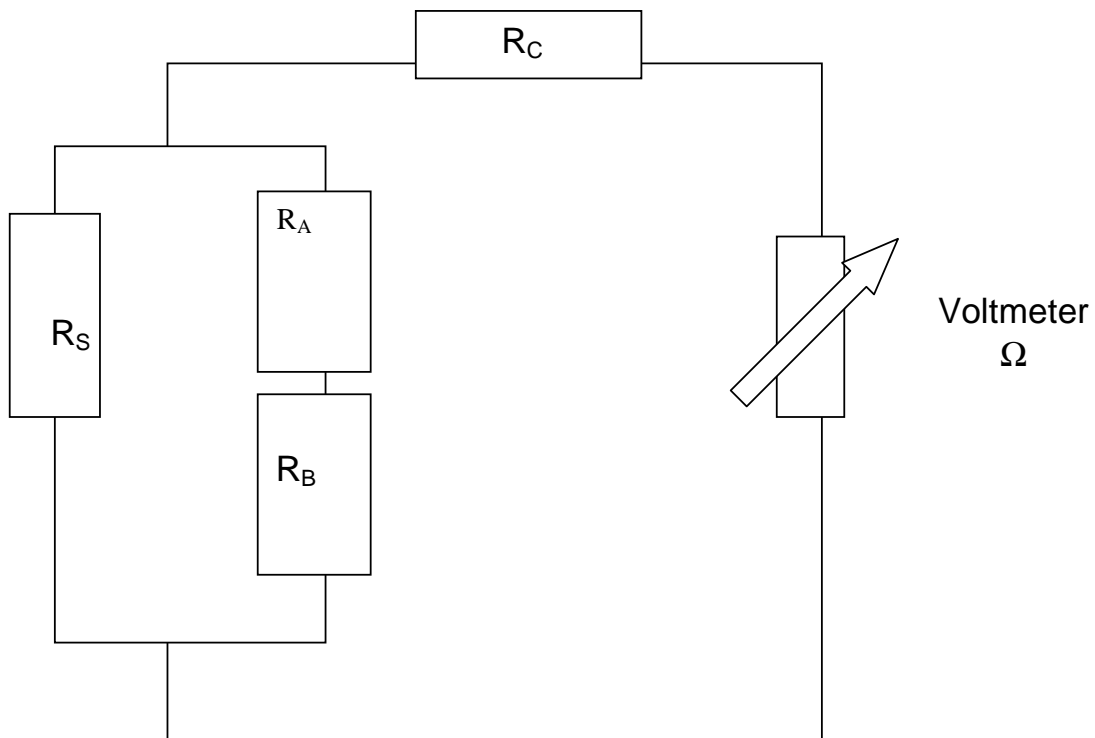
Chapter 2

Methods

2.1 Patient recruitment

Patients undergoing open bladder surgery, general anaesthetic cystoscopy or flexible cystoscopy were recruited to the study with ethical approval (from the Whittington and Moorfields Research Ethics Committee) and informed consent. Patients were classified according to their urinary symptoms in accordance with the International Continence Society classification;

Normal (No urgency and urinary frequency < 8 per day and nocturia < 2 per night.

Overactive Bladder Symptoms (Urinary urgency ± frequency (>8 voids per day) ± nocturia (>2 voids per night), without a known neurological aetiology.

Neuropathic overactive bladder symptoms (Neurological diagnosis for OAB as defined above.

Outflow Obstruction Undergoing TURP or have an enlarged prostate on cystoscopy with IPSS score >7.

The admissions lists and waiting lists were obtained each month. Patients who were on the waiting list, to undergo a rigid cystoscopy, flexible cystoscopy, TURP, TURBT, open cystolithopaxy, cystectomy and CLAM ileocystoplasty as part of their clinical management were written to with a brief lay description of the study asking whether they would be willing to consider helping. A prepaid reply envelope was enclosed along with instructions on how to respond should they be willing to consider providing a bladder biopsy for research purposes during the procedure.

The details of patients who made an affirmative response were recorded on a separate database. Where a patient agreed to provide a biopsy, they were sent a full information sheet and arrangements were made for them to sign a consent form. The usual preoperative assessment was arranged at this attendance. The admission date for biopsy was recorded in the study-monitoring diary. The study instructions relevant to the surgical team were placed in the hospital notes along with a copy of the consent form.

At the time of admission to the day-care unit the surgical staff were warned of the need for a research-related tissue biopsy and the appropriate preparations were effected. The patient's willingness to provide a gift of a tissue biopsy was re-confirmed at the time of this admission by the supervising clinician.

The biopsy sample was collected according to the instructions, which were placed in the medical notes. The collection of the specimen and the reception by the research team was recorded in the hospital notes. The investigator signed the study patient completion page and this was documented in the hospital notes.

A single research biopsy was taken from the dome of the bladder and stored in iced, calcium-free HEPES solution, buffered at pH 7.2 (mM): NaCl 105.4, NaHCO$_3$ 22.3, KCL 3.6, MgCl$_2$ 0.9, NaH$_2$PO$_4$ 0.4, HEPES 19.5 , glucose 5.4 , Na pyruvate 4.5, and experiments were commenced within one hour of collection.

Full thickness samples were taken from patients undergoing open bladder surgery and these samples were used for organ bath experiments where large samples were necessary in order for strips to be cut.

Cystoscopic samples were used for the dispersed detrusor cell experiments.

2.2 Animal Tissue Preparation

Guinea pigs

Male Duncan Hartley albino guinea pigs, weighing between 300-450 g were weighed and sacrificed using cervical dislocation or CO_2 asphyxiation. The bladder was promptly dissected and removed prior to storage in a calcium free physiological solution (Appendix 1 for organ bath work and Appendix 2 for isolated detrusor experiments) and transported on ice to the laboratory.

Rat

Male rats weighing 300 – 350g were weighed and sacrificed using CO_2 asphyxiation. The bladder was promptly dissected and removed prior to storage in a calcium free physiological solution (Appendix 2) and transported on ice to the laboratory.

Bladder preparation

The bladder was incised longitudinally, opened out and mounted under slight stretch on an agar dish using hypodermic needles. The urothelium was dissected away as a single sheet from the underlying detrusor muscle under direct microscopy (x 10 magnification) using blunt dissection with a pair of scissors taking care not to perforate the urothelium.

The urothelium was used for Ussing chamber experiments (section 3).

2.3 Detrusor organ bath experiments

Detrusor muscle strips were cut parallel to the orientation of the muscle fibres from the denuded bladder under direct light microscopy (x10 magnification). Strips were approximately 7-10mm in length and 1mm in width. The detrusor strips were fixed, in a superfusion trough, at one end to a hook and the other to an isometric force transducer (Fig 2A). The tissue was superfused with Tyrode's solution (mM): NaCl 120, NaHCO$_3$ 24, KCl 4, CaCl$_2$ 1.8, MgCl$_2$ 1, NaH$_2$PO$_4$ 0.4, glucose 6.1, Na pyruvate 5, at 37°C with 95% O$_2$-5% CO$_2$, pH 7.4, at 2 ml/minute for 3 hours to ensure equilibration prior to experimental recordings taking place. During this time contractions were generated by electrical field stimulation (EFS: 8 Hz, 3 s trains, 0.1 ms pulse width). When a consistent response was established, dose-response curves were constructed for either carbachol (≤100 µM) or oxotremorine (≤10 µM). All dose response curves were generated by a 3 minute exposure of the agonist at 2ml/minute with a subsequent 30 minutes washout period with tyrodes solution during which tension was seen to return to baseline levels prior to progression with the subsequent agonist concentration. EFS responses were elicited between 1 and 80 Hz. The selective M$_2$ antagonist methoctramine and gallamine (Sigma Aldrich Companies Limited) and the M$_3$ antagonist, 4-DAMP (Sigma Aldrich Companies Limited) were tested on different strips. They were added to the superfusate for 30 minutes after, which agonist responses and EFS were retested as described above. This sequence was repeated for increasing concentrations of antagonist (Fig 2B). On completion of the experiments the detrusor strips were weighed and their length measured.

From these two values an estimated cross sectional area for each strip was calculated. The muscle was assumed to have a density of $1g/cm^3$. Human detrusor strips were tested in tandem where sequential dose response curves were generated as described above in the absence of any antagonist to ensure that there was no significant desensitisation of the tissue during the course of an experiment.

Analysis; the peak force generated during a contraction was measured. A pEC_{50} value was calculated for each strip by curve fitting to the Hill Equation (Kaleidagraph, version 3.5, Synergy Software). For the purpose of curve fitting an assumption of competitive antagonism was made. The maximum was fixed and the slope set as $n_H = 1$. The pK_B value for each antagonist was calculated for each strip via a Schild plot when methoctramine was used as the antagonist. The shortened schild equation (log [antagonist] – log [dose ratio – 1]) was used to calculate the pK_B when 4DAMP or gallamine were the antagonist.

The overall pK_B for each antagonist was taken as the mean of the derived pK_B from each strip. The plotted points on the dose response curves shown represent the mean percentage of maximum contraction at a given agonist and antagonist concentration. The summary Schild plots were derived by calculating the mean dose ratio obtained on muscle strips for each given antagonist concentration. Significance between data sets was assessed with an unpaired t-test and statistical significance was taken as $p < 0.05$. Scatter plots comparing variables obtained from the same muscle strip were analyzed using linear regression (Kaleidagraph, version 3.5, Synergy Software) and statistical significance was tested by ANOVA.
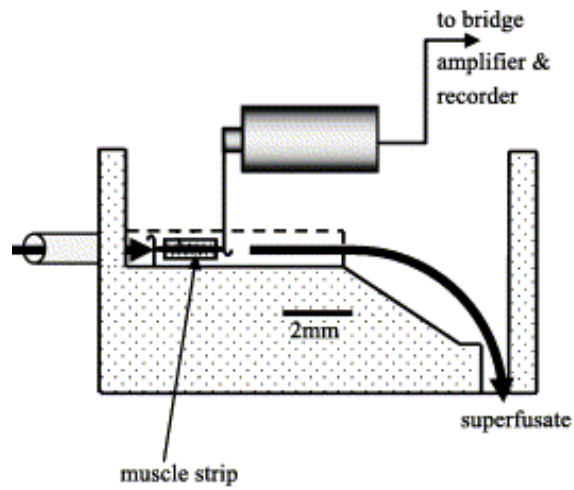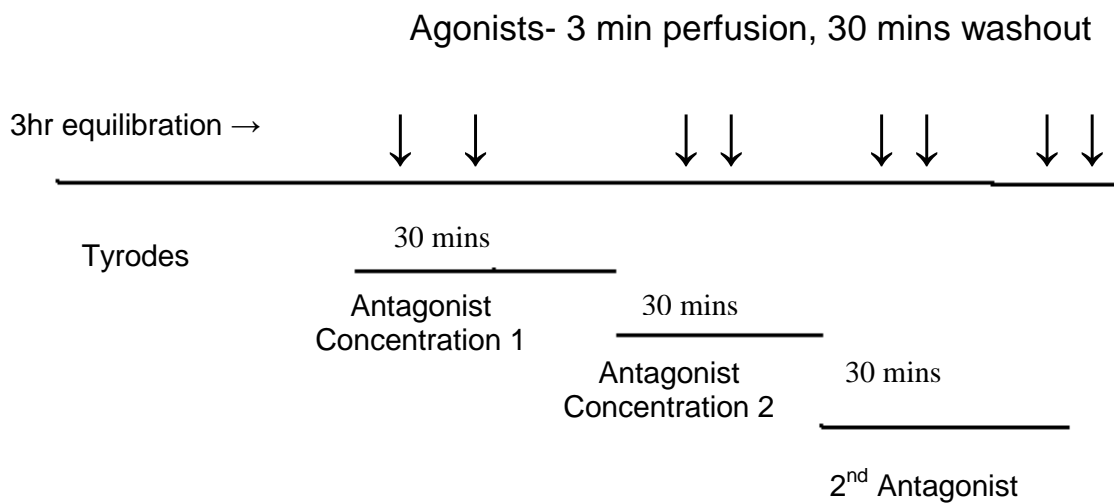
Fig 2A Organ Bath

Fig 2B Experimental Design of Agonist/ Antagonist Experiments

Agonists- 3 min perfusion, 30 mins washout

3hr equilibration →

Tyrodes

30 mins

Antagonist
Concentration 1

30 mins

Antagonist
Concentration 2

30 mins

2nd Antagonist

2.4 Single cell detrusor experiments

The following solution was used for the dispersion of detrusor cells

Calcium free Dispersion Physiological Salt Solution (DPSS):

Salt	Concentration (mM)	g/L
Sodium Chloride (NaCl)	126	7.36
Potassium Chloride (KCl)	6	0.45
Magnesium Chloride (MgCl$_2$)	1.2	1.2ml from 1M stock
Hepes	10	2.4
Glucose	11	2.0

pH made to 7.2 with 10M stock Sodium Hydroxide (NaOH).

Dispersion:

Material all obtained from Sigma-Aldrich.

- The urothelium was dissected off the bladder sample to leave the smooth muscle layer.

- The smooth muscle was cut into 0.5mm x 0.5mm pieces using sharp dissection.

- Enzyme dispersal solution was made up in 10ml DPSS, consisting of 10mg papain, 15mg collagenase type 1A, 50mg Bovine serum albumin (BSA), 2.5 mM Dithiothreitol (DTT).

- Smooth muscle pieces were added to a glass vial with enzyme dispersal solution and incubated for 30mins at 37°C.

- The smooth muscle pieces were removed from solution and washed for 5 mins in DPSS. Repeated in fresh DPSS for a further 5 mins.

- Sample left to stand for 10 mins at room temperature.

- The tissue was dispersed using 2 different wide bore Pasteur pipettes. Starting with widest to loosen up tissue and then moved on to smaller one to break up individual cells.

- The dispersed myocytes were centrifugated at 13000rpm for a short pulse

- 20µl cell suspension were pipetted onto polylysine slide. Each slide was used to test a different experimental condition. An aliquot of cells were pipetted onto a haemocytometer slide (see figure 2c, page 61) for assessment of cell counts. Cells were stained with an equal volume of trypan blue for assessment of their viability.

- 20µl of DPSS solution containing variable concentrations of Ca^{2+} (0-1mM were added to the cell suspension and the cells were allowed to equilibrate on the slides for an hour. During this period they were kept at 0°C in a humidified container.

- 20µl of DPSS solution containing the same concentration of calcium and a variable concentration of pharmacological agent was added to each slide.

- The experiment was terminated through the addition of 1% acroelin. Experiments with rat detrusor were terminated after 30 seconds whilst experiments with guinea pig and human detrusor were terminated after 3 minutes.

- Coverslips were added to the slides. The edge of the coverslips was sealed with nail varnish.

Figure 2c Haemocytometer grid

1mm
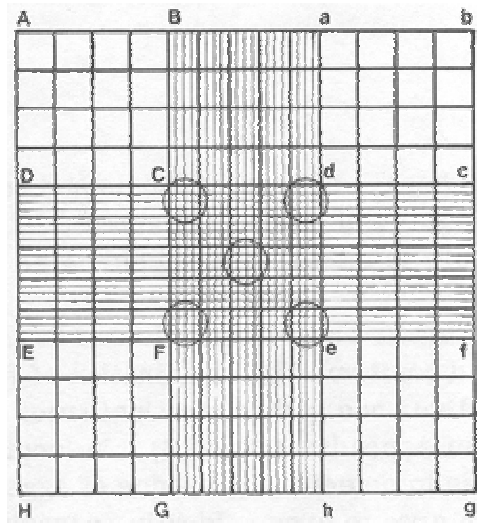
The haemcoytometer grid depicted above was used to count muscle cell numbers. The area of the grid above is 9mm^2. The chamber overlying the grid has a depth of 0.1mm therefore the total volume of the chamber is 0.9mm^3 or 0.9μl.

Data analysis

Digital photographs were taken of isolated cells under x 200 optical magnification using a Nikon 4500 camera. The aim was to include at least 100 cells per slide. For photographs the flash was turned off, the camera was set to focus to infinity and the maximum optical zoom was applied. The microscope was used to focus the image on the camera viewfinder prior to capture. A photograph of a 2mm graticule at x200 magnification was taken at the same camera setting.

Images were transferred from the camera to PC by a USB connection and opened and analysed using image J software (NIH laboratories).

Individual detrusor cells were identified by their long spindly and undulating appearance and were digitally measured using a segmented line.

These cell lengths were copied to an Excel spreadsheet and converted to an actual length using a graticule measurement for reference.

Cell lengths were analysed by PRISM 3.0 to assess their distribution, mean and SEM for statistical analysis. Differences were compared between groups using the student t test and statistical significance was taken as $p \leq 0.05$.

2.5 Urothelial Ussing chamber experiments

The opened bladder biopsies were placed mucosal surface up onto a petri dish and the entire sheet was secured under slight tension using 18g hypodermic needles. The edge of the urothelium was picked up using a pair of forceps and gently dissected way from the detrusor layer.

The urothelium was placed between the two Perspex halves of a 0.35mm lumen Ussing chamber (USS2S - Small Chamber manufactured by World Precision Instruments). The urothelium was orientated such that the luminal surface was facing the V1 test electrode and the basolateral surface was facing the V0 (zero potential reference electrode).

The voltage electrodes were placed in close proximity on either side of the urothelium and the current electrodes further away (see figure 2d).

Construction of voltage electrodes.

The voltage and current electrodes salt bridges were prepared in the same manner. A mixture of 0.5mM KCl and agar was made and this was warmed in a water bath to 80°c. The heated mixture was poured into the perspex electrode and allowed to cool and set hard. The salt bridge electrodes were stored in 0.5 mM KCl solution containing a silver pellet.

For experiments, the agar filled salt bridges were secured onto the correct port of the Ussing chamber. The luer tipped electrodes were secured into the salt bridges and connected to the amplifier.
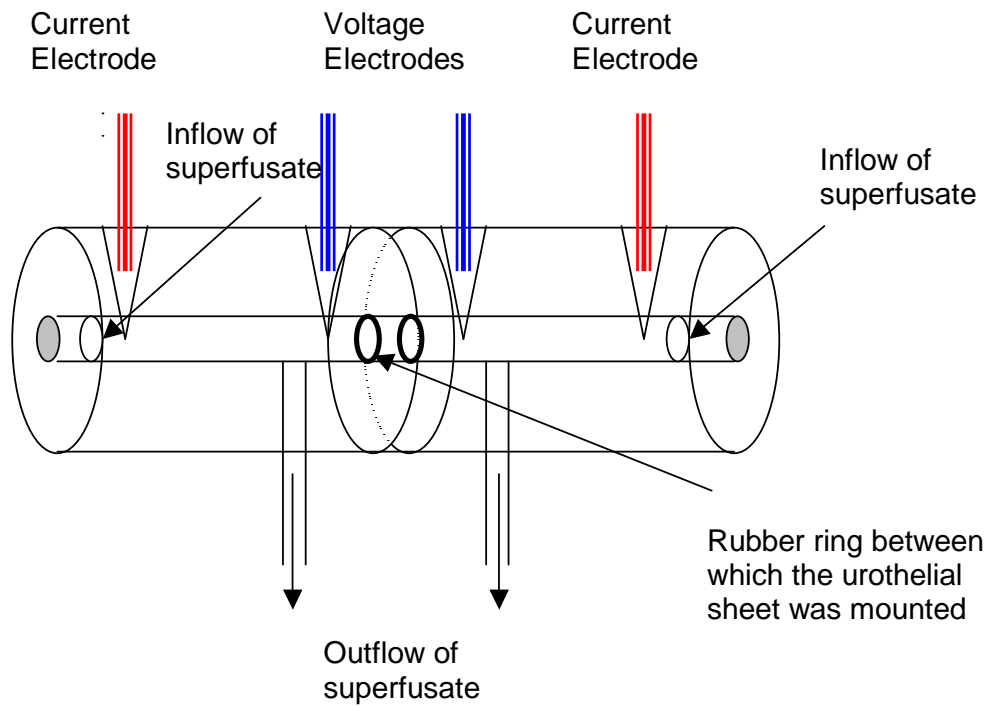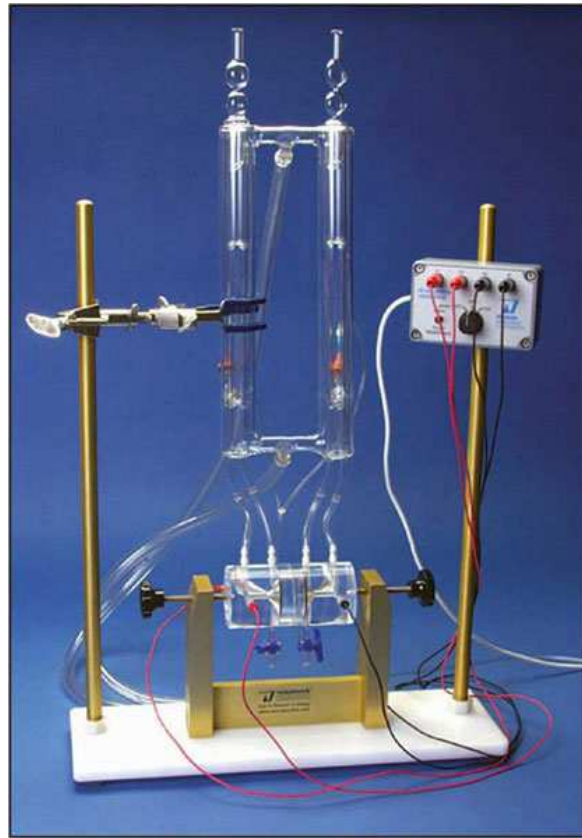
The urothelium was allowed to equilibrate for two hours. During this period each surface of the urothelium was independently perfused at the same rate

under a gravitational generated pressure gradient with tyrodes solution containing 95% O_2 , 5% CO_2 at 37°C . A World Precision Instruments DVC1000 – Two channel Voltage/Current clamp with timer function was utilised. During equilibration the voltage clamp was set to passively measure the voltage across the urothelium.

After equilibration the voltage clamp was set to cycle every 2.5 seconds between voltage and current clamping. During voltage clamping, the clamp potential was set to zero volts and current measurements were taken. During current clamp, the clamp current was set to zero and the potential difference was measured. Continuous voltage and current readings were fed from the voltage clamp to a chart recorder set at 30cm/hr and to an oscilloscope to observe the signal waveform.

During experimentation, pharmacological agents were independently added to the tyrodes solution perfusing each surface of the urothelium. The new perfusing solution was introduced without introducing bubbles into the tubing by switching to a new perfusing chamber controlled by a 3 way tap (diagram 3). Each agonist was added to the urothelium for a period of 10 minutes prior to switching back to tyrodes solution for a half hour washout. Agonists were added sequentially starting with the lowest solution prior to the addition of any antagonists.

Fig 2d Arrangement of electrodes across the Ussing chamber.

Current
Electrode

Voltage
Electrodes

Current
Electrode

Inflow of
superfusate

Inflow of
superfusate

0.0

Rubber ring between
which the urothelial
sheet was mounted

Outflow of
superfusate

Chapter 3

Results

3.1 Organ Bath Experiments- Human Detrusor.

Twelve patients were recruited to the study. Five of these had neuropathic overactive bladder and seven patients had an overactive bladder. Those with neuropathic bladder were undergoing CLAM augmentation cystoplasty (This procedure involves anastomosing small bowel on to the bladder to increase reservoir capacity and reduce voiding pressures). The overactive bladder biopsies came from patients undergoing either CLAM augmentation cystoplasty or cystectomy for cancer who described overactive bladder symptoms in accordance with the ICS definition. All biopsies were taken from the dome of the bladder.

Patients; Table 4 shows a summary of study patient details. The mean age was 45.6, ranging from 19 to 70 years of age. Four patients were male and eight were female. The mean age of the neuropathic patients was 32 and the overactive patients 53. The difference was not statistically significant. Likewise none of the other variables in table 4 differed significantly between neuropathic and overactive patients; therefore the data were pooled for analysis.

Contractile characteristics; The contractility of bladder strips was determined by taking the force generated by 100µM Carbachol divided by the cross sectional area of the strip, expressed as millinewtons mm^{-2} ($mN\ mm^{-2}$). The mean value obtained for contractility was $24mN\ mm^{-2}$ but there was wide variability. 100 µM carbachol and 10µM oxotremorine produced maximal contractions of indistinguishable amplitude (percentage difference in mean

force = 6.6% ±10.6% SEM). The pEC_{50} value (mean ± SD) obtained for carbachol and oxotremorine induced contractions were not significantly different; 5.8 ± 0.4 (n=15) strips and 6.1 ± 0.51 (n=7) strips respectively. Figure 3.0 shows an example of dose responses achieved by varying the doses of carbachol.

Repeated dose response curves (n=5) generated by carbachol did not show any significant change in EC_{50} and therefore there was no evidence of tissue desensitisation during the experimental protocol described (Fig 3.1).

M_2 inhibition; Dose-response curves were constructed for carbachol agonism in the presence of the M_2 inhibitor methoctramine at concentrations ranging from 10 -1000 nM (Figure 3.2). Schild plot analysis produced a pK_B value for methoctramine of 8.2 ± 0.3, against carbachol-induced contractions, which is compatible with M_2 inhibition (Fig 3.2b). M_2 inhibition was also tested using the allosteric inhibitor gallamine (Fig 3.3). The pK_B for gallamine against carbachol-induced contractions was derived by the shortened Schild equation and was 5.87 (n=2). This value is compatible with M_2 inhibition. Methoctomine was used to antagonise oxotremorine mediated contraction (Fig 3.4) and a pK_B value of 8.26 ± 0.7 was derived, again compatible with M_2 inhibition (Fig 3.4b)

M_3 inhibition; Similarly 4-DAMP was used to antagonize carbachol and oxotremorine contractions (Fig 3.5 and 3.6). The pK_B value for 4DAMP was

8.80 ± 0.4 against carbachol induced contractions and 9.8 ± 0.6 against oxotremorine contractions (analysis by shortened Schild equation).

Electrical Field Stimulation (EFS); In all strips the maximal response produced by EFS (60 Hz) was significantly less than that achieved by 100 μM carbachol (49% $\pm$ 16%) (Fig 3.7a). Increasing concentrations of methoctramine produced dose-dependent reduction of EFS force (Fig 3.7b). 10nM methoctramine produced a mean reduction in force of 64% at 4 Hz, 40% at 8 Hz and 34% at 40 Hz (p< 0.05 in all cases).

Association between agonist potency and M_2 activity. The potency of oxotremorine as determined by pEC_{50} values was significantly associated with the pK_B values obtained for methoctramine against oxotremorine (p<0.05, Fig 3.8). A similar comparison for carbachol points to the same trend though statistical significance was not achieved.

Age related trends; With increasing patient age, there was reduced agonist potency of oxotremorine (Fig 3.9b) and EFS became less effective at producing a response relative to the agonist (Fig 3.9d), p< 0.05 in both cases. There was a trend for reduced contractility (Fig 3.9a) and reduced pK_B of methoctramine for oxotremorine (Fig 3.9c), and increase in the half maximal frequency of stimulation (Fig 3.9e), though not statistically significant. These trends need to be treated with caution as the sample small sizes are small

and patients with neuropathic OAB are often (though not significantly so in this data set) younger.

Table 4: Summary statistics from human bladder strips.

Diagnosis	Patient no	Gender	Age	Max force mN/mm2	Carbachol Contractions				Oxotremorine Contractions			EFS MAX%
					pEC_{50}	pK_B Meth	pK_B 4DAMP	pK_B Gall	pEC_{50}	pK_B Meth	pK_B 4DAMP	
Neuropathic	1	M	20	10.5	5.63	8.29			6.26	8.13	9.7	
	2	F	65	22.1	5.36				5.37	7.31		
	2	F	65	8.5	5.52	8.45						
	3	F	19	1.4	5.52	8.15			6.02	8.13		
	4	F	25	50.4					6.63	9.26	9.64	68
	4	F	25	96.1					6.69	9.24		74
	5	F	31	2.20	5.66	7.99	8.73		6.19	8.15		40
	5	F	31	36.7	5.7							
Mean			32	28	5.6	8.2	8.73		6.2	8.4	9.67	60.6
SD			19.1	32	0.12	0.20			0.48	0.75	0.04	18.1
Overactive	6	F	43	70.9	5.73	7.81	9				9.22	
	7	F	36	9.3	5.97	8.04	8.3				10.7	
	8	M	61	9.8	5.4	8			5.5	8.02		
	8	M	61	11.6	5.43	7.95						
	9	F	42	30	5.87	8.45	8.88					56
	10	F	66	21.4	6.8							33
	11	M		2.7	6.71	8.61						
	12	M	70		5.97			5.6				36
	12	M	70	0.3	5.87			6.14				37
Mean			54.6	19.5	5.97	8.14	8.73	5.87	5.5	8.02	9.96	40.5
SD			14.3	23	0.49	0.31	0.37				1.04	10.
Overall												
Mean			45.6	24	5.8	8.2	8.7	5.87	6.1	8.3	9.8	49.1
SD			19.3	27	0.43	0.26	0.31		0.51	0.70	0.63	16.7

Fig 3.0; Agonist responses recorded from a human detrusor strip.

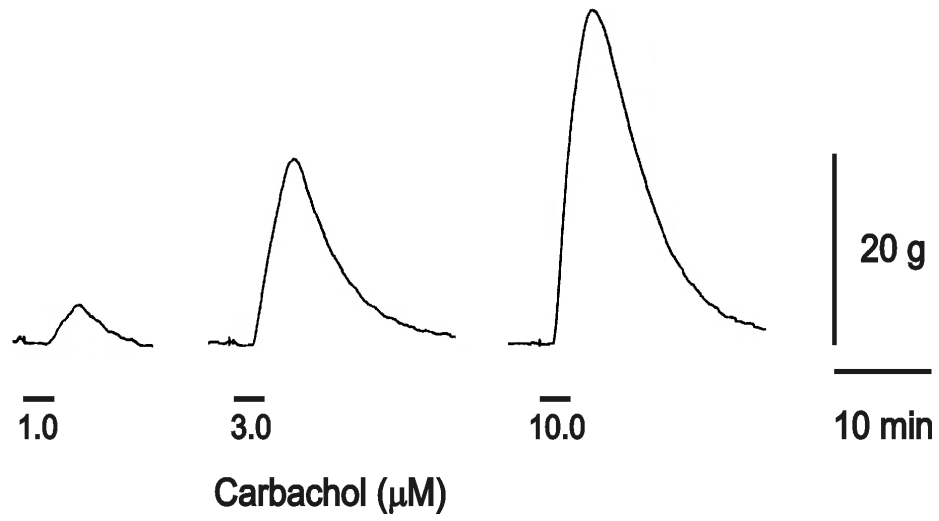1.0   3.0   10.0   10 min

Carbachol (μM)

20 g

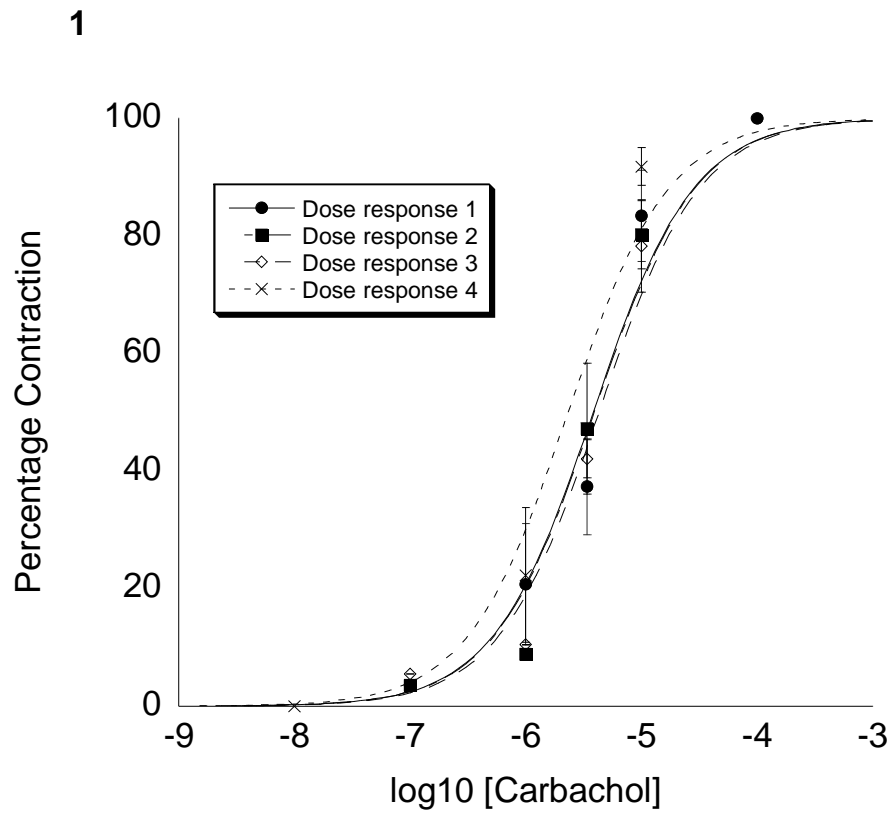Fig 3.1 Repeated Carbachol dose response curves in the absence of an antagonist (n=5).

1

Fig 3.2a Methoctramine antagonism of carbachol induced contractions (mean $\pm$SEM, n=15 muscle strips) and Schild plot, Fig 2b pK_B=8.20, H_n =0.54.
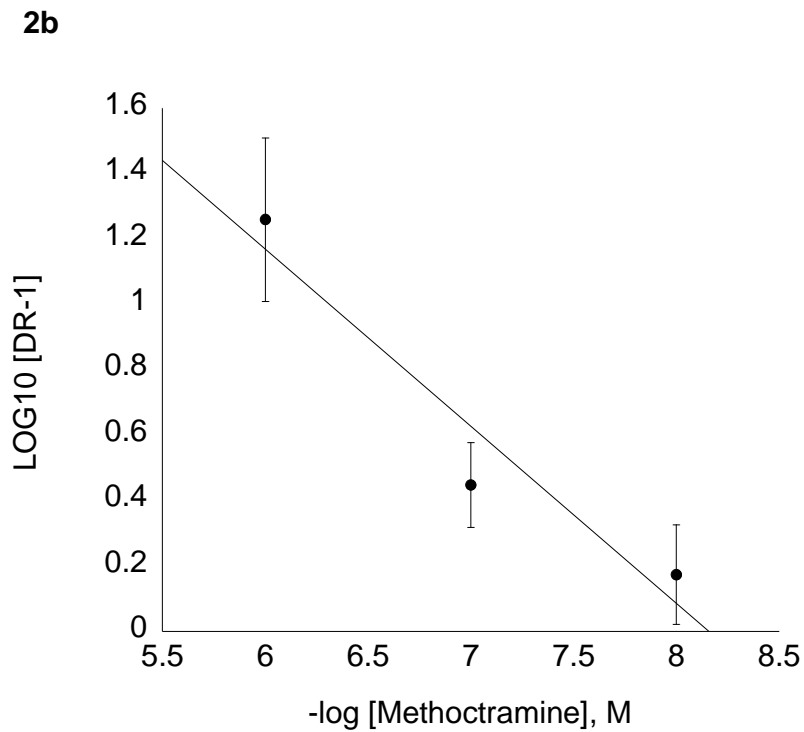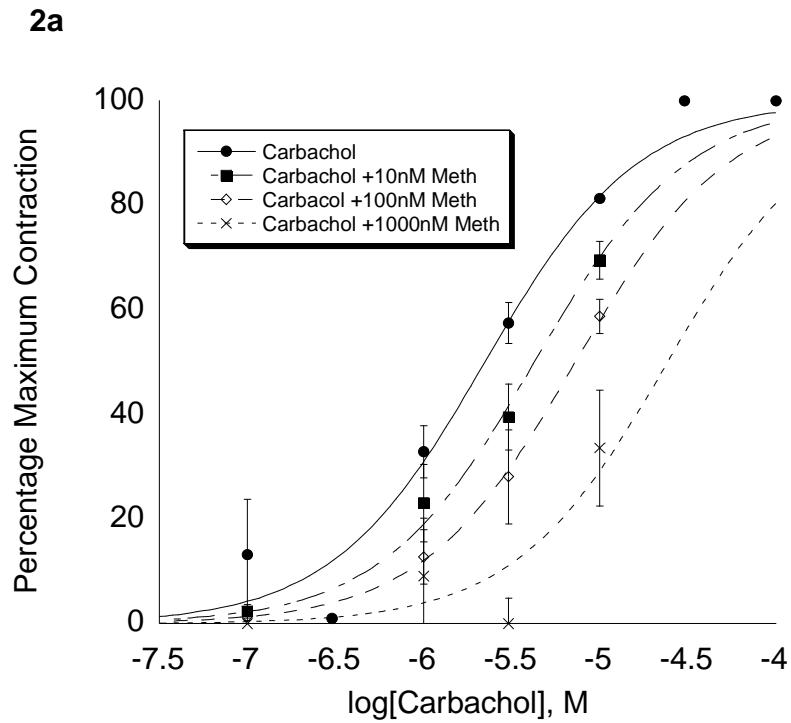
2a

2b

Fig 3.3a Gallamine inhibition of carbachol induced contractions (n=2), $pK_B=5.8$ by shortened Schild equation.

3
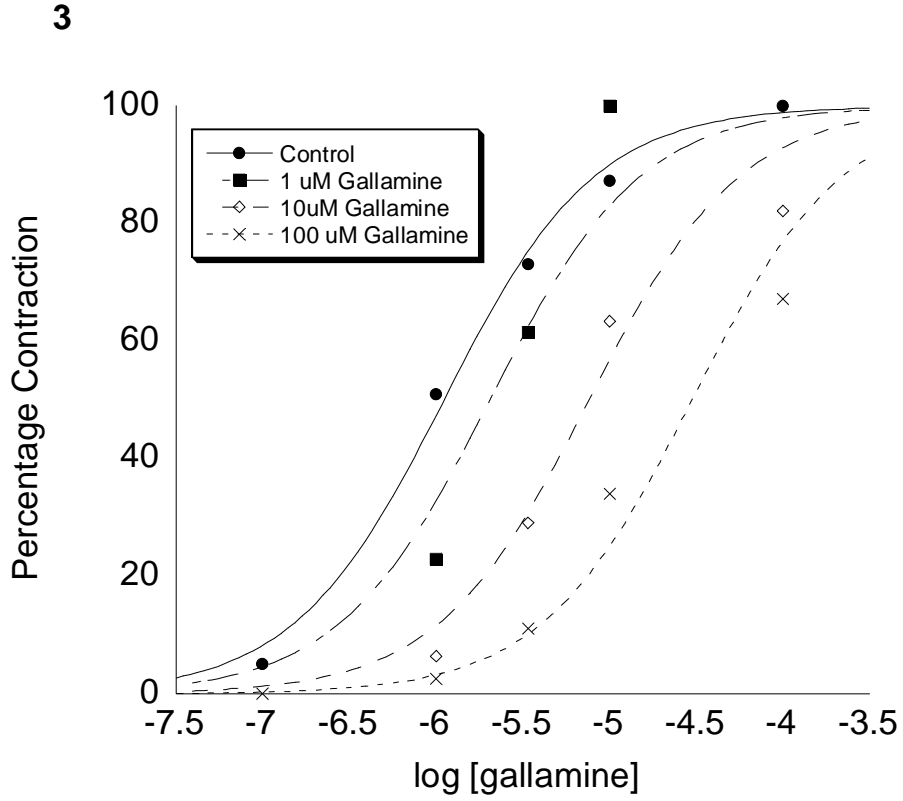
Fig 3.4a Methoctramine inhibition of oxotremorine induced contractions (mean ±SEM, n=7) and Schild plot Fig 3.4b pK$_B$=8.4, H$_n$ =0.85, N=7.

4a
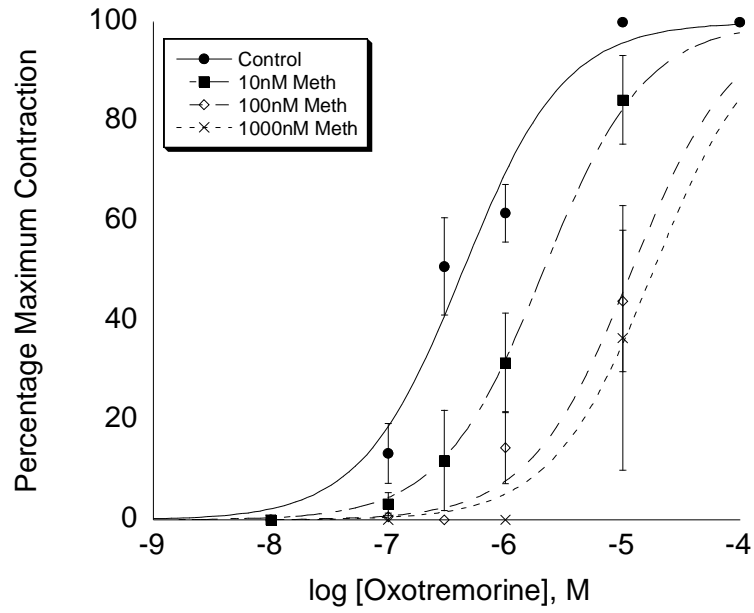
4b
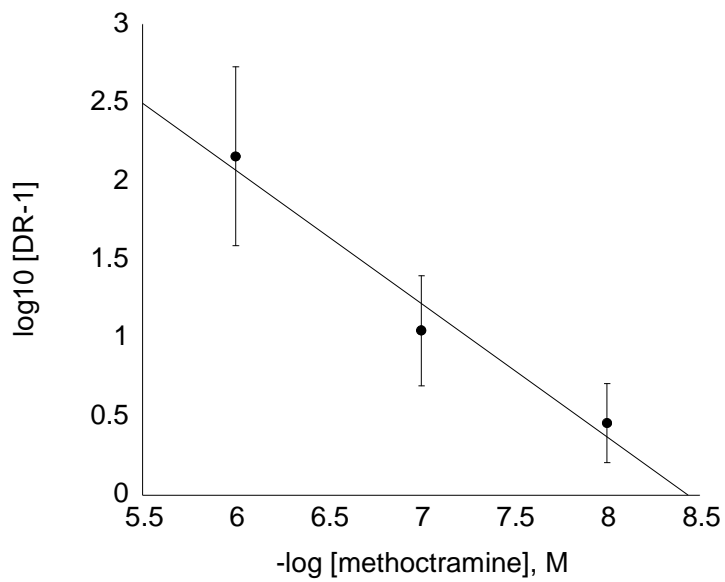
Fig 3.5; 4 DAMP inhibition of carbachol induced contractions (mean ± SEM, n=3), (pK$_B$ by shortened Schild equation=8.80.

5
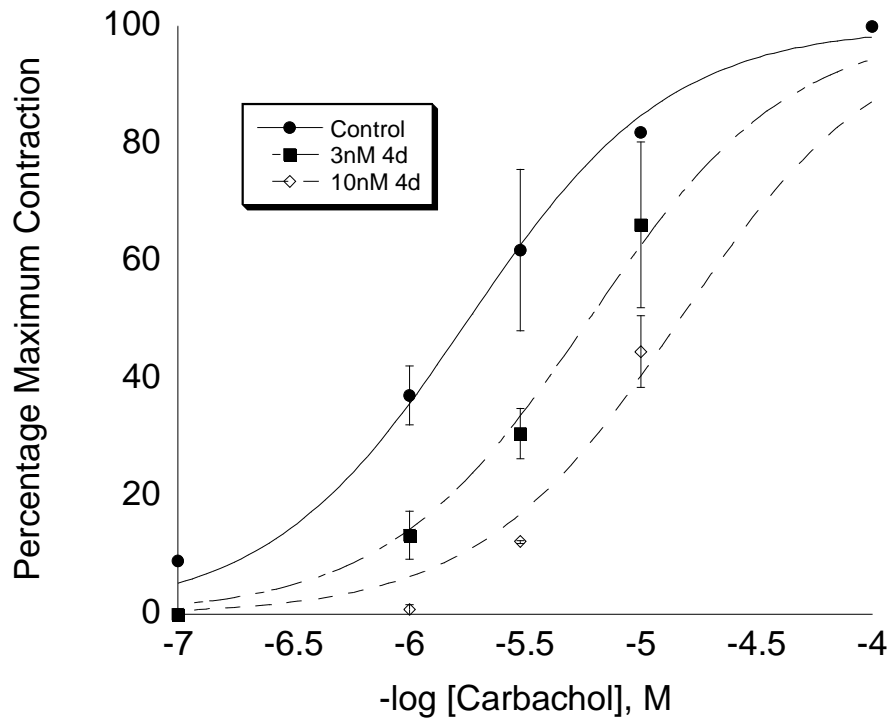
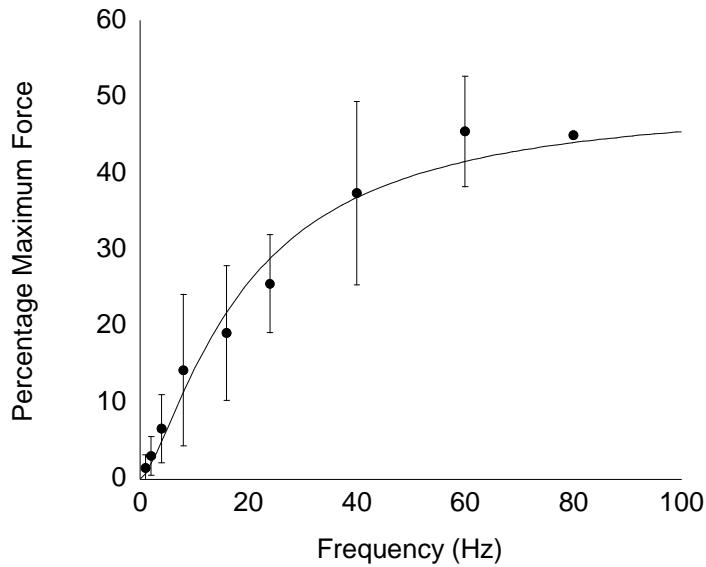Fig 3.6, 4 DAMP inhibition of oxotremorine induced contractions (mean ± SEM, n=4).

6
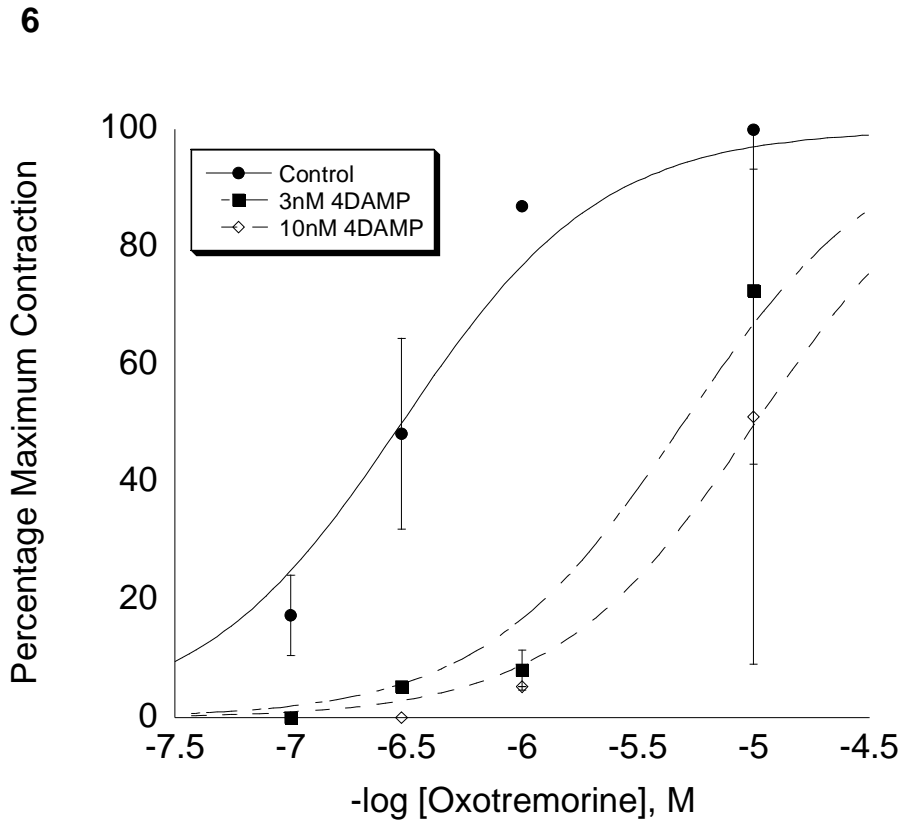
Figure 3.7a. EFS frequency responses expressed as a proportion of contractions produced by 100µM carbachol (n=8, mean ± SD). Fig 3.7b the effect of increasing concentrations of methoctramine on EFS at 4, 16 and 40 Hz (n=5, mean ± SD, force as a percentage of 100 µM carbachol contraction).
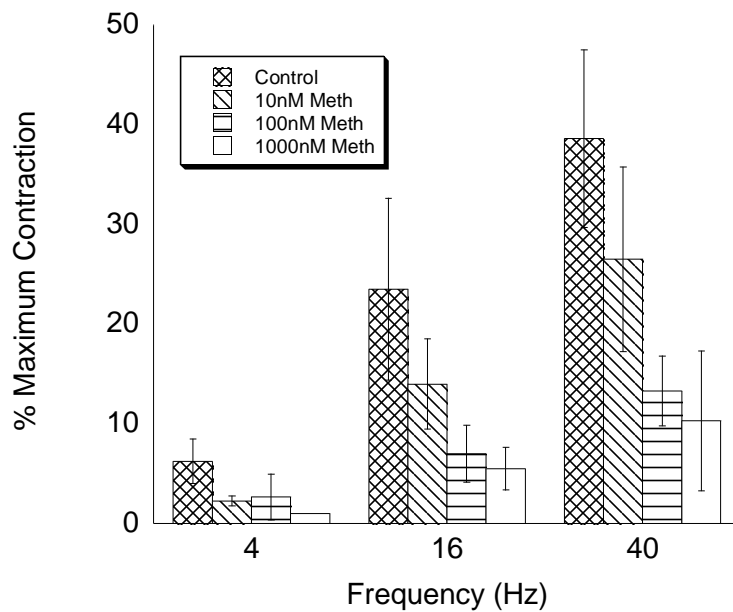
7a

7b

Fig 3.8. Agonist potency versus pK_B of methoctramine. R=0.90 for oxotremorine contractions. R=0.56 for carbachol contractions.
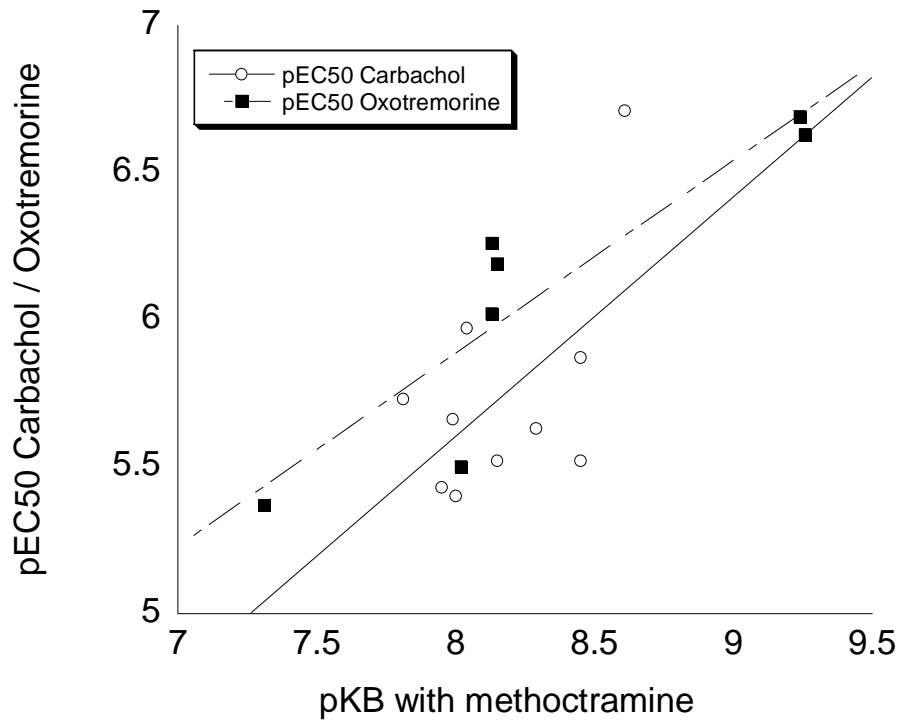
8

Fig 3.9. Age related trends in muscarinic activity; a) contractility, maximum force per cross sectional area generated by 100μM carbachol (n=15). b) Potency of oxotremorine (n=7, r=0.84, p<0.05). c) pK_B of methoctramine with oxotremorine (n=7, r=0.61).d) Change in EFS maximum response relative to 100μM carbachol (n=7, r=0.80, p<0.05). e) Frequency that produces half maximal response f50.
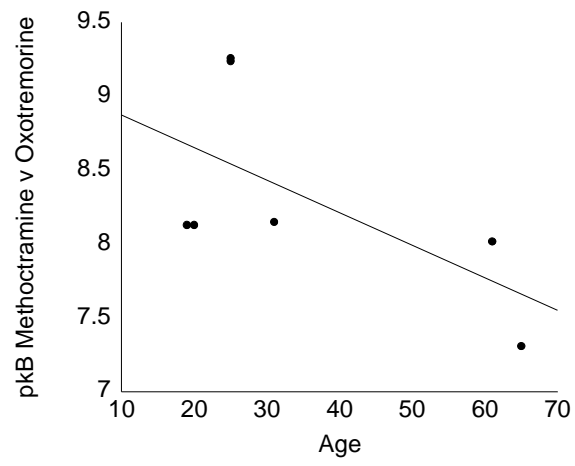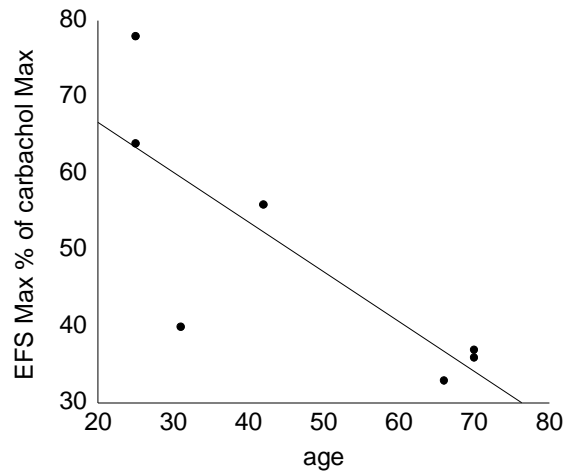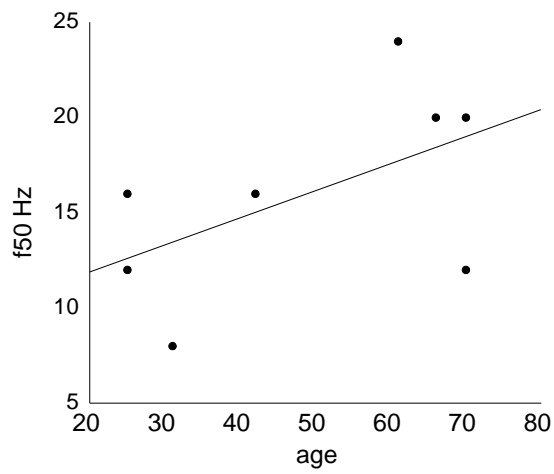
9a

9b

9c

9d

9e

92
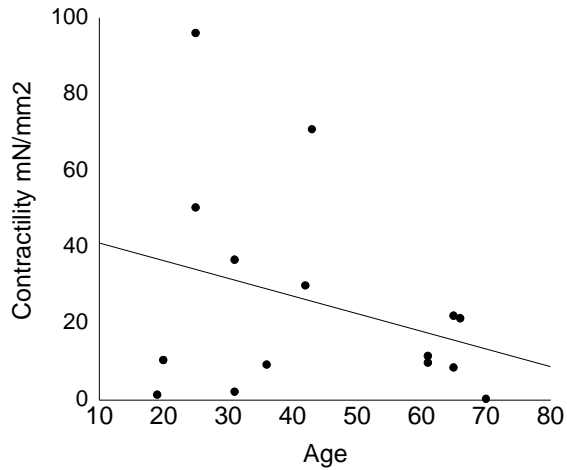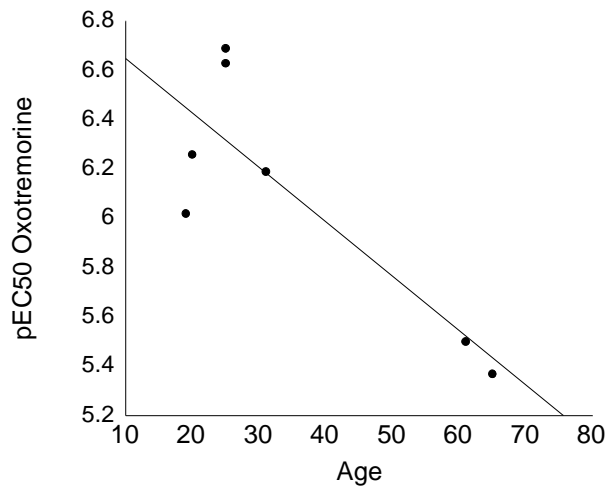
3.2 Organ bath experiments- Guinea Pig detrusor

The Guinea pig detrusor strips produced maximal contractions in response to 100µM Carbachol, with an average force per unit cross sectional area of 19.1 mN/mm^2 but there was wide variability – 3.78 to 66.6 mN/mm^2. 10uM Oxotremorine similarly elicited maximal contractions (96.6% $\pm$ 4.6% of that elicited by 100µM Carbachol, n=7). However, carbachol was a significantly less potent agonist than oxotremorine (mean $\pm$ SD; pEC_{50} = 5.34 $\pm$ 0.29 (n=19) and pEC_{50} = 6.39 $\pm$ 0.53 (n=7) respectively, p<0.05, Figure 3.10).

M_3 inhibition of carbachol contractions. Carbachol contractions could be competitively inhibited by the addition of the M_3 preferential antagonist 4 DAMP (Fig 3.11). An experimental pK_B of 8.6 $\pm$ 0.46 (n=7) with H_n = 1.16 was obtained.

M_2 inhibition of carbachol contractions. Carbachol contractions could not be competitively inhibited by either of the two M_2 antagonists, gallamine or methoctramine (Fig 3.12 and Fig 3.13),

M_2 inhibition of oxotremorine contractions (Fig 3.14)*;* Oxotremorine contractions were inhibited by the M_2 antagonist methoctramine. Near maximal inhibition was achieved with the lowest dose of inhibitor utilized. Results from shortened Schild plot analysis produced a pK_B of 8.57 $\pm$ 0.45.

Electrical Field Stimulation (EFS); Electrical field stimulation produced contractions that were on average 91.6% $\pm$ 9.7% of the maximal contraction that were achievable by 100µM Carbachol (Fig 3.15).

EFS inhibition with methoctramine; Methoctramine inhibited EFS as shown in Fig 3.15. 10nM - 1000nM had no effect on contractions elicited at 4Hz

frequency.10000nM reduced contractions by 68.4% from control. At 16hz and 40HZ frequency EFS was significantly reduced by all concentrations of methoctramine of 100nM and above (30.3% and 15.6% reduction with 100nM methoctramine at 16 and 40Hz respectively, p<0.05). At low frequency of stimulation ATP may have been the predominant neuro transmitter released and hence the relative ineffectiveness of M_2 inhibition. At higher frequencies there is greater likelihood of direct stimulation of the muscle by the electrical field stimulation and hence the reduced effectiveness of M_2 inhibition again.

EFS inhibition with 4DAMP (Fig 3.16). At 4Hz stimulation, 4 DAMP at concentration up to 100nM did not produce any significant inhibition of EFS. 3nM 4 DAMP significantly reduced contractions elicited at 16Hz and 40Hz (36.7% and 14.4% respectively). Like the experiments with methoctramine, at low frequencies 4DAMP had diminished effect, likely to be caused by the decreased cholinergic component to neurotransmission at lower frequencies. At higher EFS frequencies, there is increased direct stimulation of the muscle tissue as demonstrated by increased Tetrodotoxin (TTX) resistant component. (TTX leads to abolition of neurotransmitter release).

Inhibition of Carbachol and Oxotremorine Contractions with forskolin (Fig 3.17 and Fig 3.18)*;* 10μM Forskolin had no effect on carbachol induced contractions either when utilized in isolation or in combination with 10nM Methoctramine. However, 10μM Forskolin significantly inhibited oxotremorine contractions (Fig 3.18). This inhibition was indistinguishable from that achievable by a 10nM Methoctramine or a combination of 10μM Forskolin and 10nM Methoctramine; (pEC_{50} ± SD; Control 6.5 ± 0.28, 10μM Forskolin 6.03 ± 0.15, 10nM Methoctramine 5.93 ± 0.15, 10nM Methoctramine + 10μM

Forskolin 6.02 ± 0.17). This suggests that methoctramine and forskolin are acting by, and saturating the same mechanism to produce inhibition of contractions, namely cAMP elevation.

Combined antagonism of carbachol with 4DAMP and gallamine (Fig 3.19 and Schild plot Fig 3.20). Although gallamine had no effect on carbachol induced contractions when applied in isolation, when combined with 4DAMP it seemed to moderate the inhibition to a degree. At a concentration of 10μM gallamine, 4DAMP inhibition was slightly attenuated at $pK_B=7.95$ $H_n=1.15$ versus $pK_B=8.6$ $H_n=1.34$ when 4DAMP was used in isolation. At a higher concentration of 100 μM gallamine, gallamine seemed to synergise 4DAMP inhibition with a $pK_B=9.96$ $H_n=0.5$.

Fig 3.10 Carbachol and Oxotremorine Dose response in Guinea pig detrusor.
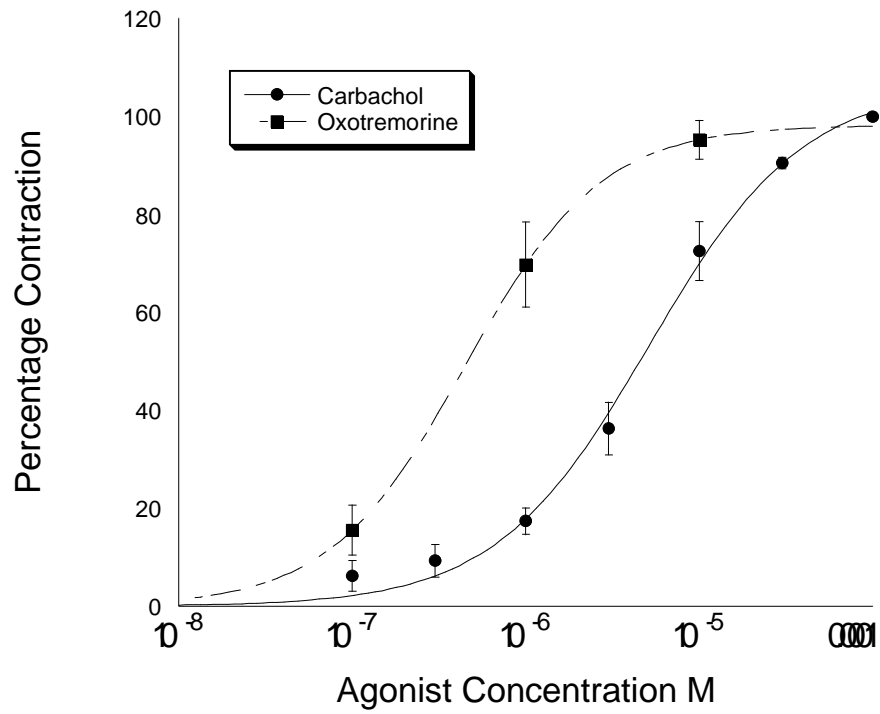
Fig 3.11; The effect of 4 DAMP on Carbachol induced contractions (n=7) pK_B =8.6, H_n=1.15.

The effect of 4DAMP on carbachol contractions
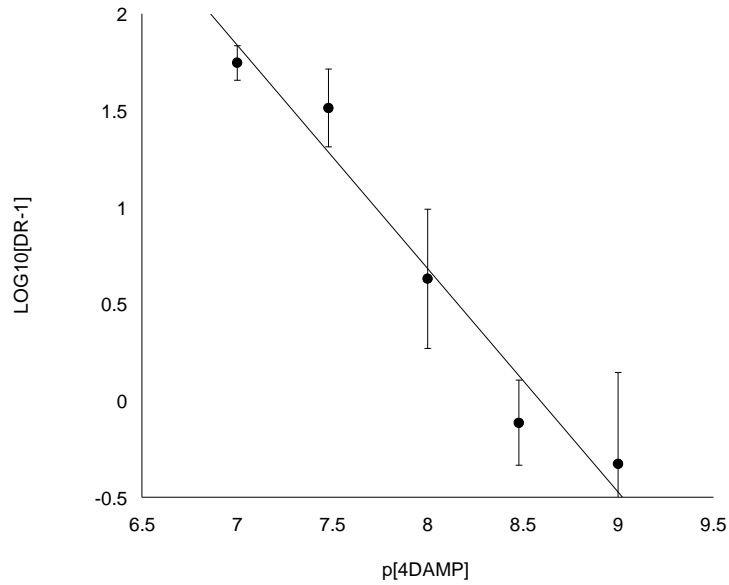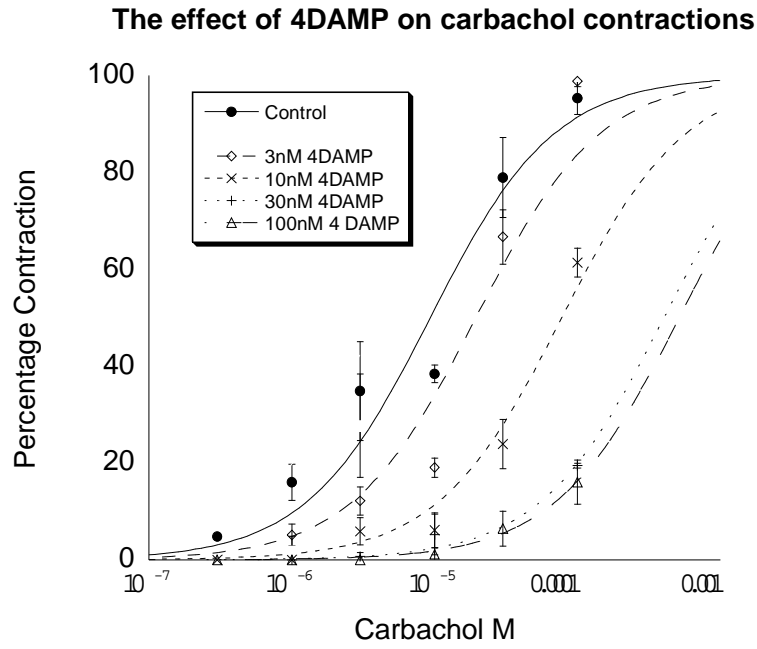
Fig 3.12; The effect of gallamine on carbachol induced contractions (n=7).
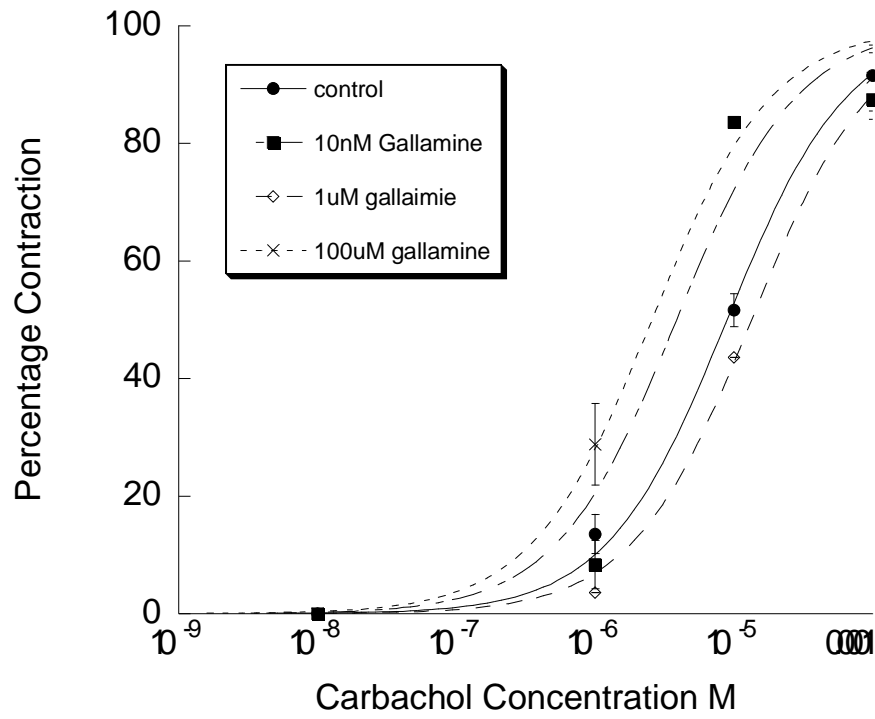
Fig 3.13. The effect of methoctramine on Carbachol Contractions.
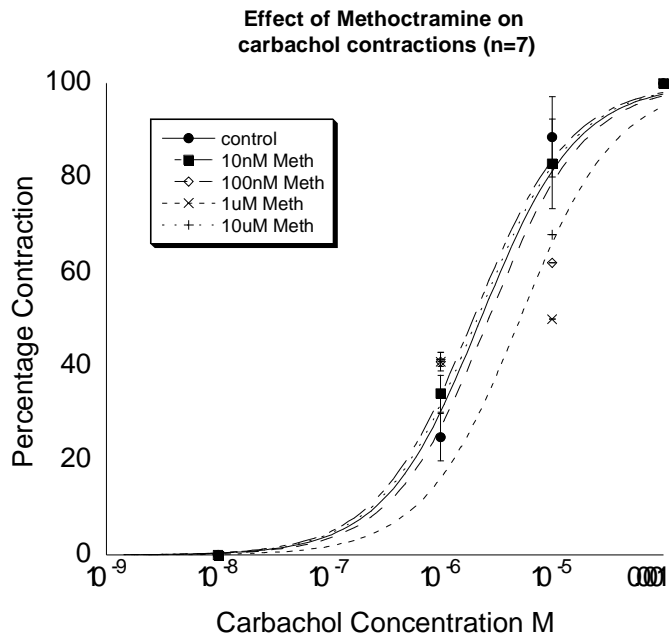
Effect of Methoctramine on carbachol contractions (n=7)

Fig 3.14a Methoctramine inhibition of oxotremorine induced contractions.

Rffect of methoctranine on Oxotremorine Contractions (n=7)
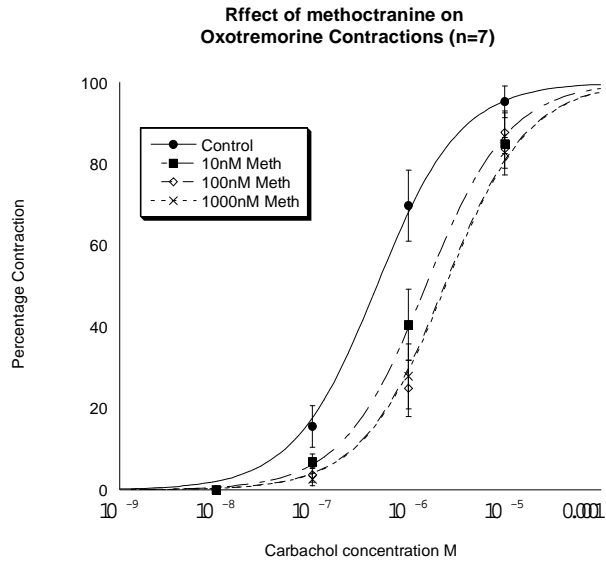
Fig 3.14b Schild analysis of methoctramine inhibition of oxotremorine induced contractions, pkb=8.57 $\pm$ 0.45 (SD) by shortened schild equation.

The efect of methoctramine on oxotremorine (n=7)
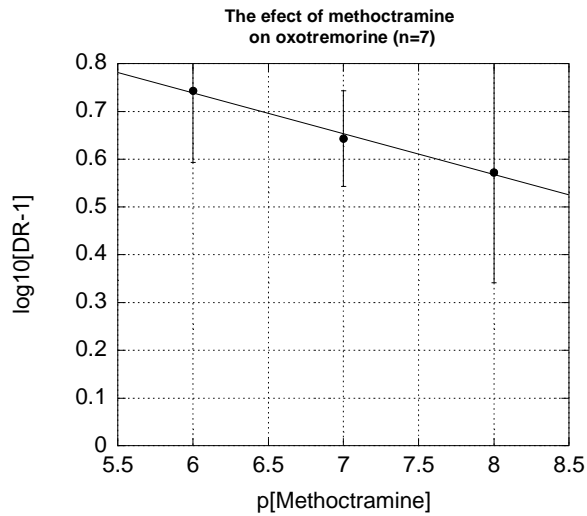
Fig 3.15; Effect of methoctramine on EFS (n=6).

Effect of Methoctramine on EFS
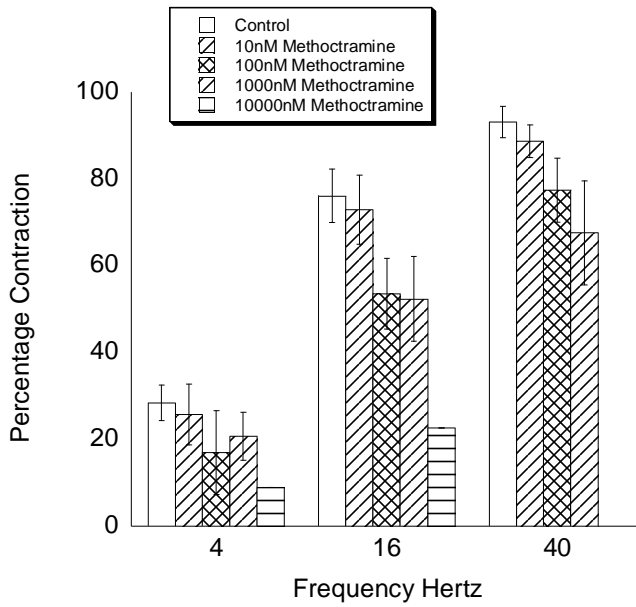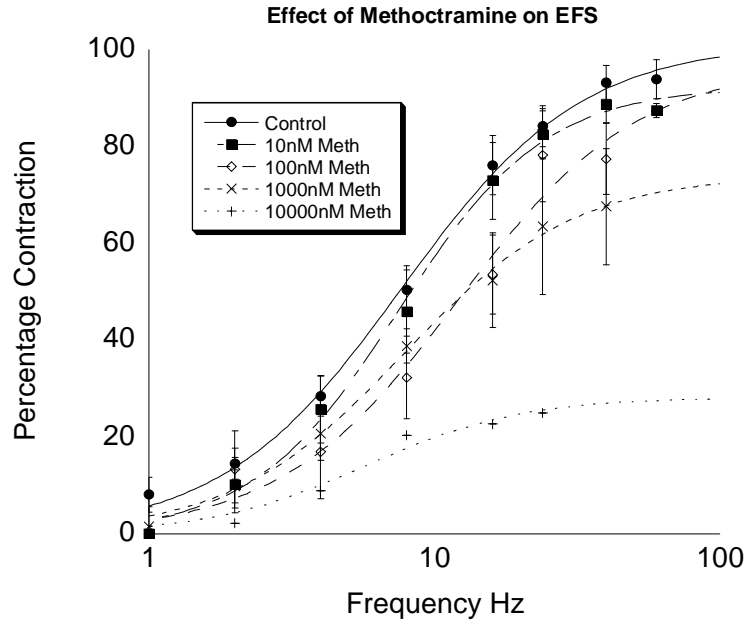
Fig 3.16; The effect of 4DAMP on EFS (n=6).

4 DAMP inhibition of EFS
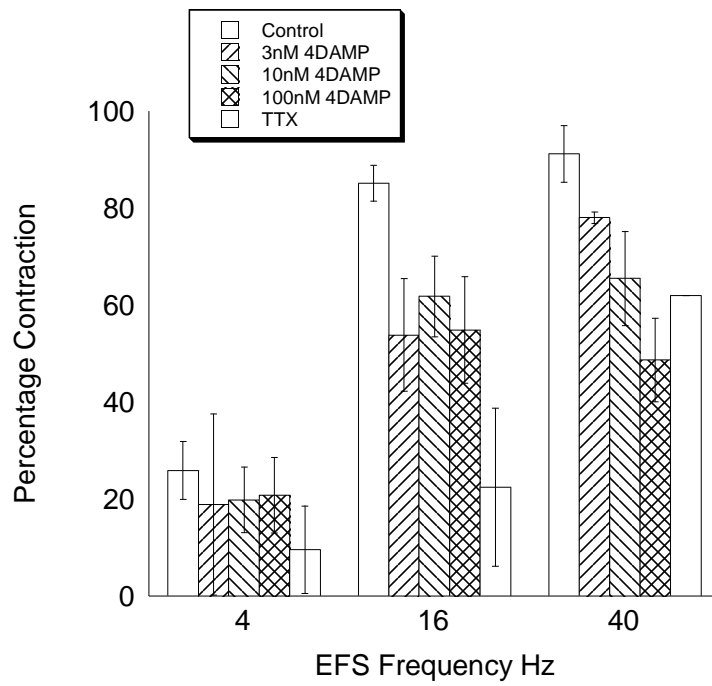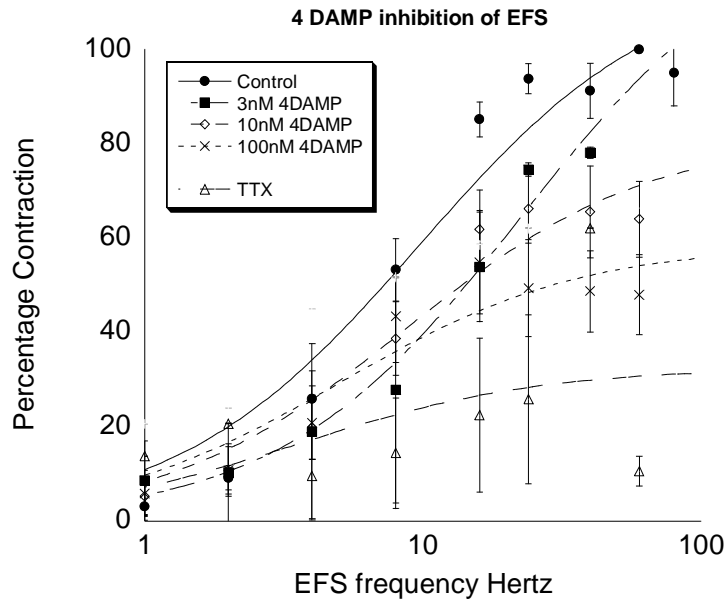
Fig 3.17 The effect of forskolin on oxotremorine contractions (n=5).
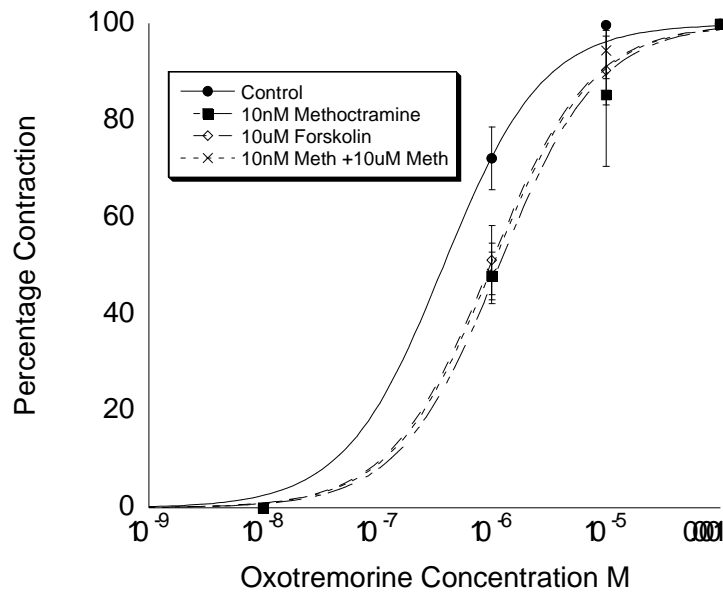
Fig 3.18; The effect of forskolin on carbachol contractions (n=4).
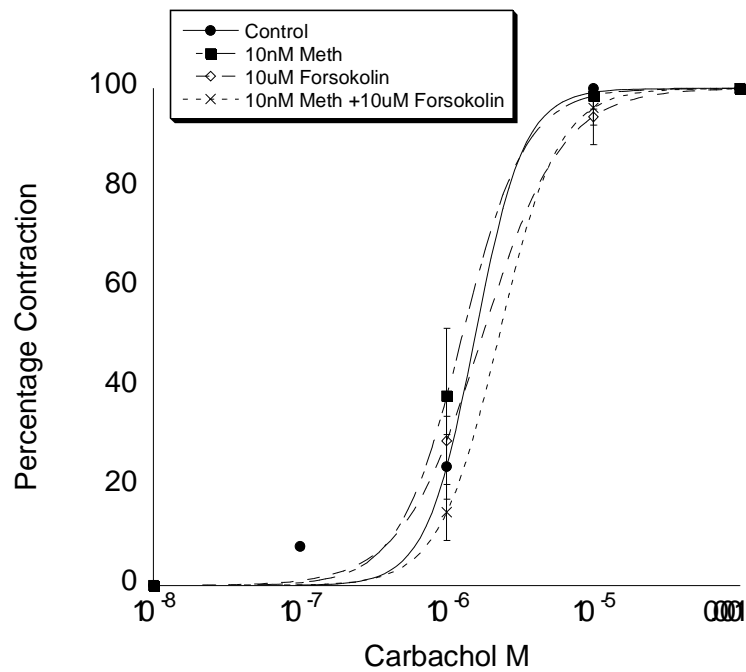
Fig 3.19; Combined antagonism of gallamine and 4-DAMP.

a) 10 μM gallamine (n=4)
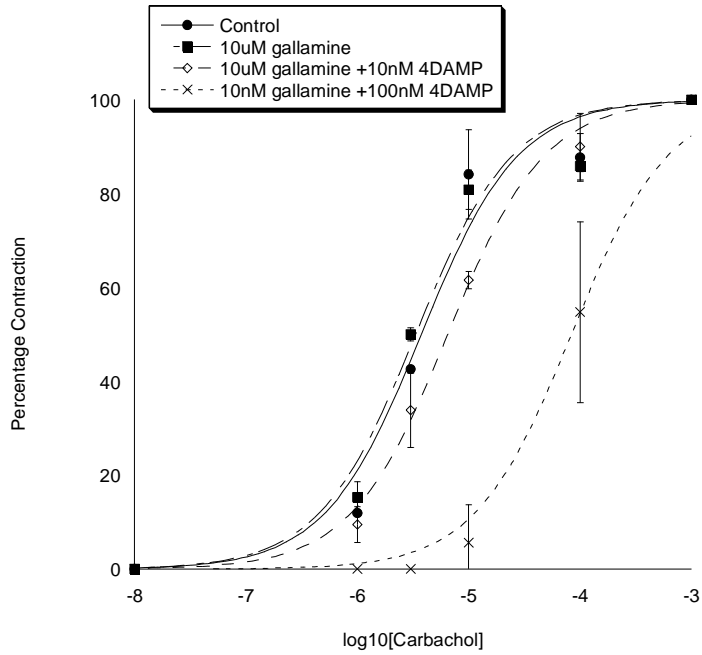
b) 100 μM gallamine (n=2)
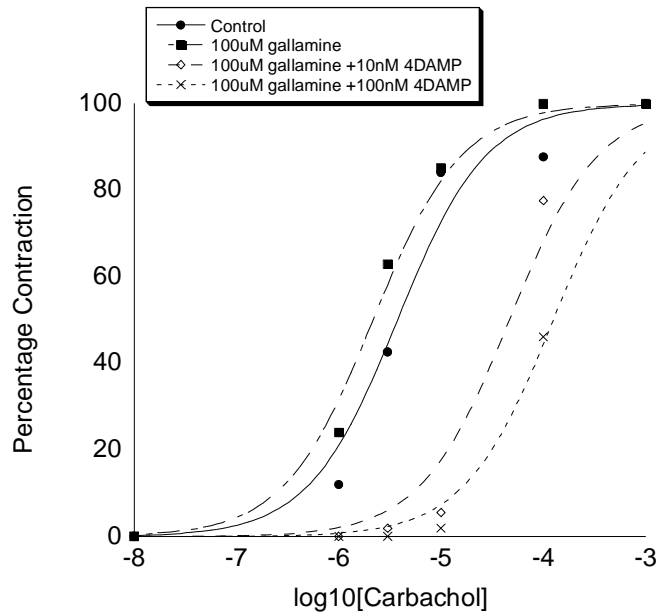
Fig 3.20 Schild plot of the antagonism of carbachol with 4DAMP in the absence of gallamine, the presence of 10µM gallamine and 100µM gallamine. pK_B=7.95 H_n=1.15, pK_B=8.6 H_n=1.34 and pK_B=9.96 H_n=0.5 respectively.
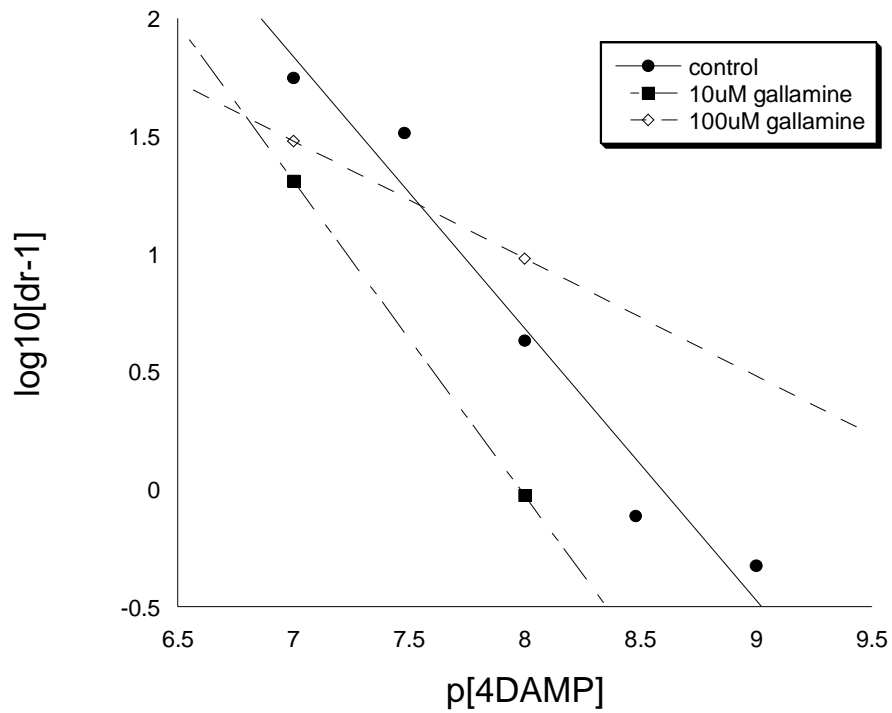
3.3 Isolated detrusor cell experiments

Fig 3.21 shows a typical micrographic image of isolated human detrusor myocytes, obtained from a patient with overactive bladder symptoms. Typically cell counts of over 100 were sought for each experimental condition. The micrograph demonstrates the variability in length and girth of isolated mycocytes and hence the need for large sample sizes. Figure 3.22 shows rat detrusor myocytes stained by H+E (x400 magnification).

Figure 3.23 shows a comparison of detrusor cell lengths between human, guinea pig and rat detrusor tissue carried out in the absence of Ca^{2+}. Values are shown on a relative scale but demonstrate that cell lengths were normally distributed. Guinea pig cells (mean $\pm$ sem = 258 $\pm$ 11) were significantly longer than rat cells (223 $\pm$ 11), which were significantly longer than human cells (193 $\pm$ 8), all $p<0.05$.

Figure 3.24 demonstrates the effect of altering $[Ca^{2+}]$ between 0 and 0.5mM on resting cell lengths. An increase of $[Ca^{2+}]$ to 0.25mM and 0.5mM results in a significant shortening of resting cell lengths from 248 $\pm$ 11 to 198 $\pm$ 9 (relative units $p<0.05$).

Figure 3.25 demonstrates carbachol dose response curves carried out in rats (n=3). A pEC_{50} of 6.1 was obtained which is slightly lower than that obtained in organ bath experiments pEC_{50} = 5.34 $\pm$ 0.29 albeit in the guinea pig.

Figure 3.26. Methoctramine inhibition of carbachol mediated contractions in the presence of 0.5mM Ca^{2+} (n=1). A pK_B of 9.23 was obtained, however, the plot does not demonstrate parallel shift that would be indicative of competitive inhibition.

Figure 3.27; Oxotremorine dose response curves (n=2) carried out in the presence of 0.5mM Ca^{2+}. The greatest degree of muscle cell relaxation was obtained in the presence of 0 mM Ca^{2+} and 10^{-5} M Ca^{2+} channel blocker nicardapine, perhaps indicating the presence of Ca^{2+} ions in the dispersal solution that have arisen from an intracellular source either due to trans membrane efflux or cell lysis as a result of the dispersal process or perhaps due to contaminants in the solution.

A comparison between carbachol (n=3) and oxotremorine (n=2) dose response curves in the rat detrusor is shown in figure 3.28. Oxotremorine was slightly more potent pK_B=6.21 versus 5.05. This is a similar trend to that which was established in organ bath experiments both in human and guinea pig detrusor strips.

Human detrusor experiments: Carbachol dose response curve in 0.5 mM Ca^{2+} and 0.5mM Ca^{2+} and 10nM methoctramine (Figure 3.29, n=1, patient symptomatic with OAB). pK_B =5.68, this was very similar to the value previously obtained with organ bath experiments of 5.8 $\pm$ 0.4). However, no evidence of M_2 inhibition was manifest in this patient, as 10nM methoctramine did not produce any discernable shift in the dose response curve.

Figure 3.30 shows the resting and contracted detrusor cell lengths from 26 patients who provided cystoscopic biopsies. All experiments were carried out in 0.5mM ca^{2+}. The resting length was determined when no other drug was added. The fully contracted length was determined by the addition of 100 μM Carbachol. Patients with outflow obstruction had significantly longer resting cell lengths than OAB patients and controls (mean ± sem; 64.6. ± 1.45, 56.5 ± 1.67 and 48.6 ± 1.67 respectively). Patients with OAB showed a decreased contractile response with carbachol indicating that patients with OAB may either contract less or have a higher resting tone i.e. the resting muscle cell length was precontracted and closer to the fully contracted state but this needs to be confirmed through relaxing the muscle with antagonists.

Fig 3.21 Micrograph of human detrusor cells (unstained) and graticule x200 magnification.
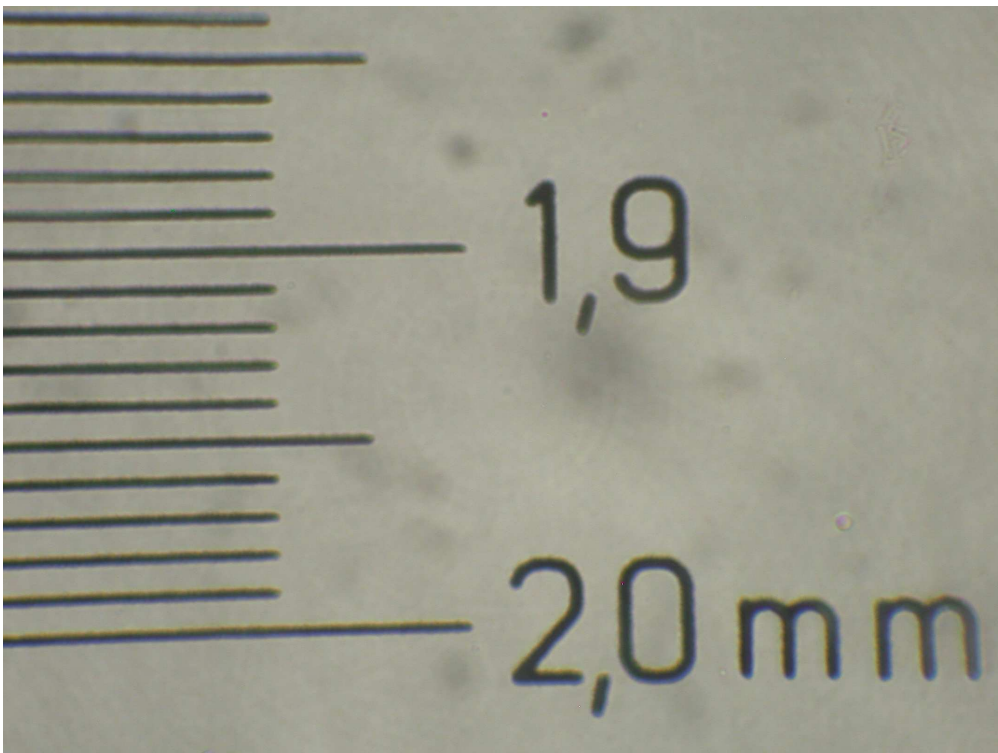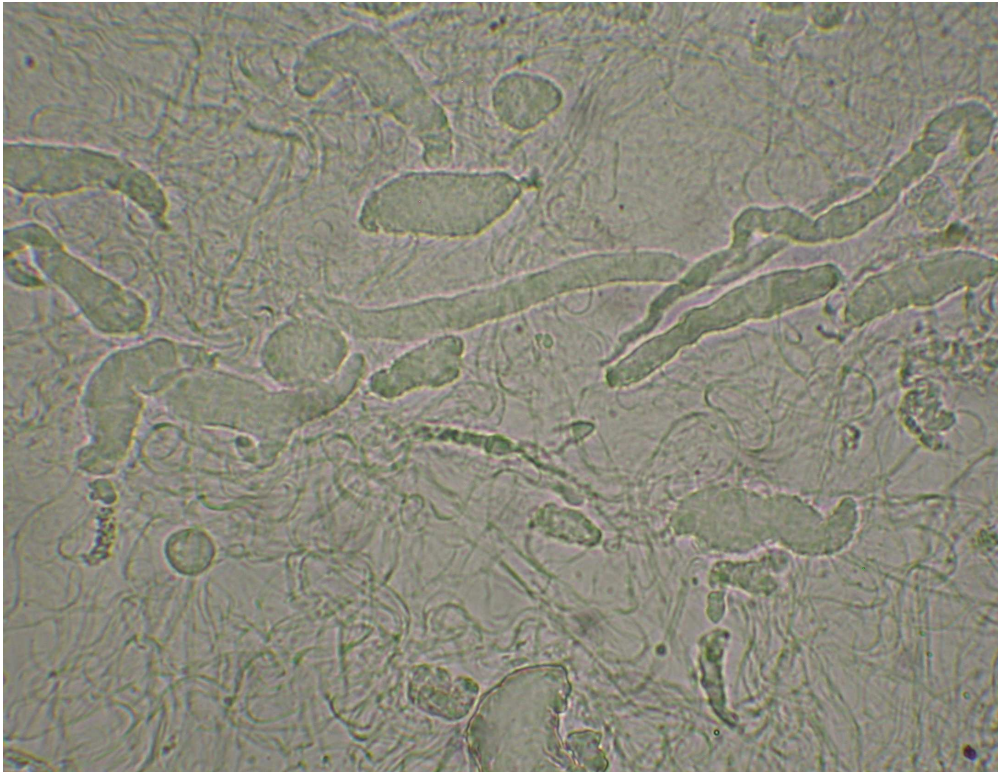
Fig 3.22 gram stain of rat detrusor cells, x 400 magnification (carried out by Linda Churchill).
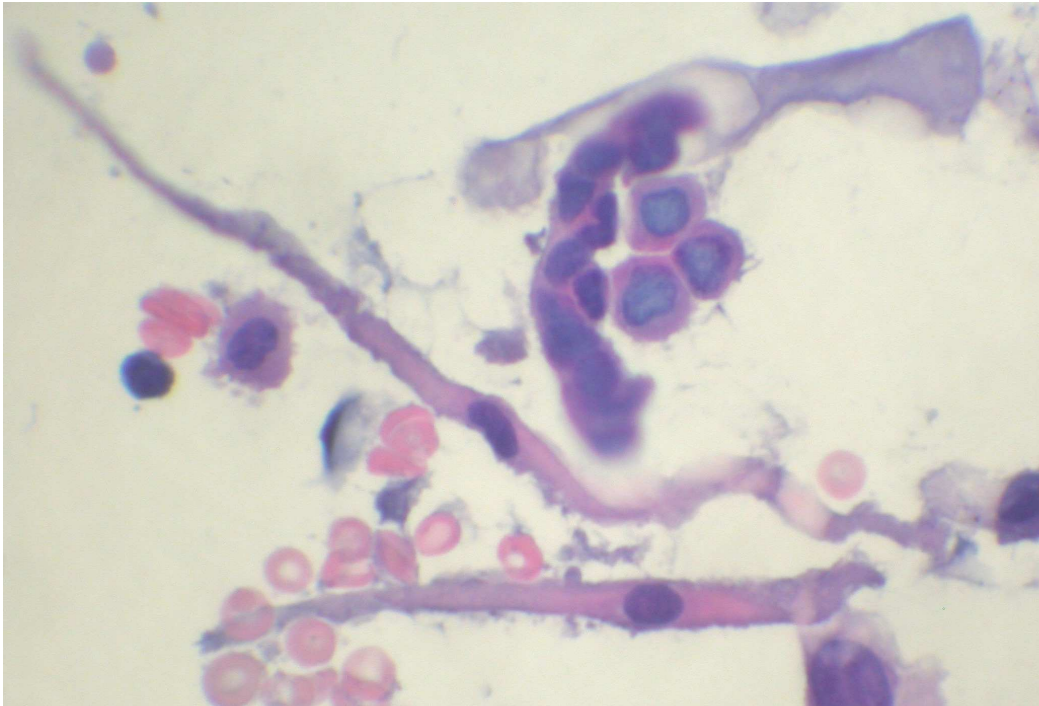
Fig 3.23. Comparison of the resting detrusor muscle lengths between human, guinea pig and rat tissue (carried out by Sebastian Clark).
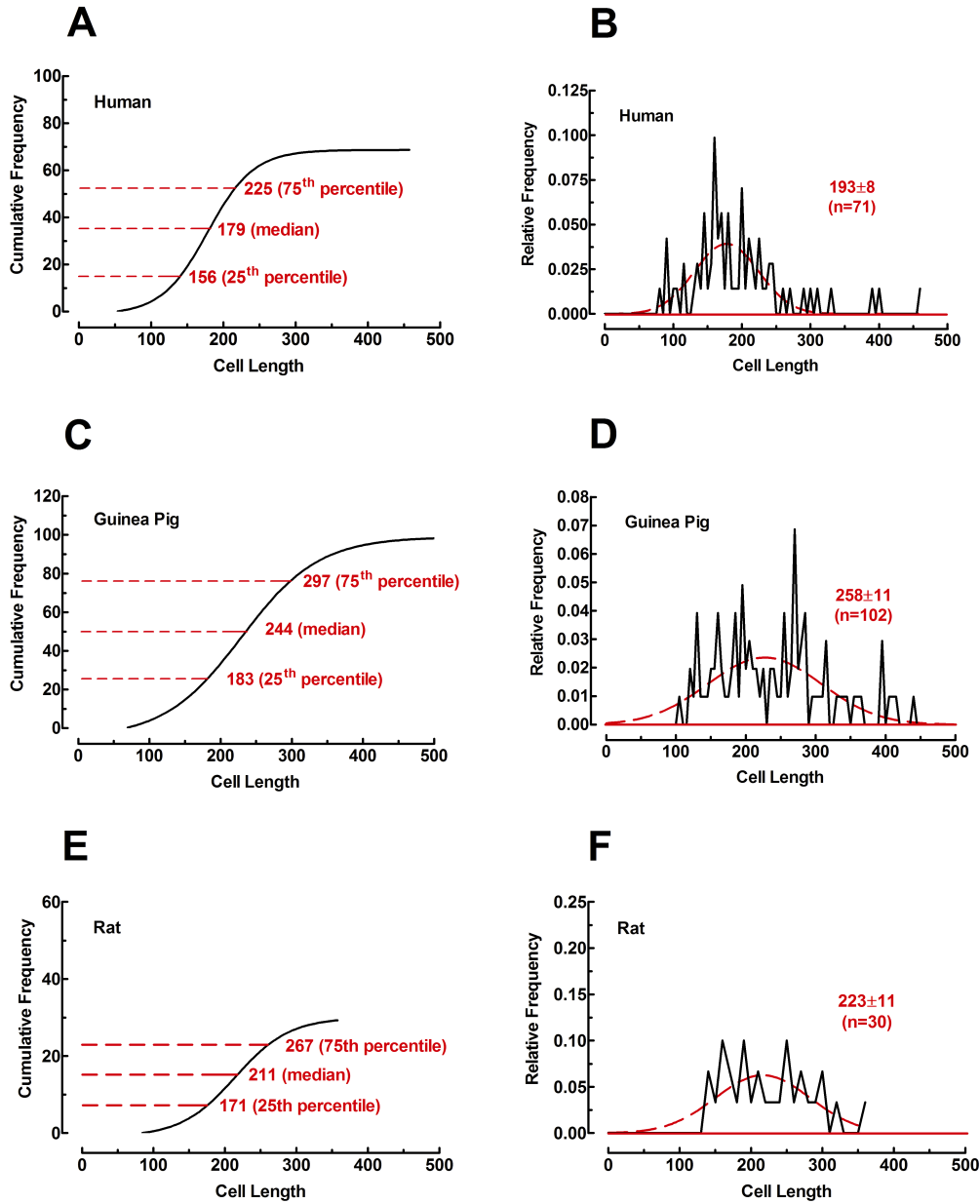
Fig 3.24. The effect of increasing Ca^{2+} concentrations on detrusor cell length (carried out by Sebastian Clark).
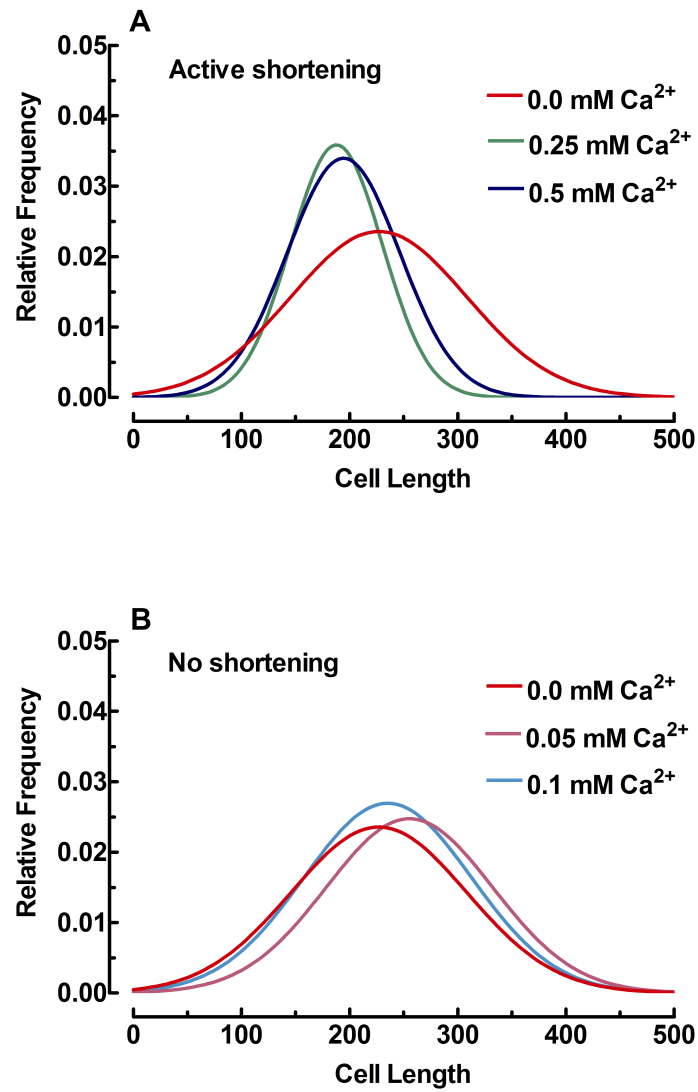
Fig 3.25 Carbachol dose response curve in rat detrusor tissue (n=3, mean ± sem, fit to raw data) EC_{50} =6.1.

Fig 3.26

Methoctrtamine inhibition of carbachol mediated contractions in rat detrusor, pKb =9.23

(y-axis) Percentage maximum contraction

(x-axis) log10[carbachol]

Legend:
- Control
- 10nM methoctramine
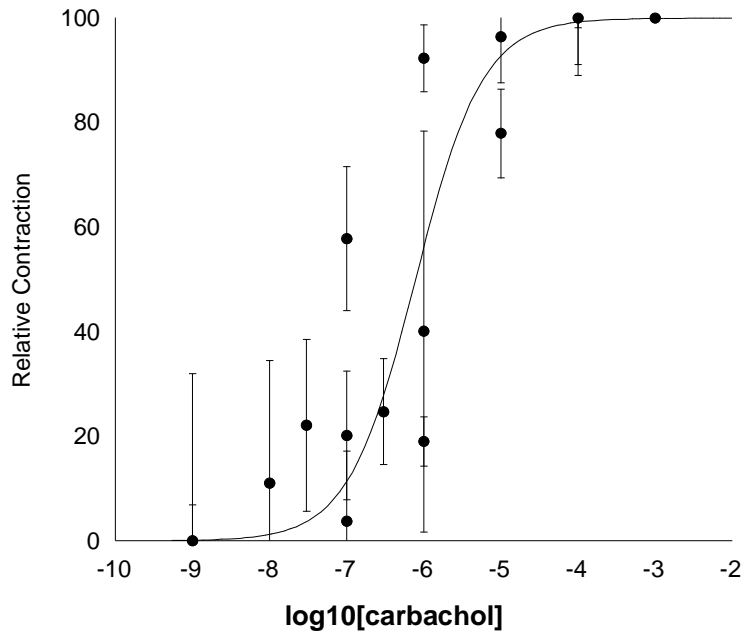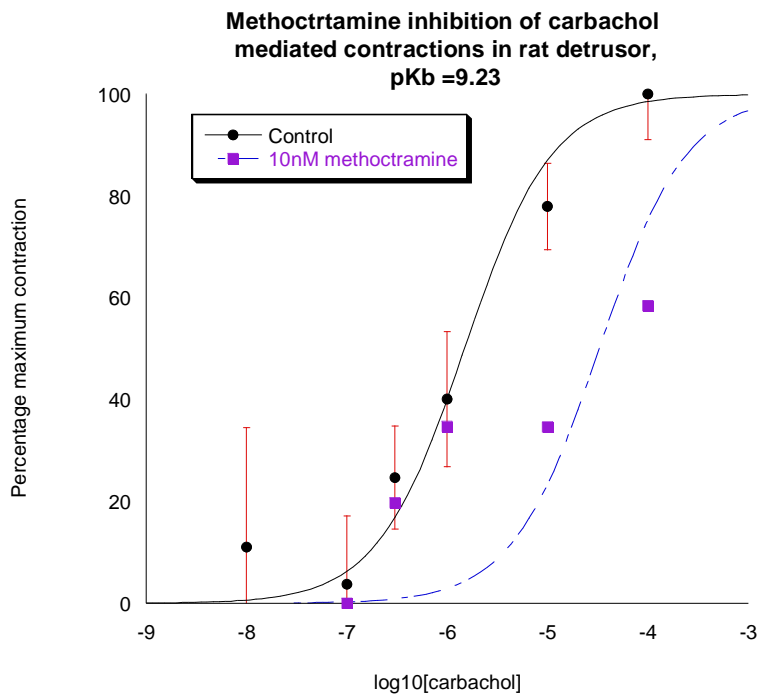
Fig 3.27 the effect of Ca^{2+} and Oxotremorine dose response in the presence of 0.5mM Ca^{2+} in isolated rat detrusor cells (n=2).

The effect of Ca^{2+} and oxotremorine on single cell resting lengths

The effect of Ca^{2+} and oxotremorine on single cell resting lengths
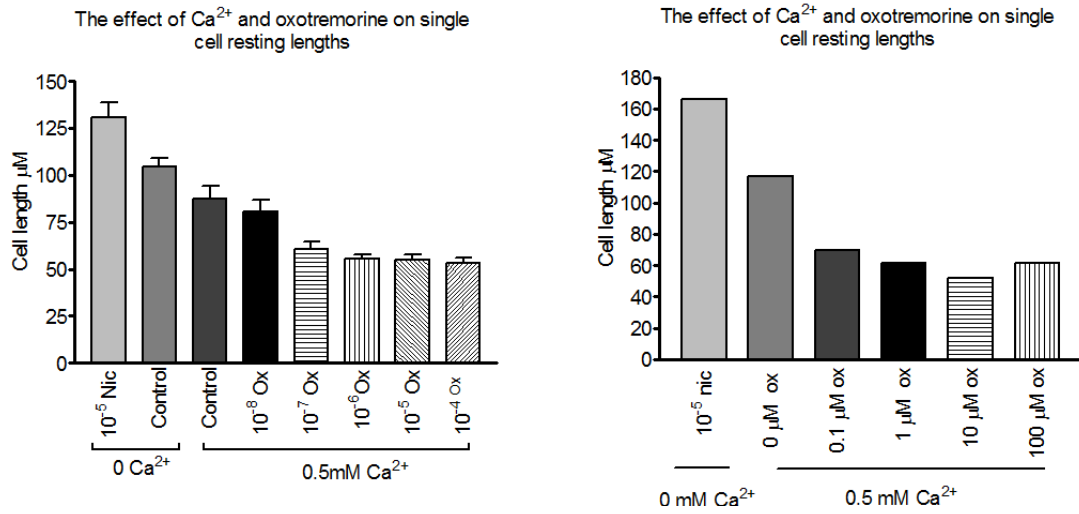
Fig 3.28 Comparison of carbachol dose response (n=3) and oxotremorine dose response (n=2).

Dose response in 0.5mM Calcium

- Carbachol
- Oxotremorine
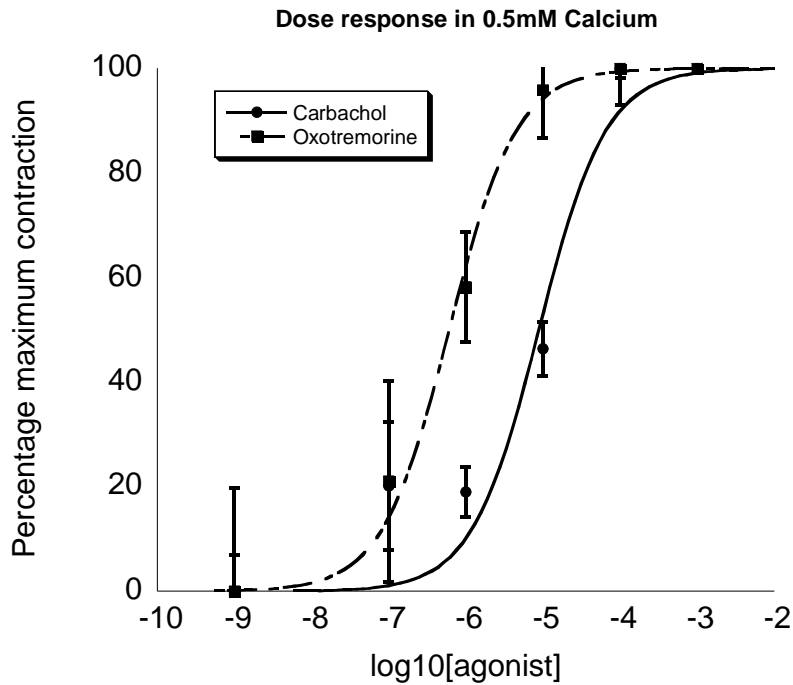
Fig 3.29 Carbachol dose response curve ; control and in the presence of 10 nM methoctramine.
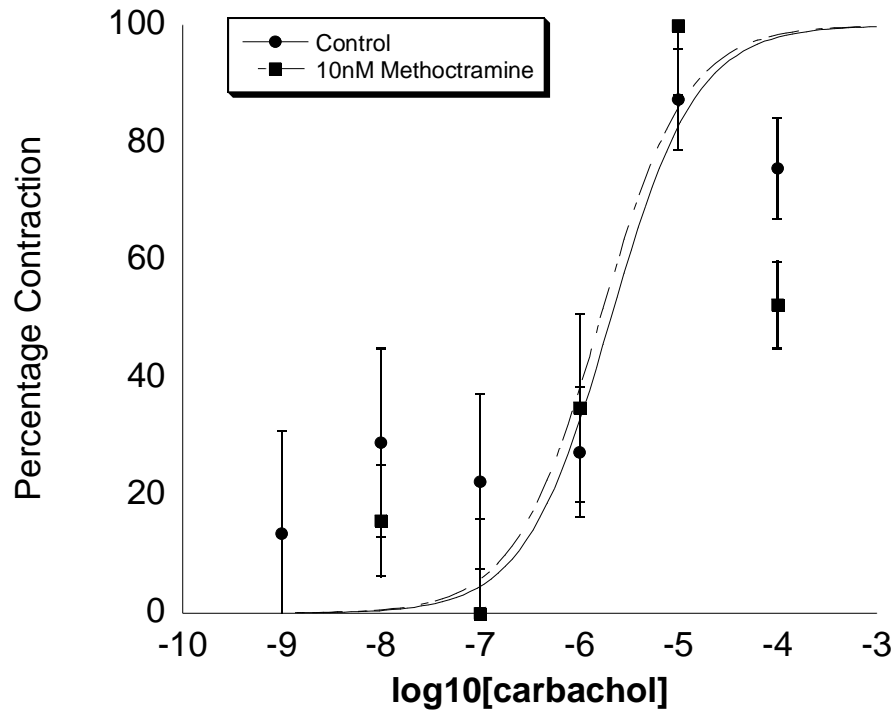
Fig 3.30 Evidence of detrusor muscle plasticity in outflow obstruction.

Bladder muscle plasticity
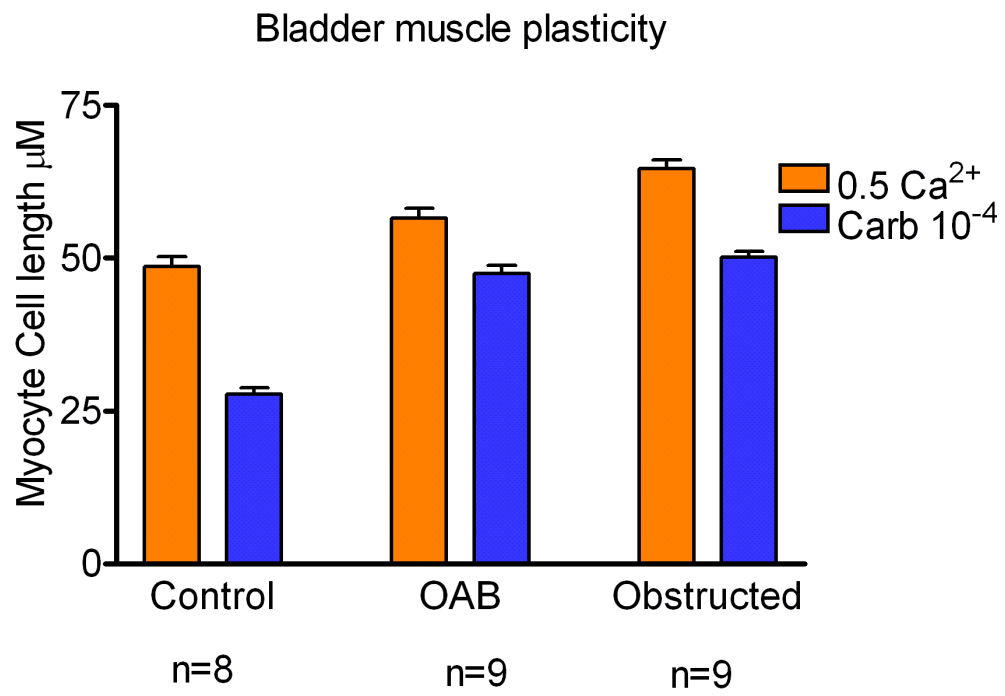
3.4 Ussing chamber experiments

Purinergic and muscarinic function

Measurement of baseline electrical properties; The baseline transepithelial potential (TEP), short circuit current (SCC) and conductance were measured as shown in Figure 3.31.

Baseline measurements (n = 56 guinea pigs) yielded a TEP of -4.86 ± 0.32 mV and SCC of 2.08 ± 0.27 μA, with a mean membrane resistance of 2.34 ± 0.30 kilohms (KΩ) Fig 3.32. These values are similar to published values. Here, the Ussing chamber was circular in profile with a diameter of 3.5mm and therefore a surface area of 0.123 cm^2. The mean baseline conductance per cm^2 of the preparations was 3.47 m.siemens per cm^2, which is similar to the value of 5 m.siemens per cm^2 obtained by Ferguson (Ferguson et al. 1997). The consistent baseline negativity of the transepithelial potential is indicative of an asymmetry of membrane ion transport between the luminal and basolateral surfaces of the urothelium. This could arise due to increased permeability to a cation such as Na$^+$ at the luminal urothelial membrane (Fig 3.41). Na$^+$ permeability at the luminal layer is potentially under the control of P$_2$X purinoreceptors.

Figure 3.33 shows that there was a weak positive correlation between TEP and SCC. Figure 3.34 and 3.35 show the frequency distribution of TEP and SCC respectively indicating that both of these parameters have a positive skew distribution. The highest SCC and TEPs may have been associated with leaky membranes as a result of damage to the urothelium during the dissection process.

A range of experiments were carried out to ascertain the effect of purinergic and cholinergic agonists on the luminal and basolateral urothelium. The addition of ATP to the luminal urothelium resulted in increased negativity of the TEP, and increased SCC. This appears to be self-limiting as the effect was maximal at a concentration of 10^{-6} M, ΔTEP = -2.85 $\pm$ 0.15mV and ΔSCC = 0.4 $\pm$ 0.1μA (n=19, p<0.05) (Fig 3.36) and diminished at higher concentrations. This may be due to luminal ATP having a complex effect on ionic transport. However, the addition of exogenous ATP to the basolateral urothelium did not have any effect on TEP or SCC and therefore flux of Na^+ across the urothelium (n=7) (Fig 3.37).

Some preliminary experiments to assess the effect of a longer acting ATP analogue (ATPγS) on the apical urothelium were carried out. This was not found to have a significant effect, however the experiments were only carried out on two occasions and require further clarification (Fig 3.38).

The addition of the non-specific cholinergic agonist carbachol at the basal (Fig 3.39) and luminal epithelium (Fig 3.40) had no discernable effect on transepithelial potential or short circuit current.

Ion flux that results in TEP

We sought to clarify the ion that was being transported across the urothelium in response on the addition of exogenous ATP.

The NaCl concentration at both the luminal and basolateral membranes was independently altered. The addition of 140mM NaCl (effectively doubling the

Na$^+$ on one side) to the solution perfusing the basolateral urothelium resulted in a trend for increased negativity of TEP (ΔTEP = 1.71 $\pm$ 1.02 mV, n =8) and increased SCC (ΔSCC = 0.093 $\pm$ 0.069) (Fig 3.42). Addition of 140mM NaCl to the apical urothelium resulted in a reduction in negativity in TEP (ΔTEP = 2.65 $\pm$ 1.0 mV, $p < 0.05$, n=20) (Fig 3.43). These results indicate that the baseline TEP is complex and dependent on the concentrations of several cations, and that Cl$^-$ may have an important influence as well. However these experiments were not carried out in iso-osmolar conditions, as increasing the NaCl concentration on one side of the urothelium increases the osmolarity on one side and this could influence the effect of transport of other ions.

Combination of Purinergic agonists with ionic gradient

We tested the addition of a combination of ATP 10^{-4} and 140mM NaCl to the luminal urothelium. Luminal ATP tends to increase the negativity of TEP but luminal NaCl decreases it. We found that under these circumstances the effect of NaCl predominated and TEP was increased (Figure 3.44 n=2, Δ TEP = 0.49. The magnitude of this increase was less than that achieved by NaCl alone (ΔTEP = 2.7 $\pm$ 0.9 mV from above and Fig 3.43).

When combining the ATP analogue ATPγs with NaCl a trend for similar rise in TEP was noted (Figure 3.38), though statistical significance was not achieved.

Isolating ionic flux

We have carried out further experiments to clarify the influence of either Na$^+$ or Cl$^-$ on TEP. An iso-osmolar solution where NaCl was replaced by Na glucoronate was added independently to each half of the Ussing chamber

(this contained a reduced Cl^- ion concentration of 9.6mM but a physiological concentration of Na^+ of 140 mM). This experiment, which was carried out once, demonstrated that reducing the Cl^- concentration on the luminal side resulted in an increased negativity of TEP (Δ TEP = -4.5mV and ΔSCC of 0.05 µA). When reduced Cl^- concentration was effected on the basolateral layer there was a corresponding slight increase in TEP (ΔTEP =0.05mV). This experiment needs to be repeated but it indicates that Cl^- flux from the basolateral to the luminal surface is an important determinant of TEP. The fact that altering the Cl^- concentration on the luminal side had a greater effect may indicate that there is greater permeability for Cl^- at the luminal side of the urothelium.

By adding 140mM Na Pyruvate to the tyrodes solution perfusing the luminal half of the Ussing chamber, an attempt was made to increase the Na^+ gradient without increasing the Cl^- gradient (Fig 3.45). A trend for increased positivity of TEP was noted which was inconsistent with increased transport of Na^+ from the luminal half of the Ussing chamber; perhaps indicating that pyruvate ion transport may have had a major effect on TEP in this experiment. This experiment unlike the Na-glucuronate experiment was not carried out under iso-osmolar conditions and the effect on the membrane conductance is similar to the addition of NaCl where the osmolarity was also effectively doubled on one side of the urothelium.

The effect of nicotine

The addition of nicotine to either the basolateral (figure 3.46, n=3) or luminal half of the Ussing chamber (figure 3.47) had no discernable effect on TEP or SCC indicating that nicotinic receptors do not have an important effect on TEP.

Fig 3.31 Typical Ussing chamber trace; short circuit current (SCC) in red and transepithelial potential (TEP) in blue. Long arrows show the baseline reading of TEP (-ve) and SCC (+ve). Short arrows indicate the change in SCC (Δ SCC) and change in TEP (Δ TEP) in response to the addition of NaCl to the basal urothelium.
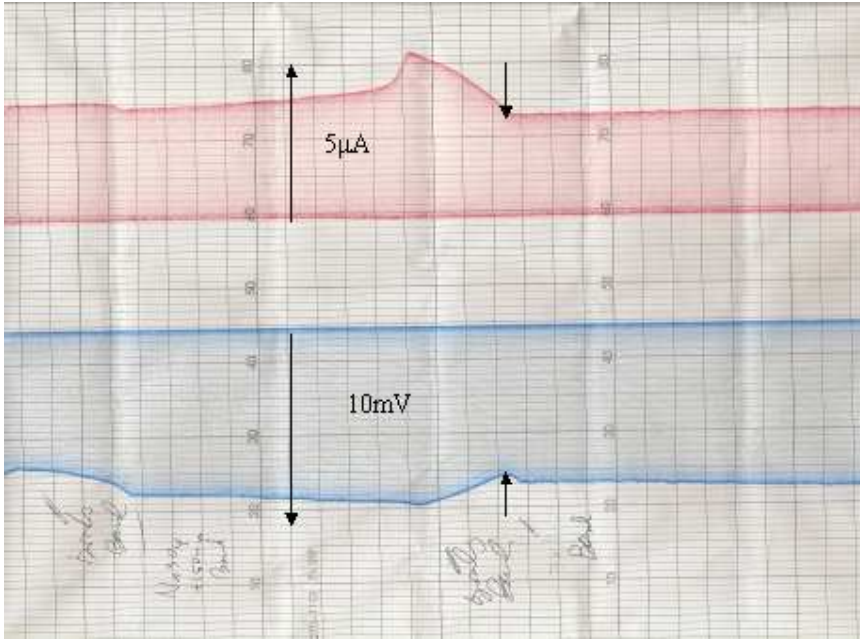
Fig 3.32

Baseline a) transepithelial potential (TEP), b)
short circuit current (SCC) and c) resistance
from Ussing Chamber Experiments
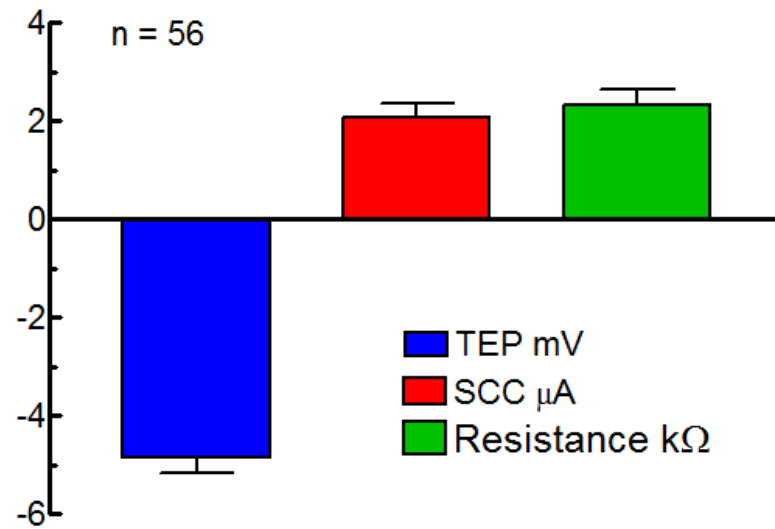
Fig 3.33

Relationship between transepithelial potential
and short circuit current
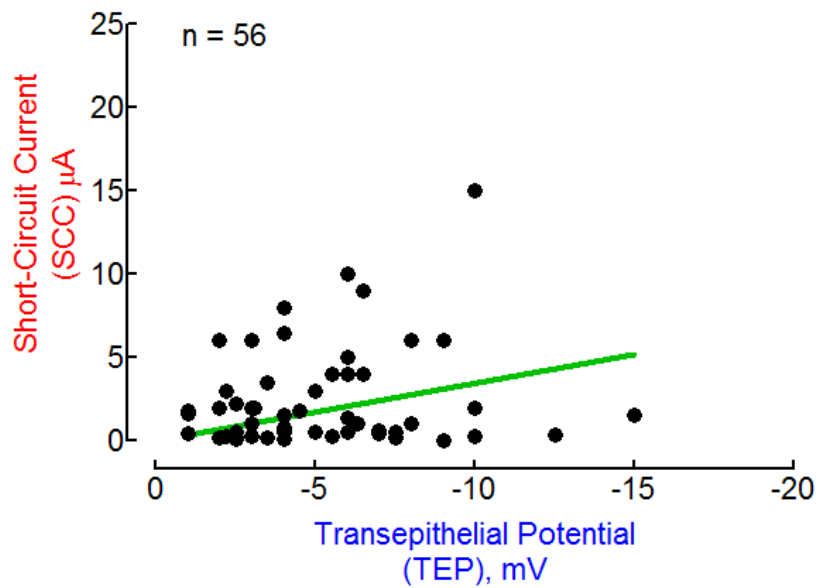
Fig 3.34

Frequency distribution histogram of baseline transepithelial potential (n=86)
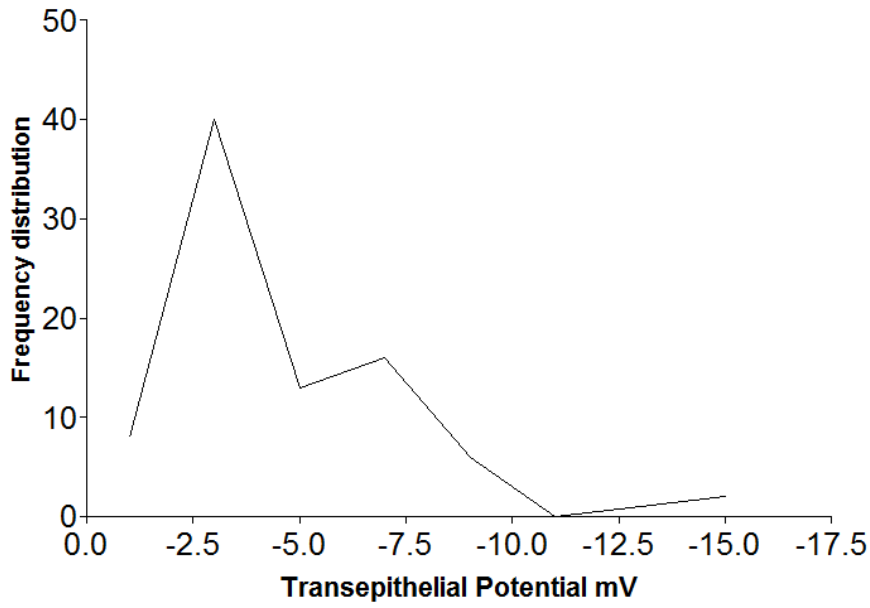
Fig 3.35

Frequency distribution of baseline short circuit current (n=86)
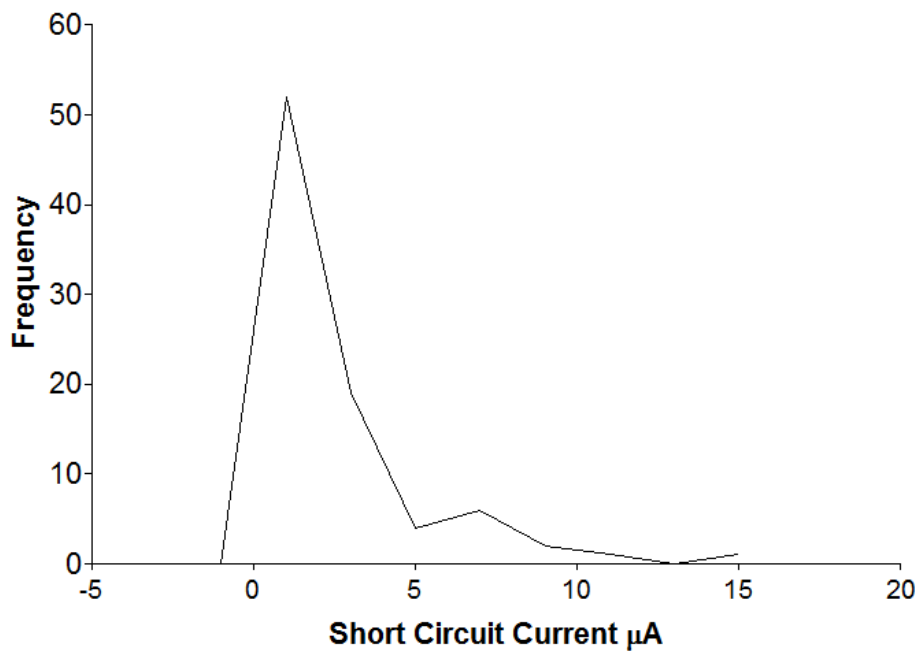
Fig 3.36

The effect of ATP when added to the apical urothelium a) Transepithelial Potential (TEP)
b) Change in TEP c) Short Circuit Current (SCC) d) Change in SCC (n=19).
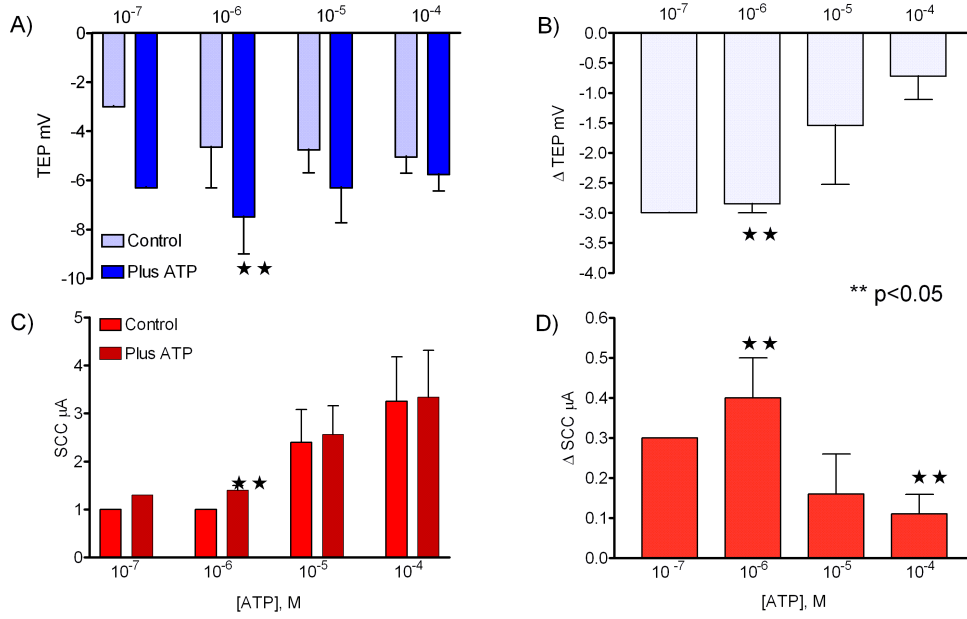
Fig 3.37

The effect of ATP when added to the basal urothelium on a) Transepithelial Potential (TEP) b) Δ TEP c) Short Circuit Current (SCC) and d) Δ SCC (n=7).
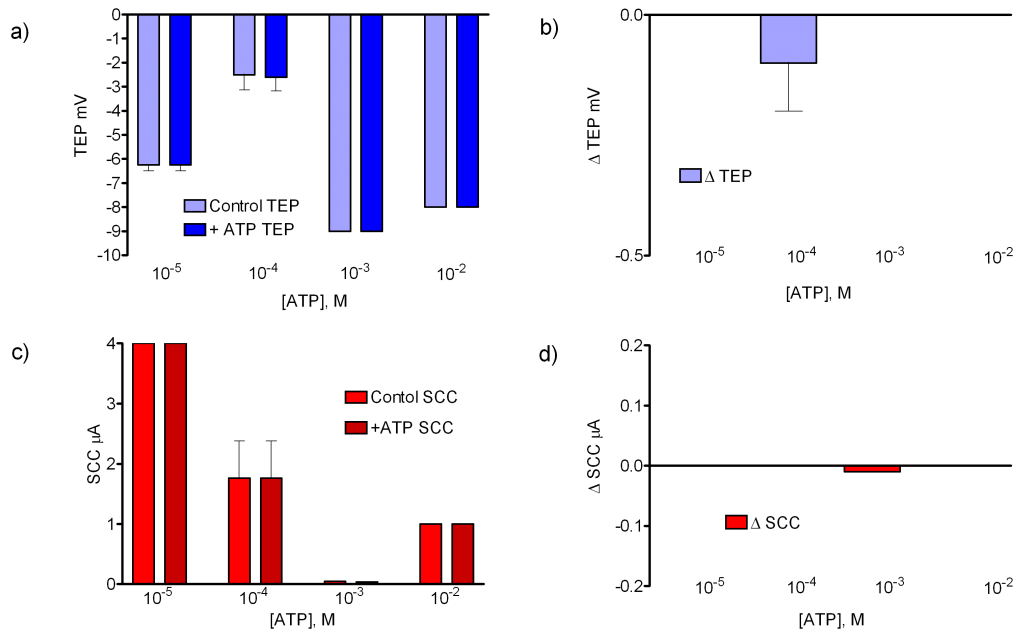
Fig 3.38

The effect of the addition of ATPγS ±NaCl to the apical urothelium on a) Transepithelial Epithelial Potential (TEP), b) Short circuit current (SCC) and c) ΔTEP and d) Δ SCC (n=2).
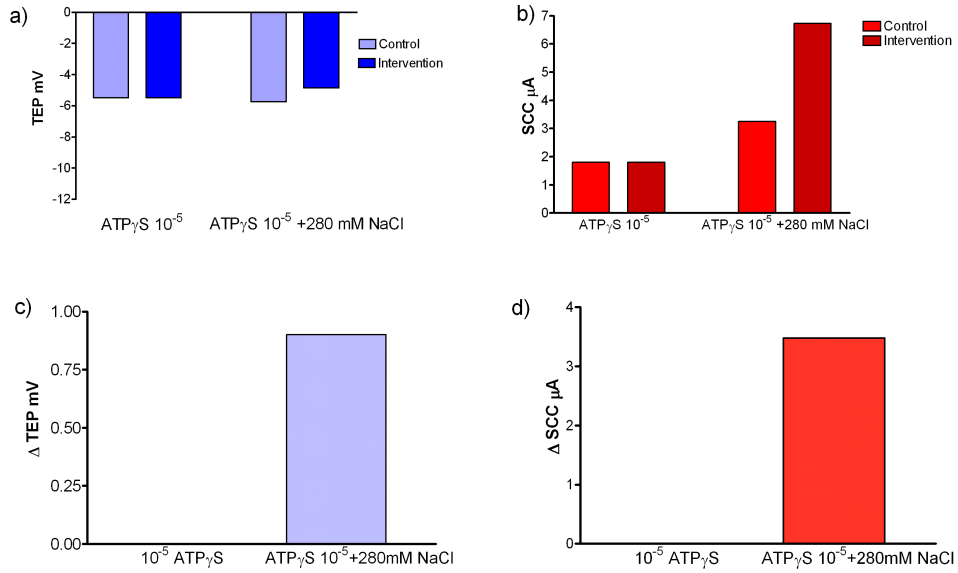
Fig 3.39

The effect of adding Carbachol to the basal epithelium on a) transepithelial potential (TEP) b) Change in TEP c) Short Circuit Current (SCC) d) Change in SCC (n=4).
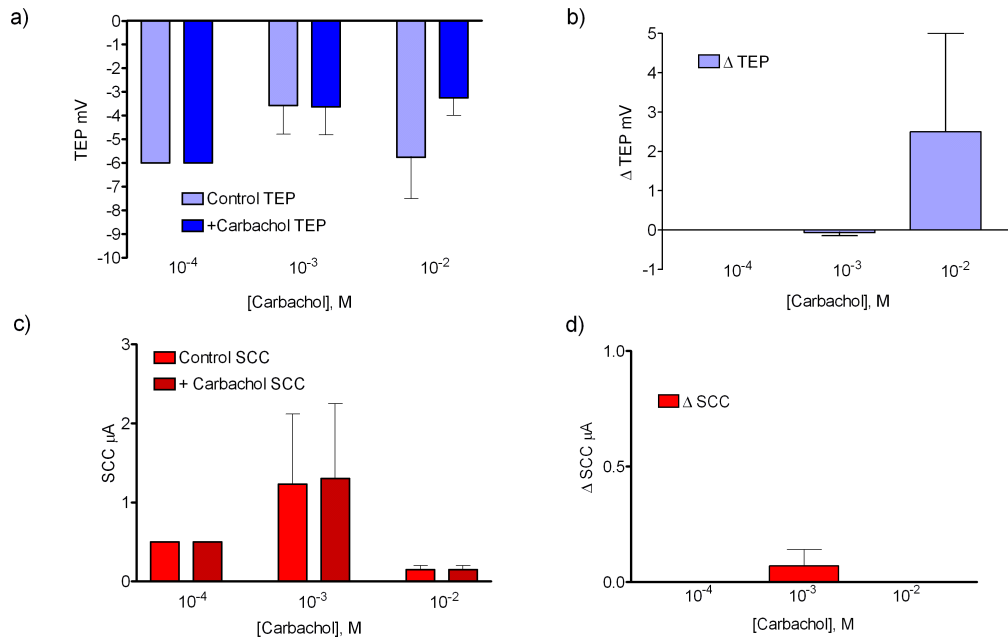
Fig 3.40

The effect of the addition of Carbachol to the apical urothelium on a) Transepithelial Potential (TEP) b) Change in TEP c) Short Circuit Current (SCC) d) Change in SCC (n=5).
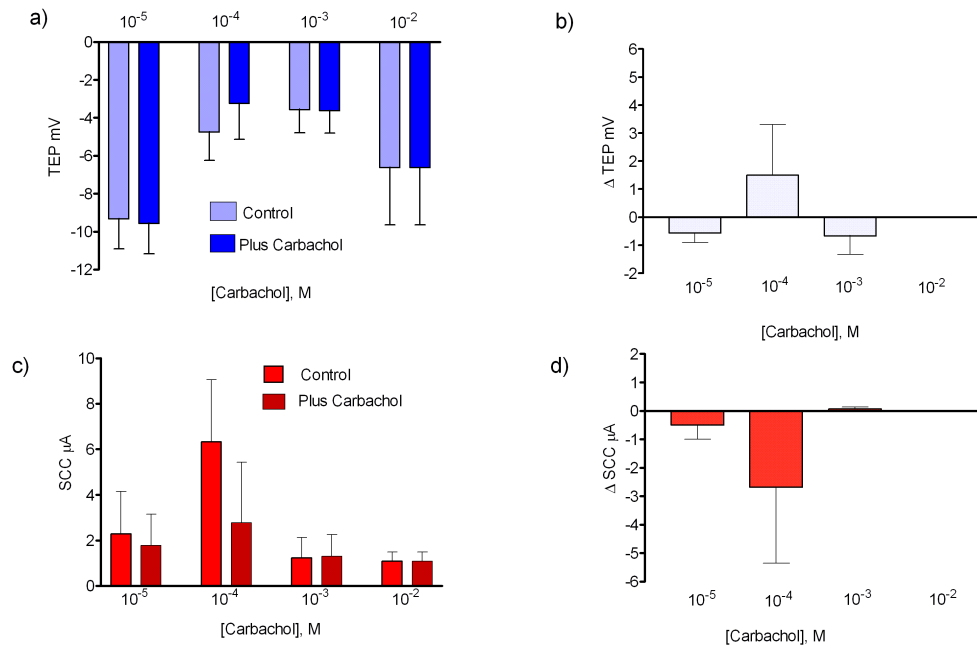
Fig 3.41 Proposed mechanism of action of ATP on the urothelium; luminal ATP release leading to depolarisation of the urothelium which spreads form the luminal to the basal surface, leading to ATP release from the basolateral surface and activation of afferent nerves.
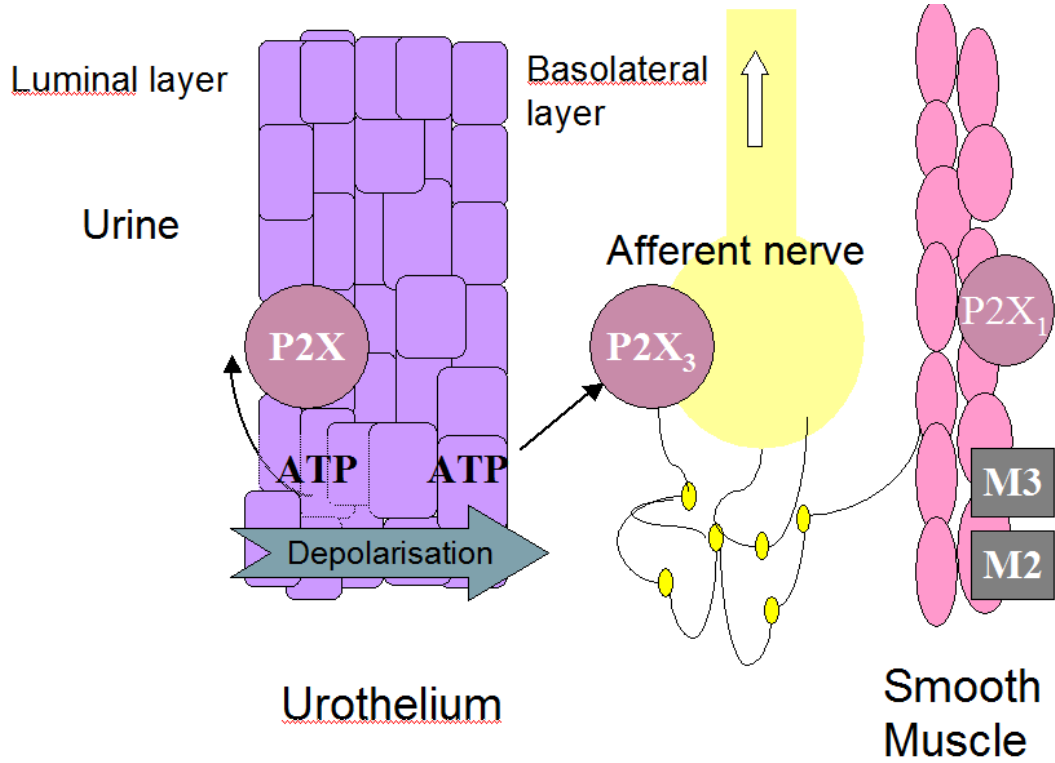
Fig 3.42

The effect of the addition of NaCl to the basal urothelium to a concentration of 280 mM on a)
Transepithelial Potential (TEP), b) Short circuit current (SCC) and c) ΔTEP and d) Δ SCC (n=7).
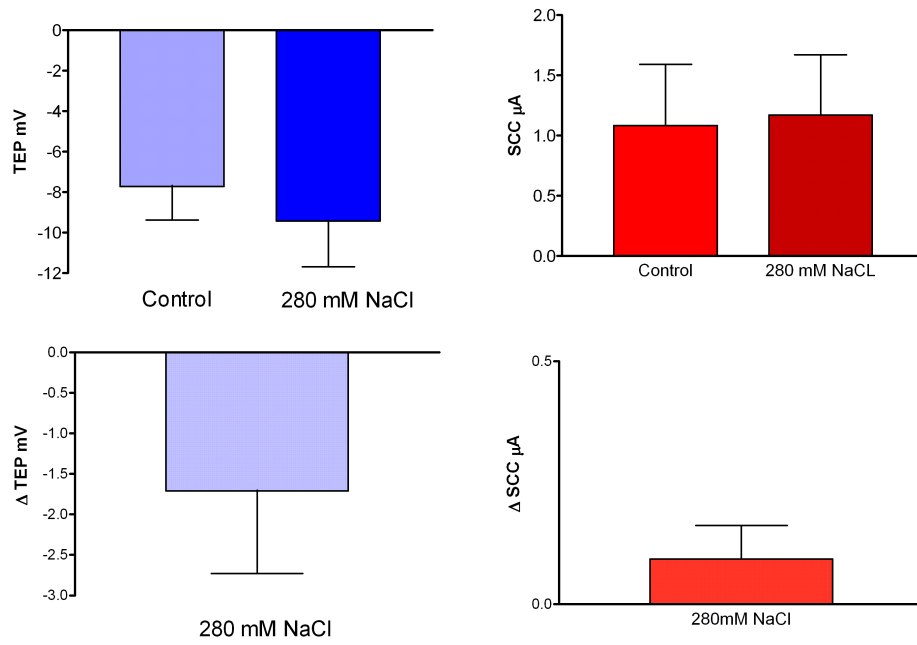
Fig 3.43

The effect of the addition of NaCl to the apical urothelium to a concentration of 280 mM on a) Transepithelial Potential (TEP), b) Short circuit current (SCC) and c) ΔTEP and d) Δ SCC (n=20).
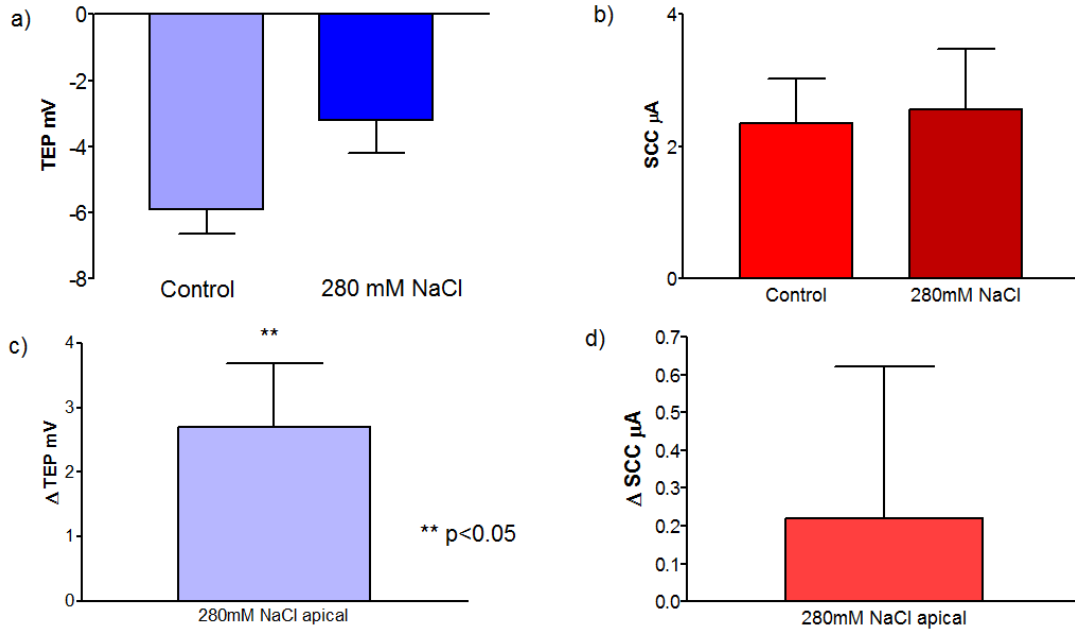
Fig 3.44

The effect of the addition of ATP ±NaCl to the apical urothelium on a) Transepithelial Epithelial Potential (TEP), b) Short circuit current (SCC) and c) ΔTEP and d) Δ SCC (n=2).
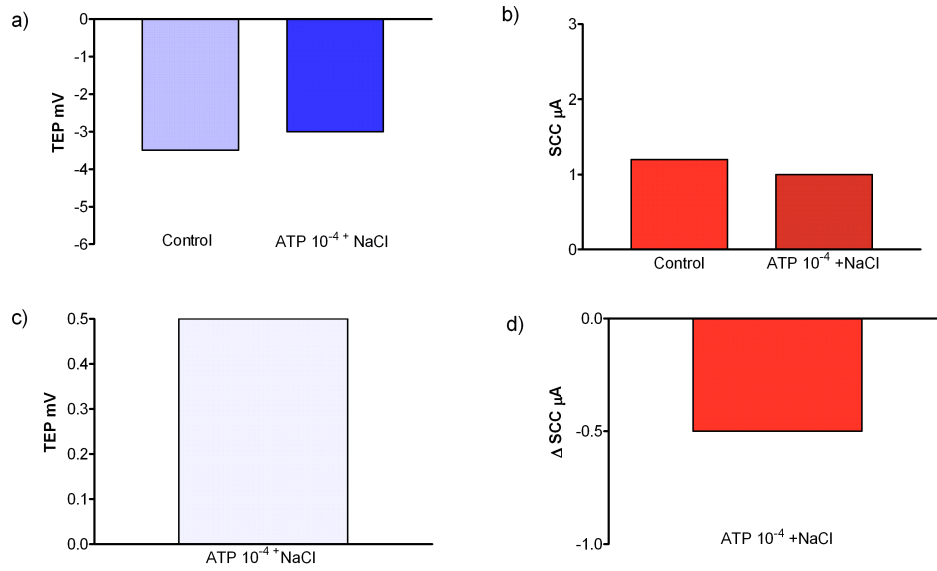
Fig 3.45

The effect of the addition of 280 mM Na^+ and 140mM Pyruvate in the the apical urothelium on a)
Transepithelial Epithelial Potential (TEP), b) Short circuit current (SCC) and c) ΔTEP and d) Δ SCC (n=2).
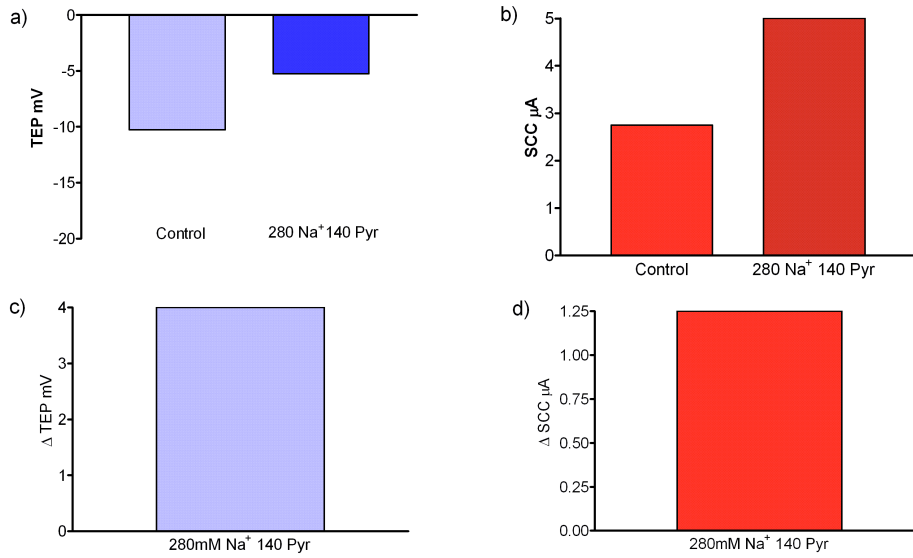
Fig 3.46

The effect of adding Nicotine to the basal epithelium on a) transepithelial potential (TEP) b)
Change in TEP c) Short Circuit Current (SCC) d) Change in SCC (n=1).
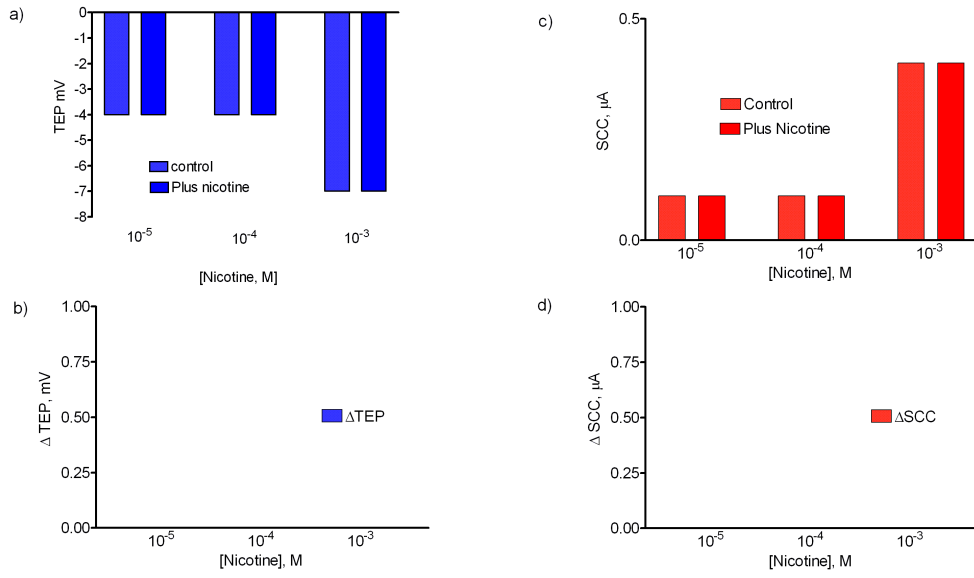
Fig 3.47

The effect of adding Nicotine to the apical epithelium on a) transepithelial potential (TEP) b) Short Circuit Current (SCC) c) Change in TEP   d) Change in SCC (n=3).
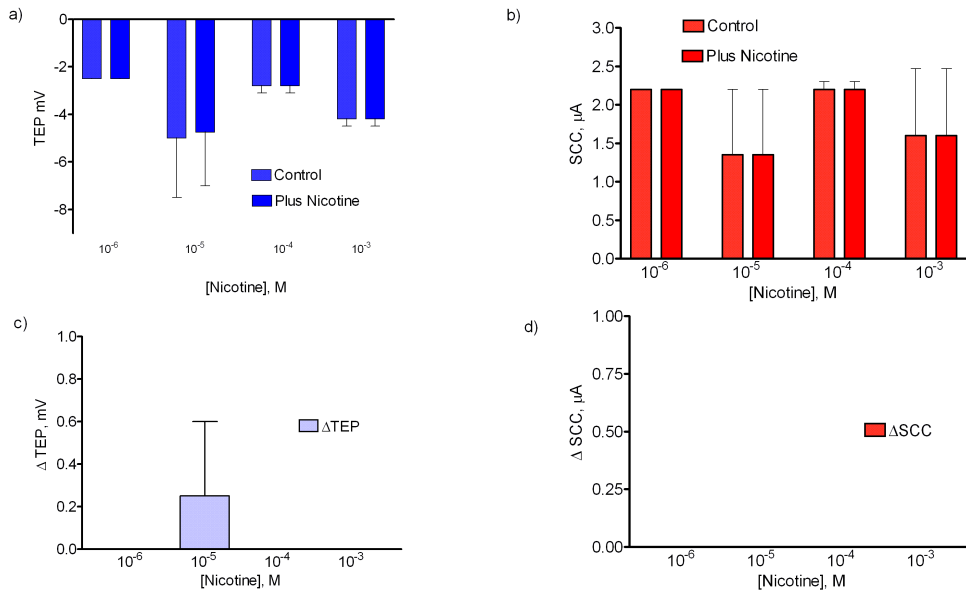
Chapter 4

Discussion

4.1 Clinical impact of OAB

Overactive bladder symptoms (OAB), defined by the International Continence Society as the presence of urinary urgency ± frequency ± nocturia ± urinary incontinence has a high prevalence, which is likely to increase in a population that continually achieves greater longevity. However, it is not a disorder that is restricted to the elderly and has an incidence approaching 10% in younger women (Irwin et al. 2006). OAB may cause considerable morbidity to its sufferers and place a strain on their working and social lives. Incontinence is associated with a multitude of other health problems in the elderly such as falls and depression (McGrother et al. 2006). The burden that the management of incontinence places on the UK National Health Service is significant. The cost of nursing care and social services to manage the problem greatly exceeds the direct costs attributable to drug therapies. Improved symptomatic management with drug therapies have the potential to greatly reduce the indirect social costs attributable to urinary incontinence.

A number of treatments are available for managing overactive bladder symptoms. The therapy that is initiated is dependent on symptom severity, the patient's preferences and their functional status. Treatment is usually initiated in a stepwise fashion starting with the simplest and least invasive. Prior to commencing any drug treatment, the National Institute for Clinical Excellence (NICE) recommends bladder retraining and lifestyle measures such as reducing caffeine or alcohol intake. Bladder retraining aims to help patients who are complaining of urinary frequency and is achieved through the patient voiding at particular time intervals, which they may eventually lengthen until a

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satisfactory gap between voids is achieved. Its effectiveness demonstrates that voiding is in part a behaviour that is under the influence of higher centres. Failure to respond to these measures will lead to the consideration of drug therapies. Antimuscarinic drugs are the mainstay of therapy of which there are several available. They differ slightly in their degree of muscarinic receptor subtype specificity (Appendix Table 1) and degree of tissue specificity for the bladder or other organs. The effect on other organs may lead to unwanted side effects such as dry mouth, constipation, blurred vision and cognitive impairment. As an example tolterodine has greater specificity for the M_3 receptor relative to the M_2 receptor, determined by its pK_B values, however oxybutynin is known to be more likely to cause dryness of the mouth (due to inhibition of M_3 receptors within the salivary gland) than tolteridine as a result of increased tissue specificity for the salivary gland. There is no compelling evidence to recommend one anticholinergic over another (oxybutynin has shown marginally superior efficacy to tolteridine in a single RCT (Novara et al. 2008)).

The use of anitmuscarinics has revolutionised the treatment of OAB and spared many patients the need for bladder augmentation surgery. However not all patients are able to tolerate their side effects or attain a sustained or satisfactory response to treatment.

For these patients, many clinicians would undertake urodynamics assessment to confirm the presence of detrusor overactivity characterised by the presence of involuntary detrusor contractions during the filling phase as a prelude to

further treatment. There is not always concordance between urodynamic findings and patients symptoms and the appropriateness of patient selection for botulinum toxin treatment on the basis of symptoms alone has been demonstrated (Ghei et al 2006).

Botulinum toxin is utilised in patients with overactive bladder symptoms who have not responded to anticholinergics. It is administered locally into the bladder using a fine injecting needle at cystoscopy. The duration of action of botulinum toxin A is approximately 6-12 months. It has demonstrated an efficacy superior to that achievable by antimuscarinics alone (Ghei et al 2006) and its use has successfully been demonstrated in idiopathic and neuropathic detrusor activity and in interstitial cystitis and is likely to lead to further reductions in the number of patients requiring bladder augmentation surgery.

Botulinum toxin is known to act through the inhibition of SNAP 25, a type of SNARE protein, which is essential for the exocytotic release of neurotransmitters. By analogy with its paralytic action on skeletal muscle it is assumed that its primary target is the detrusor muscle, however, its ability to inhibit neurotransmitter release from the urothelium and stop firing of the afferent sensory nerves is unevaluated. The superiority of botulinum toxin to antimuscarinic drugs in alleviating patient's symptoms poses a number of physiological questions and presents a new benchmark for the pharmacological industry. Many patients will prefer oral medication to repeated cystoscopic injections and the question arises as to whether the same degree of efficacy can be attained with an oral medication.

To achieve this, firstly a much better understanding of the neurotransmitter signalling mechanisms of both the urothelium and the detrusor is required, particularly in the diseased state. The ideal oral drug or combination drug would have sufficient efficacy at the target receptor subtype(s) and sufficient tissue specificity to avoid unwanted side effects.

There is some evidence of an associated change in neurotransmitter signalling both within the detrusor and within the urothelium. Atropine resistant contractions of the detrusor have been demonstrated in human and animal studies. There appears to be a smaller atropine resistant component in human tissue but it has been demonstrated that there is an increase in the atropine resistant component of nerve stimulated detrusor contractions with advancing age (Yoshida, Miyamae, Iwashita, Otani, & Inadome 2004). OAB symptoms increase with advancing age and atropine resistance or a change in neurotransmission from cholinergic to purinergic transmission is a plausible explanation for this. There is also evidence for a change of muscarinic receptor subtype. Detrusor contractions have largely been demonstrated to be mediated by the M_3 receptor, which accounts for about 25% of the total population of muscarinic receptors within the detrusor. It has been demonstrated through the decreased effectiveness of darifenacin (M_3 selective inhibitor, appendix B) at inhibiting contractions that there is a tendency for reduced M_3 signalling amongst patients with neuropathic overactivity relative to controls.

The urothelium is believed to be important as the primary relay of sensory signalling from the bladder via the afferent nerves. ATP has been demonstrated to be released from the bladder in response to bladder stretch and noxious stimuli. Increased urothelial ATP release has been found to be associated with interstitial cystitis (Sun et al. 2001) and UTI (Osterberg, Hallander, Kallner, Lundin, & Aberg 1991). Furthermore there is evidence for increased expression of urothelial $P2X_3$ (Tempest et al. 2004) receptors amongst patients with interstitial cystitis. Inhibition of ATP transmission from the urothelium may be an important effect of botulinum toxin inhibition and therefore there is reason to believe that a selective ATP inhibitor may provide a useful therapy in OAB.

4.2 Comparison of organ bath experiments with isolated detrusor cell method.

Organ bath experiments and isolated detrusor cell length measurements are two methods, which we have used to investigate the neurotransmitter regulation of muscle contractility. They are both assays of whole cell function and can provide only limited information about cellular mechanisms. Their utility is that they give a good corollary for in vivo drug efficacy and the use of these types of assays is likely to continue for the foreseeable future; given the large number of unknowns in intracellular function, it is not currently possible to construct a model of whole cell function based on the interactions of individual molecules.

The organ bath experiments carried out here, perhaps offer a superior reproduction of in vivo conditions. Tissue preparation is relatively atraumatic and minimally disrupts cellular architecture. The matrix of muscle fibres is maintained and there is minimal cell death. By contrast, the process of isolating cells is likely to lead to the death of a fair proportion of the detrusor cells and the release of ions and neurotransmitters such as Ca^{2+} and ATP into the extracellular fluids. These have the potential to affect cell measurements. These effects were manifest in our experiments by the fact that even in 0 Ca^{2+} solution the addition of the Ca^{2+} channel blocker nicardipine led to an increase the resting cell lengths. The addition of suramin to the cell dispersal solution did not produce a further relaxation in myocyte lengths in the absence of the addition of any purinergic agonists, suggesting the presence of insignificant quantities of ATP in the cell dispersal solution. The organ bath experiments

were carried out under conditions of high O_2 tension, controlled pH and temperature and constant perfusion. Constant bathing maintains the concentration of metabolites perfusing the tissue and washes away any by products of metabolism that may accumulate and have an inhibitory effect on contractility. It is possible that this methodological difference between these organ bath experiments and those of other authors may account for the observed difference in the effect of methoctramine on contractility. Whilst the isolated cell experiments were carried out under constant pH, temperature control and perfusion with O_2 was not carried out. As the cells were suspended as a droplet on a slide there is very little barrier to diffusion of O_2 from room air into the suspension and into the isolated cells.

Organ bath experiments uniquely allow for the assessment of the effect of endogenous neurotransmitters via the use of electrical field stimulation. By comparing the effect of an exogenous agonist and electrical responses it may be possible to draw some conclusions about presynaptic signalling. Acetylcholine analogues were used, as acetylcholine itself is unstable in solution. As exogenous acetylcholine analogues such as carbachol and oxotremorine are long acting due to their resistance to cleavage by acetylcholine esterase, their action on the postsynaptic membrane is likely to overwhelm any effect they may have on the release of endogenous neurotransmitters.

An advantage of the isolated cell technique is that it allows confirmation that it is the detrusor cells themselves that are responding to cholinergic agonism, which is what we have observed. In a detrusor strip there are other cells such

as interstitial cells of cajal and neuronal cells. It is theoretically possible that an observed contraction could be due to an alternative neurotransmitter such as ATP being released by an intermediary cell in response to a cholinergic agonist and this diffusing to act on the detrusor cells.

The isolated detrusor cells that are suspended in solution offer very little barrier to diffusion of an exogenous agonist and hence the observed EC_{50} for pharmacological agonists may be much lower than that achieved with much thicker detrusor strips and closer to the true working agonist concentrations within the neuromuscular junction.

There are several other advantages of the isolated cell technique, principally that very small biopsy samples such as that obtained from flexible cystoscopic biopsies may be utilised which means that a much greater number of biopsies can be collected from a broader group of individuals, including controls. No controls were collected for the organ bath experiments, as biopsies were restricted to the subset of patients who were undergoing open bladder surgery. Unfortunately all of the patients who were undergoing cystectomy for bladder cancer were also describing overactive bladder symptoms, as were all of the patients undergoing clam cystoplasty be it of neuropathic or idiopathic origin; as would be expected of any individual requiring this surgical intervention. The ability to use flexible cystoscopic biopsies aditionally means that the research could be carried out in any laboratory affiliated to a district general hospital where flexible cystoscopic biopsies are routinely undertaken.

The isolated detrusor experiments demonstrated remarkable statistical power as hundreds of cell lengths could be measured from a single biopsy sample

and therefore significant results could be obtained from a single biopsy sample.

Utilising the isolated detrusor technique it is possible to measure changes in muscle cell plasticity. Changes in muscle length due to pathology are not detectable in organ bath experiments, as they may not alter the force generated per cross sectional area.

4.3 Organ bath experiments

The experiments on human detrusor muscle strips provide evidence of significant M_2 receptor signalling in human detrusor from patients complaining of both neuropathic overactivity and OAB. This was manifest by 10 nM methoctramine producing sizeable inhibition of contractions induced by carbachol, oxotremorine and electrical field stimulation. 10nM is compatible with M_2 rather than M_3-dependent inhibition (Appendix, Table 1). This is the first time that M_2 activity has been demonstrated in patients with detrusor overactivity and the first time that evidence for M_2 activity has been demonstrated directly in human tissue using an M_2 inhibitor. It is not possible to use an M_2 agonist, as no such selective ligand is available. M_2 activity in human detrusor has previously been inferred from the relative ineffectiveness of darifenacin, a selective M_3 ligand in inhibiting muscarinic agonists amongst patients with neuropathic detrusor overactivity (Pontari, Braverman, & Ruggieri, Sr. 2004). We do not know why M_2 activity has not been demonstrated previously when an M_2 inhibitor was used to inhibit detrusor contractions in the human bladder. It is possible that this is due to methodological differences. Firstly, these experiments were carried out with a 30-minute washout period in-between agonist exposure rather than a cumulative dose response carried out without a washout. We have demonstrated that this does not desensitise the agonist responses during the experimental period. Secondly all experiments were carried out under continuous perfusion. This has the benefit of removing any products of metabolism, which may accrue in the tissues and have a deleterious affect on function. The disadvantage of this technique is that it is much more time

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consuming and fewer pharmacological interventions can be applied within a given experimental time frame.

Significant M_2 activity was present in all the patients studied. All twelve patients had overactive bladder symptoms, albeit from different aetiologies. They were undergoing open bladder surgery so were not representative of the general population but may be representative of many symptomatic patients who may benefit from treatment with anticholinergic drugs.

The study is limited by two factors; firstly the overall patient numbers and the lack of control patients. Previous experience of human tissue strip work within the laboratory indicate an average of 45% difference between mean sample variables, x_1-x_2, (where x_1 and x_2 denote the mean of two distinct samples groups) with sample standard deviations about 40% of the value. For a significant difference at p ≤0.05 , the standardised normal deviate, $u_{0.05}$ =1.96. The desired sample number, n, is $n > 2(u_{0.05}.s/x_1-x_2)^2$ =6.1, which can be rounded up to 7. Whilst there was the potential to collect full thickness biopsies from patients who were undergoing cystecomies; all of these patients were symptomatic when questioned. As there was also no discernable difference demographically or experimentally between the two groups, the overactive and neuropathic overactive patient groups were pooled together and thus analysis was of a single larger heterogenous group who all complained of OAB symptoms regardless of the aetiology. This is not ideal, as it does not allow investigation of the pathological differences between the two groups, but within this data set it has allowed for unequivocal documentation of M_2 activity (which is a noteworthy finding) by increasing the sample size

above the desired level. However, we cannot answer the question of whether M_2 activity would have been found in controls using our experimental protocol. Furthermore the summary table on page 81 shows that not every experiment was carried out on every patient and some experiments were carried out fewer than 7 times. The patient numbers allow for demonstration of agonist efficacy and M_2 activity but there are not sufficient numbers to distinguish between the OAB and neuropathic OAB groups let alone controls.

This is only the second study to collect detrusor biopsies from patients who are documented to have overactive bladder symptoms not due to a neuropathic aetiology (Stevens, Chapple, & Chess-Williams 2007). The findings here contrast with that study in that no evidence of M_2 activity was found in their study amongst any of the patient groups, be they control patients (undergoing cystectomy for cancer), OAB patients or neuropathic overactive patients, as methoctramine only inhibited carbachol contractions when it was used in the micromolar range in muscle strips from all of the patient groups. This is consistent with its inhibition of the M_3 receptors at higher concentrations. The reason for the differences in findings between the two studies remains uncertain, however it may be due to the differing methodologies used, as discussed above.

We however, have found that OAB patients like the neuropathic patients have significant M_2 activity. Our results support Pontari's findings that there is significant M_2 activity amongst neuropathic patients. Since it was not possible

to study normal controls here, the pathological deviation from normality cannot be accounted for in this study.

Methoctramine (10 nM) produced significant inhibition of EFS responses in addition to agonist-induced responses. EFS is both more physiological and complex, resulting in the release of not just acetylcholine but other neurotransmitters (Bayliss, Wu, Newgreen, Mundy, & Fry 1999). Methoctramine could potentially have a dual action on EFS, by facilitating pre-synaptic acetylcholine release and inhibiting its post-synaptic actions, as there are believed to be inhibitory prejunctional M_2 receptors (Braverman et al. 1998). However no evidence of this effect was witnessed here, as the magnitude of EFS inhibition was similar to that induced by agonists. As an example, 10nM methoctramine reduced contractions elicited by 1μM oxotremorine (pEC$_{50}$=6.1) by 48% (from Fig 3.2a) and contraction elicited by 16Hz EFS by 39% (from Fig 3.7b).

Although oxotremorine is often described as a non selective agonist, Griffin in his study of the recombinant human M_2 and M_3 receptor in Chinese hamster ovary cells demonstrated that oxotremorine had a pEC$_{50}$ of 7.44 for inhibition of cAMP accumulation (an M_2 effect) and pEC$_{50}$ of 6.23 for phosphoinosotide hydrolysis (an M_3 effect) (Griffin et al. 2003). A stronger association between agonist pEC$_{50}$ of oxotremorine and the pK$_B$ of methoctramine against oxotremorine has been demonstrated. This means that patients with detrusor strips that were more sensitive to oxotremorine could also be inhibited to a greater degree by methoctramine i.e. had greater M_2 activity. This would be

compatible with M_2 receptor signalling increasing the sensitivity of the detrusor to acetylcholine

An age-associated reduction in the potency of oxotremorine, but not of carbachol, and reduction in maximal EFS response relative to an agonist was recorded. Diminished EFS responses would be compatible with a reduction in nerve function with advancing age. Nonetheless, these findings accord with previous studies which have described decreased cholinergic and increased purinergic activity in older persons (Yoshida, Miyamae, Iwashita, Otani, & Inadome 2004). However the dataset accumulated here is clearly much smaller and caution needs to be exercised in interpreting the age related trends.

Anticholinergic medications are the most common treatment employed for patients with overactive and neuropathic overactive bladders. The data suggest that M_2 receptor blockade may be beneficial. M_3 targeting amongst the latest generation of anticholinergics such as darifenacin and solifenacin, is frequently cited, without evidence, for offering therapeutic advantage. However the present study suggests otherwise. Avoiding inhibition of cardiac M_2 receptors, however, may be desirable, although the most common side effect causing discontinuation of these drugs is dryness of the mouth, an M_3 effect.

Activated M_2 and M_3 receptors preferentially couple to different G proteins, M_3 to Gq/11 and M_2 receptors to Gi/o. One potential mode of action of M_2 activation is Gi/o-mediated inhibition of adenylyl cyclase and reduction of

cAMP levels (Somlyo & Somlyo 2003). This could increase force by increasing myosin light chain phosphorylation either by protein kinase A inhibition or rho kinase activation (Zholos & Bolton 1997) or facilitation of extracellular calcium entry (Eglen & Watson 1996). Our experiment encourages further scrutiny of these mechanisms.

In conclusion we believe that the M_2 receptor plays a significant role in modulating contractions in the overactive human detrusor.

We have demonstrated significant M_2 activity in guinea pig detrusor contractions. However, this could only be demonstrated when oxotremorine rather than carbachol was used as an agonist. Oxotremorine and carbachol are both full agonists however oxotremorine is significantly more potent. The potency of oxotremorine in the presence of methoctramine is similar to that of carbachol, which is unaffected by any of the M_2 acetylcholine inhibitors used here. Forskolin had a similar effect to methoctramine, i.e. no effect on carbachol contractions and inhibition of oxotremorine to the same degree as that obtained by 10nM methoctramine. It is tempting to conclude that in this preparation, oxotremorine is producing G_{i0} mediated inhibition of adenylate cyclase whereas carbachol does not. Previous studies examining the effect of recombinant porcine M_2 receptor activation have found no inherit difference between the activatory function of carbachol and oxotremorine (Vogel, Sheehan, & Schimerlik 1997), as both agonists are capable of inducing IP_3 activation and adenylate cyclase inhibition albeit at different concentrations; carbachol favouring IP_3 production and oxotremorine favouring adenylate

cyclase inhibition. Though we can find no equivalent study in guinea pig detrusor.

However, the experiments with oxotremorine demonstrate that forskolin and methoctromine have equivalent effects and that inhibition of cAMP is an important step in M_2 signalling that has a significant modulatory effect on detrusor contractility. This equivalence of action is illustrated below. Forskolin has previously been demonstrated to reduce the frequency of spontaneous action potentials in guinea pig detrusor in a Ca^{2+} independent manner however this has not been previously been shown to be mediated by the M_2 receptor (Hashitani et al. 2004).
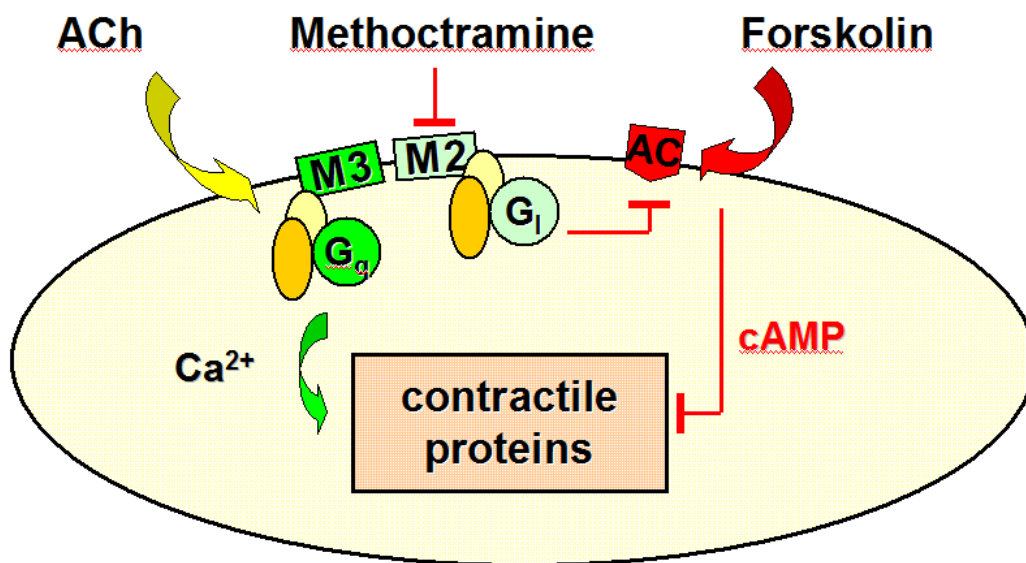
Parallel shift in dose response was not attained when methoctramine was used to inhibit oxotremorine dose responses in guinea pig detrusor and thus the pK_B of methoctramine was calculated utilizing the shortened version of the schild equation. A simple competitive binding model thus cannot explain the observed inhibition of oxotremorine-mediated contractions by methoctramine in the guinea pig detrusor. Possible explanations include the unknown additive effect of simultaneous M_2 and M_3 receptor activation, oxotremorine and methoctramine acting on a high affinity binding site which carbachol does not activate or the differential effect of carbachol and oxotrmorine on M_2 and M_3 hetero and mono dimers.

Methoctramine produced inhibition of EFS at concentrations of 100nM and above but not at 10nM where as 4DAMP produced inhibition at 3nM and above. These results are inconclusive in terms of determining the proportional effect of M_2 and M_3 receptor activation as at these concentration 4DAMP and methoctramine will each be inhibiting M_2 and M_3 receptors. It is likely that both receptor populations contribute to contraction with the M_3 receptor predominating.

In these experiments, M_2 activation was much easier to demonstrate in human detrusor (albeit pathological) than in the guinea pig. Nonetheless M_2 signalling contributes directly to detrusor contraction in both tissues.

These experiments also indicate that caution should be exercised in extrapolating results from animal models to humans particularly when only one acetylcholine analogue is used in experimentation. We like some

previous authors have failed to demonstrate M_2 acetylcholine activity in the (guinea pig) detrusor when carbachol alone was used as an agonist (Chess-Williams et al. 2001).

4.4 Isolated detrusor cells

In 1979 a technique was used to assess smooth muscle contractility which involved the dispersion of individual viable cells from a small sample of stomach tissue (Bitar, Zfass, & Makhlouf 1979). Isolated gastric smooth muscle cells were prepared from the stomach of Bufo marinus by successive incubation in collagenase without added trypsin.

Since this time, the approach has been applied to various tissues, particularly the gastrointestinal tract, from a number of mammalian species, including humans (Bitar & Makhlouf 1982). These preparations have been used to study the mechanical and biomechanical properties of the cells (Seidel & Johnson 1983; Collins & Gardner 1982).

The isolated detrusor experiments conducted here based on Bitar's method represent the first time that this technique has been used on human detrusor tissue. As detailed previously, there are few studies investigating detruosr contractility in human tissue and most conclusions have been drawn from animal studies. The great potential for this technique is that detruosr contractility can be measured using tiny biopsy samples that can be obtained from cystoscopies rather than the full thickness bladder biopsies which are required from organ bath experiments that can only be obtained from open bladder surgery such as cystectomy and clam ileo-cystoplasty. This provides the opportunity for many more samples to be collected from a greater number of patients who may be better representative of patients with lower urinary tract symptoms.

The method proved successful in producing a large yield of viable detrusor cells for the purpose of pharmacological probing. A small biopsy weighing 0.1 g could yield > 10000 cells of which 70-80% were alive as determined by trypan blue staining.

Furthermore, through counting a large number of cell lengths from a single sample, statistically significant results could be obtained on each sample though each experiment was repeated at least 3 times in order to obtain a mean of means.

A comparison between the three species studied; human, rat and guinea pig reveal similar resting cell lengths of 80-100μm. Resting cell lengths were normally distributed. A good range of contractility could be demonstrated with an almost 50% reduction in cell length brought about by the addition of 100μM carbachol, when compared with the maximally relaxed state in the presence of zero Ca^{2+} and the Ca^{2+} channel antagonist nicardipine. Addition of Ca^{2+} ions produced an incremental shortening of detrusor cell lengths with the near plateau effect achieved at 0.5mM. Somewhat surprisingly cells were significantly more relaxed when nicardipine was added to a zero calcium solution. There are several possible explanations for this; firstly that there is release of intracellular calcium into the extracellular space as result of equilibration of ionic concentrations across the cell membrane, secondly that there is increased calcium release due to break down of cell membranes brought about by the cell separation process, and thirdly there may have been a small concentration of Ca^{2+} contaminating the Tyrodes solution. This possibility may have been excluded through the addition of the Ca^{2+} chelator EDTA to the cell suspension solution. Cell membrane breakdown may lead to

the release of other neurotransmitters such as ATP. To explore this possibility the non-selective purinergic antagonist suramin was added to the cell dispersal solution, however this did not produce a further relaxation of the cells when used alone or in conjunction with nicardipine.

It was aimed to get the cells in a state of maximal relaxation prior to the addition of any of any contracting agent without inhibiting their contractility so that the greatest change of length could be produced and therefore the highest signal relative to noise. Nicardipine and zero calcium solutions were clearly able to relax the cells, however both of these conditions would inhibit contractility and therefore agonist reactions were carried out in 0.5mM Ca^{2+} and the absence of nicardipine. As suramin had no relaxatory effect it was elected not to use this to relax the cells simultaneously with agonist addition.

The maximal effect of the agonists carbachol and oxotremorine on isolated detrusor cell contractions were comparable just as the case with the organ bath experiments.

The values of EC_{50} for agonist response with carbachol and oxotremorine utilising the dispersed cell method are comparable to that obtained by the organ bath experiments indicating that the diffusional barrier to exogenous agonists activating muscarinic receptors on the detrusor muscle, may have not been a factor in the organ bath experiments.

The time series experiments carried out here, indicate that rat detrusor myocytes contract faster than human detrusor myocytes, reaching peak

shortening within 30 seconds and after 45 seconds, there is diminished agonist induced shortening thereafter, indicating receptor desensitisation.

Some M_2 activity was demonstrated in the rat detrusor on the single occasion when methoctramine was used against carbachol, however parallel shift was not achieved. An estimated pK_B of 9.23 was obtained. The analogous organ bath experiment was not carried out in rat detrusor. In the case of guinea pig detrusor M_2 inhibition could not be detected when methoctramine was used against carbachol but only when methoctramine was used against oxotremorine. M_2 activity was demonstrated in organ bath experiments involving human detrusor where methoctramine was used against carbachol. In this case an experimental pK_B achieved which was higher than the values achieved in human organ bath experiments.

Using the single cell dispersal technique we can assess cell lengths from different patient groups. Surprisingly, this has never been done before. Other groups have looked at changes in muscle cell diameter but not length, as the single cell dispersal technique has not been used before on human detrusor tissue for the purpose of measuring cell length. In a histological section through a muscle biopsy, the orientation of the muscle cells is random and there is greater likelihood by chance of obtaining transverse sections across muscle cells, as they are long and thin. It has been shown that in patients with bladder outflow obstruction, that muscle cells hypertrophy and become thicker (Uvelius, Persson, & Mattiasson 1984). However we do know that bladder

adaptation can lead to changes in bladder capacity and the single cell

technique allows us to measure cell length and diameter

Outflow obstruction in men due to prostatic enlargement is a slow process,

which occurs over many years resulting in changes to bladder function.

Initially, outflow obstruction may result in voiding with increased pressures, as

the bladder must do more work to overcome the resistance. This can lead to

overactive symptoms, similar to those experienced by patients with idiopathic

overactivity. With time the bladder gradually enlarges in some men and it is

not unusual to see patients with prostatic enlargements having bladders of 1 –

2 litres capacity. This is much more than the normal male bladder capacity of

500- 600ml. Enlargement of the bladder could arise in one of three ways;

either by the muscle cells becoming longer or by the cells becoming interlaced

with fibrous tissue and thus becoming more spread out (Gosling & Dixon

1980;Inui et al. 1999) or by an increase in cell numbers (de Castro Sasahara

et al. 2007; Nielsen et al. 1995). Furthermore patients with long standing

chronic retention tend to void at lower bladder pressures and are said to have

atonic bladders. Laplace's law describes the relationship between wall

tension and pressure in a spherical elastic container and predicts that the

pressure of the liquid is proportional to the wall tension but inversely

proportional to the radius. Therefore an atonic bladder may produce lower

pressures simply because it is larger and not because there is any reduction

in contractile force of the muscle. Three hypotheses that were tested were:

1) Patients with outflow obstruction have longer bladder cells.

2) Muscle cells from patients with outflow obstruction undergo the same

percentage shortening as control and overactive patients.

3) Detrusor cells from OAB patients undergo a lower percentage lengthening when an agonist (muscarinic and purinergic) is added as the cells are already partially contracted in their resting state.

All of the above comparisons were affected by describing a working muscle range for each patient group. A resting length was measured by adding a physiological concentration of Ca^{2+} and nothing else to cells on one slide; the aim was to represent the muscle length during the storage phase when the efferent nerves to the detrusor are quiescent and the detrusor muscle is exposed to low concentrations of endogenous agonists. A fully contracted length was achieved by adding a high dose of non selective muscarinic agonist carbachol to another slide and a fully relaxed length could be assessed by adding a high concentration of antagonists, both muscarinic (atropine) and purinergic (suramin) to cells on another slide.

The antagonists might have been expected to have an effect because within the cell dispersal solution, the endogenous neurotransmitters acetylcholine and ATP will be present. Some of this may be due to physiological release but a significant proportion will be due to cell disruption as a result of the dispersal process. Furthermore, it is possible that an antagonist can have a direct effect on a receptor if it has inverse antagonistic function. About 85% of antagonists are estimated to promote inverse antagonism (Kenakin 2001)

The data in figure 4.30, would support hypothesis 1 and 2 . The nine patients with OAB and the nine control patient would appear to demonstrate a resting length which is closer to a contracted length that was achieved with 100 μM carbachol. However as detrusor muscle in OAB has shown to exhibit increased atropine resistance then these muscle cells need to be contracted

with ATP as well to see if they can be further shortened. These data

demonstrates for the first time that detrusor cells can elongate in response to

outflow obstruction. Clearly this finding needs to be corroborated in a large

group of patients, relating detrusor cell length to residual volume and bladder

capacity.

4.5 The urothelium

In summary ATP when released from the luminal surface of the urothelium may result in a P2X mediated flux of cations from the urine into the urothelium, which is manifest by increased SCC and increased TEP. However, this mechanism appeared to be self-limiting because higher ATP concentrations reverse this effect. No ionic flux was noted when ATP was added to the basolateral surface or when cholinergic or nicotinic agonists were added to either surface. The luminal purinergic response reflects an asymmetry in the distribution of P2X receptors across the urothelium.

The effect of altering NaCl concentration indicates that the baseline TEP is not simple and is dependent on the concentrations of several cations and that Cl^- fluxes have an important influence.

 Measurement of baseline electrical properties; the baseline transepithelial potential (TEP), short circuit current (SCC) and conductance were made.

Baseline measurements (Figure 3.32, n = 56 guinea pigs) yielded a TEP of - 4.86 ± 0.32 mV and SCC of 2.08 ± 0.27 µA with a mean membrane resistance of 2.34 ± 0.30 Kiliohms (kΩ). The consistent baseline negativity of the transepithelial potential is indicative of an asymmetry of membrane ion transport between the luminal and basolateral surfaces of the urothelium. This could arise due to increased permeability to a cation such as Na^+ at the luminal urothelial membrane. Na^+ permeability at the luminal layer is potentially under the control of P_2X purinoreceptors.

A range of experiments was carried out to ascertain the effect of purinergic and cholinergic agonists on the luminal and basolateral urothelium. We originally hypothesised that exogenous ATP would principally act at the basolateral urothelium and allow the efflux of positive ions. However we were unable to demonstrate that the addition of exogenous ATP to the basolateral urothelium had an effect on TEP or SCC and therefore flux of Na^+ across the urothelium (Figure 3.36, n=7). However, the addition of ATP to the luminal urothelium resulted in increased negativity of the TEP, and increased SCC. The effect appears to be self-limiting as the response was maximal at a concentration of 10^{-6} M, ΔTEP = -2.85 $\pm$ 0.15mV and Δ SCC = 0.4 $\pm$ 0.1μA (Figure 3.37 n=19, p<0.05), and diminished at higher concentrations. This may be due to luminal ATP having a complex effect on ionic transport, perhaps having different effects on different classes of purinergic receptors. We carried out some pilot experiments to assess the effect of an ATP analogue ATPγS on the urothelium. This was not found to have a significant effect however the experiments were only repeated on two occasions and needs further clarification.

The addition of non-specific cholinergic agonist carbachol at the basal and luminal epithelium (Figure 3.39 and 3.40) had no discernable effect on transepithelial potential or short circuit current. Likewise the addition of nicotine (Figure 3.36 and Figure 3.47) had no discernable effect on TEP and SCC. The lack of action of muscarinic agonists indicates ionic flux is governed by a mechanism independent of acetylcholine /muscarinic receptors. This is not to say that muscarinic signalling does not have a role in conveying a

sensory signalling just that it may not alter urothelial cell transmembrane voltage and therefore could not be detected by this assay. The lack of action of nicotine suggests that nicotinic receptors do not play an important role in urothelial function, as activation of nicotinic receptors leads to opening of an ion channel and this effect is likely to lead to an appreciable change in TEP and SCC which was not detected here.

The effect of luminal ATP and lack of effect of basolateral ATP on the urothelium is of interest and has caused us to proffer an alternative model of how we believe the urothelium is functioning as a microsensor relaying sensory signals to the afferent nerves located in close proximity (Figure 3.41). ATP has been shown to be independently released from both surfaces of the urothelium (Lewis & Lewis 2006) and therefore both of these effects need to be addressed. These experiments suggest that basal ATP release may not have an important feedback function on the urothelium and may primarily function as a direct feed forward on to afferent nerves and suburothelial cells. However the role of luminal ATP release has never been addressed before and therefore the question arises as to what is the purpose of release of ATP from the luminal urothelium into the urine when the sensory nerves are located on the other side. The experiments here suggest that luminal ATP release acts as feed forward, working directly on the urothelium so as to increase the flux of cation form the urine into the urothelium. We have interpreted this as a sensory mechanism. The urothelium is a thick epithelial surface of about 5 – 7 cells deep and this mechanism may be a means of conveying a sensory signal such as bladder stretch or inflammation which primarily occurs at the luminal side of the urothelium, from the luminal to the

basal side by causing a depolarisation of the urothelium which starts at the luminal surface and spreads to the basal surface. This could lead to release of ATP and other neurotransmitters from the basolateral layer of the urothelium that may act on the sensory nerves and propagate this sensory signal. We believe this model offers a logical explanation of urothelial electrical properties, as firstly ATP is controlling Na^+ flux on the same side that it is acting and signal transduction proceeds sequentially from inflammatory signal (urinary side) to the afferent sensory nerves on the basolateral surface. We have not clarified the purinergic receptor that is mediating the ionic flux but we believe that activation of P_2X receptors which control Na^+ current is a likely candidate. The P_2X_3 receptor expression has been found to be increased in interstitial cystitis which is an inflammatory disorder characterised by symptoms of urinary urgency, frequency and pain (Sun & Chai 2004).

The increased presence of ATP has been found in the patients suffering with inflammatory conditions such as UTI (Osterberg et al. 1991) and interstitial cystitis (Caulfield 1993). It is possible that symptoms may be mediated by excessive signalling form the urothelium and therefore there exists the potential for effecting a treatment through the use of specific purinergic inhibitors. The lack of effect of muscarinic agonists does not necessarily mean that acetylcholine has no important effect on the urothelium merely that an effect could not be detected by this functional assay and therefore ion flux or membrane depolarisation may not be part of its mode of action. The lack of action of nicotine suggests that nicotinic receptors do not play an important role in urothelial function, as activation of nicotinic receptors leads to opening

of an ion channel and this effect is likely to lead to an appreciable change in TEP and SCC.

Ion flux that results in TEP

We sought to clarify the ion that is being transported across the urothelium in response on the addition of exogenous ATP.

We altered the NaCl concentration on both the luminal and basolateral membranes. Addition of 140mM NaCl (effectively doubling the Na^+ on one side) to the perfusing solution to the apical urothelium resulted in a reduction in negativity in TEP (ΔTEP = 2.65 $\pm$ 1.0 mV, $p<0.05$, n=20) (Fig 3.43) and addition of 140mM to the basolateral urothelium resulted in a trend for increased negativity of TEP (ΔTEP = 1.71 $\pm$ 1.02 mV, n =8) and increased SCC (ΔSCC = 0.093 $\pm$ 0.069) (Fig 3.42). These results indicate that the baseline TEP is complex and dependent on the concentrations of several cations, and that Cl^- have an important influence as well.

Further experiments to clarify the influence of either Na^+ or Cl^- on TEP were carried out. An iso-osmolar solution where NaCl was replaced by Na-glucoronate was added independently to each half of the ussing chamber (this contained a reduced Cl^- ion concentration of 9.6mM but a physiological concentration of Na^+ of 140 mM). This experiment was carried out once but it demonstrated that reducing the Cl^- concentration on the luminal side resulted in an increased negativity of TEP (Δ TEP = -4.5mV and ΔSCC of 0.05 μA). When reduced Cl^- concentration was effected on the basolateral layer there was a corresponding slight increase in TEP (ΔTEP =0.05mV). This

experiment needs to be repeated but it indicates that Cl^- flux from the basolateral layer to the luminal is an important determinant of TEP.

Concluding message

These data support the hypothesis that luminal release of ATP results in depolarisation of the urothelium starting from the luminal surface and spreading to the basolateral surface, potentially leading to increased neurotransmitter release from the basolateral surface. We believe that this constitutes a mechanism whereby a sensory signal, such as that generated by inflammation of the luminal urothelium, can be conveyed to the basolateral urothelium where sensory nerves are in proximity. The experiments regarding the ionic flux, which is contributing to TEP do provide some insight. The results suggest that it is complex and more than one ion contributes and in particular the permeability of Cl^- is important. The next steps are to identify of the purinergic receptor subtype(s) that are mediating the ATP induced change in TEP. There is potential that identification will lead to novel treatments for patients suffering with overactive bladder symptoms.

Chapter 5

Future plan of investigations

Future Plan of Investigations

Detrusor

The experiments here highlight a role for M_2 receptors in the direct modulation of detrusor contractility. This study demonstrates M_2 activity using M_2 preferential inhibitors – methoctramine and gallamine. This the most direct means possible of demonstrating M_2 activity as there are no muscarinic agonists with sufficient specificity for the M_2 over and above the M_3 receptor Clearly there is much to be elucidated in clarifying its mechanism of action of the M_2 receptor. The experiments with forskolin suggest that adenyly cyclase inhibition and reduction of cAMP concentrations is an important function of M_2 activation that is capable of increasing the force of contraction in a Ca^{2+} independent manner. These experiments were only carried out on guinea pig detrusor and it would be worth carrying out these experiments on a range of human tissues particularly as M_2 activity was easier to demonstrate in human detrusor preparations.

Isolated detrusor cell experiments

The isolated detrusor cells experiments have demonstrated the potential of using small cystoscopic samples to assess detrusor contractility and plasticity in diseased states. There are a huge range of experiments yet to be done, from clarifying the role of cAMP inhibition mentioned above to clarifying the role that ATP has in mediating detrusor contraction and using specific ATP inhibitors such as TNP ATP and RB2 to identify the purinergic subtype mediating contractility where atropine resistant contractions occur.

The hypothesis that detrusor cells from atonic bladders maintain contractility and failure to void may largely be due to the failure of a large bladder to generate pressure needs to be further explored. To demonstrate this hypothesis detrusor samples need to be taken from patients with chronic obstruction and larger bladder volumes of 1-4 litres. This work could be continued in an experimental animal model whereby bladder hypertrophy and urinary retention is induced in animals by occluding the urethra. An attempt at restoring voiding function in such animals could be made through excising, tying off, or stapling off part of the bladder so as to reduce bladder volume. If detrusor cells in these animals still have good contractile function then voiding function could be restored. If this were the case then the ramifications for many patients would be quite profound, as the changes in bladder function due to outflow obstruction have so far been demonstrated to be largely irreversible. However if the only change is one of size rather contractility then this could be reversed by a surgical reduction of the bladder. Currently patients who have developed chronic retention due to outflow obstruction may not be able to void even after the obstruction in the outflow tract is surgically relieved and therefore require lifelong indwelling or intermittent self catheterisation in order to void.

Furthermore, the mechanism of detruosr hypertrophy could be explored. It has been largely assumed that bladder hypertrophy results passively in response to pressure, however it is plausible too that there are physiological control mechanisms. As previously documented here the bladder releases a host of neuropeptides such as ATP and acetylcholine when it is stretched. It is possible that that these are growth as well as sensory signals and that

inhibiting these neurotransmitters through the use of cholinergic and purinergic inhibitors could reduce the rate of bladder hypertrophy. Furthermore some patients with OAB and neuropathic bladders have low capacity, poor compliance and elevated pressures. An alternative to bladder augmentation surgery for these patients could be induction of bladder muscle elongation if the growth signals are elucidated.

Urothelium

Although ATP was demonstrated to have an effect when it was added to the luminal half of the Ussing chamber we need to clarify the effect of ATP on the luminal and basal urothelium at low concentration and clarify the receptor subtype that is mediating contraction using ATP inhibitors such as suramin ppads, TNP ATP, RB2. It would be also be instructive to attempt to block apical Na channels by adding luminal amiloride as this would support our hypothesis. The experiments with ATPyS and ATPyS combined with NaCl need to be repeated to determine if the same effect as that produced by ATP is yielded as would have been expected.

It would be desirable to clarify ion transport generating TEP. To achieve this, Cl- and Na+ replacement solutions of fixed osmolarity using a non-transported anion and cation respectively need to be added to each side of the urothelium. Chloride ion transport could be further elucidated through the

use of Cl- channel blockers, e.g. - indanyloxyacetic acid (IAA-94) or 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS).

The relationship between TRPV channels and transepithelial potential could be explored through the addition of capsaicin or resinferatoxin to the luminal urothelium, to inhibit TRPV channels and determine whether these are involved in ATP release.

We would like to further test the hypothesis that luminal ATP release result in basolateral release after the urothelium becomes depolarised by; 1) Measuring ATP in the effluent in each half of the ussing chamber in response to the addition of ABMA on each side. If this is true that basal ATP release is stimulated most by the addition of luminal ABMA. 2) Demonstrating that Urothelium is impermeable to ATP through the addition of radiolabled ATP to each half of ussing chamber and testing the effluent in each half for the presence of the radiolabelled ATP.

Chapter 6

Appendices

Table 1 Muscarinic Antagonists and their specific pKB values for each receptor

(Caulfield 1993),(Hou, Hirshman, & Emala 1998),(Eglen & Watson 1996)

Antagonist	M_1	M_2	M_3	M_4	M_5
atropine	9.0 – 9.7	9.0 – 9.3	8.9 – 9.8	9.1 – 9.6	8.9 – 9.7
pirenzipine	7.8 – 8.5	6.3 – 6.7	6.7 – 7.1	7.1 – 8.1	6.2 – 7.1
4 DAMP	8.6 – 9.2	7.8 – 8.4	**8.9 – 9.3**	8.4 – 9.4	8.9 – 9.0
Methoctramine	7.1 – 7.8	**7.8 – 8.3**	6.3 – 6.9	7.4 – 8.1	6.9 – 7.2
Gallamine		**6.2**	4.5		
Oxybutynin	8.5	7.8	8.7	8.2	7.6
Tolteridine	8.5	8.4	8.5	8.1	8.6

Table 2 Purinergic receptors, summary of distribution, agonists and antagonists (Burnstock 2007).

Receptor	Main Distribution	Agonists	Antagonists	Transduction Mechanisms
P1 (adenosine)				
A_1	Brain, spinal cord, testis, heart, autonomic nerve terminals	CCPA>R-PIA=S-ENBA>NECA; CVT-510	DPCPX, N-0840, MRS1754, N-0840, WRC-0571	G_i/G_o $\downarrow$ cAMP
A_{2A}	Brain, heart, lungs, spleen	NECA>CGS 21680=CVT-3146	KF17837, SCH58261, ZM241385, KW 6002	G_S $\uparrow$ cAMP
A_{2B}	Large intestine, bladder	NECA (nonselective)	Enprofylline, MRE2029-F20, MRS17541, MRS1706	G_S $\uparrow$ cAMP
A_3	Lung, liver, brain, testis, heart	IB-MECA>NECA>2-Cl-IB-MECA; DBXRM; VT160	MRS1220, L-268605, MRS1191, MRS1523, VUF8504	G_i/G_o, G_q/G_{11}, $\downarrow$ cAMP, PLC-β activation
P2X				
$P2X_1$	Smooth muscle, platelets, cerebellum, dorsal horn spinal neurons	ATP = 2-MeSATP$\geq$$\alpha$,$\beta$-meATP=L-$\beta$,$\gamma$-meATP (rapid desensitization)	TNP-ATP, Ip_5I, NF023, NF449	Intrinsic cation channel (Ca^{2+} and Na^+)
$P2X_2$	Smooth muscle, CNS, retina, chromaffin cells, autonomic and sensory ganglia	ATP$\geq$ATPγS$\geq$2-MeSATP$\gg$$\alpha$,$\beta$-meATP (pH + zinc sensitive)	Suramin, isoPPADS, RB2, NF770, NF279	Intrinsic ion channel (particularly Ca^{2+})
$P2X_3$	Sensory neurons, NTS, some sympathetic neurons	2-MeSATP$\geq$ATP$\geq$$\alpha$,$\beta$-meATP$\geq$$Ap_5A$ (rapid desensitization)	TNP-ATP, PPADS, A317491, NF110, Ip_5I, phenol red	Intrinsic cation channel
$P2X_4$	CNS, testis, colon	ATP$\gg$$\alpha$,$\beta$-meATP=2-MeSATP=CTP; Ivermectin potentiation	TNP-ATP (weak), BBG (weak), phenolphthalein	Intrinsic ion channel (especially Ca^{2+})
$P2X_5$	Proliferating cells in skin, gut, bladder, thymus, spinal cord	ATP=2-MeSATP=ATPγS>α,β-meATP	Suramin, PPADS, BBG	Intrinsic ion channel
$P2X_6$	CNS, motor neurons in spinal cord	(Functions poorly as a homomultimer)		Intrinsic ion channel
$P2X_7$	Apoptotic cells in, for example, immune cells, pancreas, skin	BzATP$\geq$2-MeSATP$\geq$ATP$\gg$$\alpha$,$\beta$-meATP	KN62, KN04, MRS2427, O-ATP Coomassie brilliant blue G, RN6189, Az11645373, A-740003	Intrinsic cation channel and a large pore with prolonged activation
P2Y				
$P2Y_1$	Epithelial and endothelial cells, platelets, immune cells, osteoclasts	MRS2365>2-MeSADP =ADPβS>2-MeSATP =ADP>ATP	MRS2179, MRS2500, MRS2279, PIT	G_q/G_{11}; PLC-β activation
$P2Y_2$	Immune cells, epithelial and endothelial cells, kidney tubules, osteoblasts	2-thio-UTP>UTP=ATP>UTPγS; INS 37217; INS 365	Suramin > RB2, AR-C126313	G_q/G_{11} and possibly G_i/G_o; PLC-β activation
$P2Y_4$	Endothelial cells	UTP>ATP>Up_4U>UTPγS; INS 37217	RB2 > suramin	G_q/G_{11} and possibly G_i; PLC-β activation
$P2Y_6$	Some epithelial cells, placenta, T cells, thymus	3-Phenacyl-UDP>UDPβS >UDP>UTP$\gg$ATP	MRS2578	G_q/G_{11}; PLC-β activation
$P2Y_{11}$	Spleen, intestine, granulocytes	AR-C67085MX>BzATP=ATPγS> ATP; NF546	Suramin>RB2, NF157, 5′-AMPS, NF340	G_q/G_{11} and G_S; PLC-β activation
$P2Y_{12}$	Platelets, glial cells	2-MeSATP$\geq$2-MeSADP >ADP>ATP	CT50547, AR-C69931MX, INS49266, AZD6140, PSB0413, ARL66096, 2-MeSAMP	$G\alpha_i$, inhibition of adenylate cyclase
$P2Y_{13}$	Spleen, brain, lymph nodes, bone marrow	ADP=2-MeSADP$\gg$2-MeSATP>ATP	MRS2211, 2-MeSAMP	G_i/G_o
$P2Y_{14}$	Placenta, adipose tissue, stomach, intestine, discrete brain regions	UDP glucose$\geq$UDP-galactose		G_i/G_o

Shown are receptor subtypes for purines and pyrimidines: distribution, agonists, antagonists, and transduction mechanisms. BBG, Brilliant blue green; BzATP, 2′- & 3′-O-(4-benzoyl-benzoyl)-ATP; CCPA, chlorocyclopentyl adenosine; CPA, cyclopentyladenosine; CTP, cytosine triphosphate; IP_3, inosine triphosphate; Ip_5I, di-inosine pentaphosphate; 2-MeSADP, 2-methylthio ADP; 2-MeSATP, 2-methylthio ATP; NECA, 5′-N-ethylcarboxamido adenosine; PLC, phospholipase C; RB2, Reactive blue 2. P2X receptor subtype agonist potencies are based on rat preparations, while P1 and P2Y receptor subtype agonist potencies are based on human preparations. [Updated from Burnstock (271) with permission from Elsevier.]

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Figure 5 The role of calcium in mediating detrusor contractions.

Major signal transduction pathways that modulate myocyte tone.

(+) denotes a positive or stimulatory effect; (–) denotes a negative or inhibitory effect. M_2 and M_3- muscarinic receptors. BK_{Ca}, the calcium-sensitive K channel (maxi-K), although clearly there are several types of K channels known to be present in detrusor myocytes; SR, sarcoplasmic reticulum; RyR, ryanodine receptor; PLC, phospholiapse C; DAG, diacylglycerol; PKA, protein kinase A; IP_3, inositol trisphosphate; MLC, myosin light chain; A, agonist; R, receptor; CPI-17, protein kinase C-potentiated protein phosphatase-1 inhibitory protein.
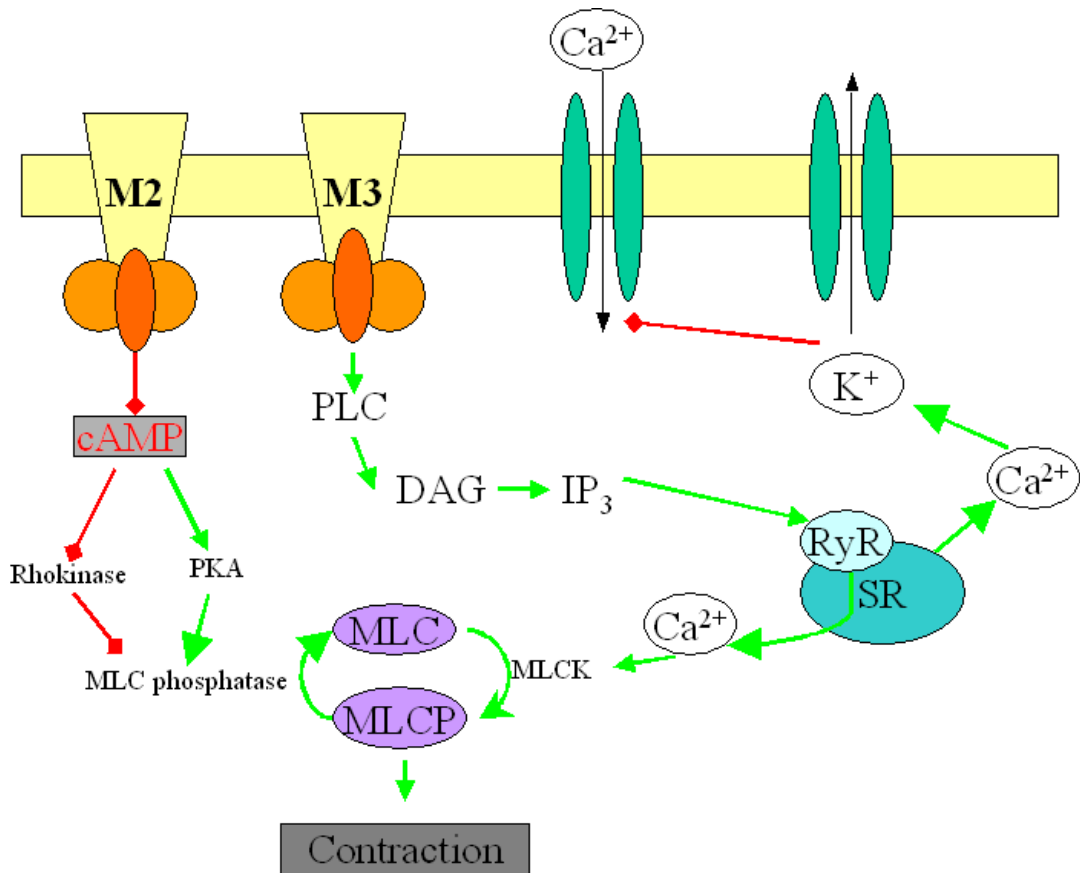
Equations used for curve fitting and their derivation;

Hill equation;

A = agonist concentration, R = free receptor concentration

A R = concentration of receptor and agonist complex

R_T = Total concentration of all receptors regardless of state

n = number of agonists that need to bind to activate each receptor complex.

K_B = dissociation constant for AR

$A + nR \leftrightarrow A R$

From the law of mass action at equilibrium $K_B = A^n.R/AR$ (1)

Assuming measured response, as ratio of maximal response is equal to

fractional binding.

Relative response = Y

$Y = AR/R_T$

$Y = AR/(R+AR)$

Substituting for AR from (1) $AR = A^n.R/K_B$

$$Y = \frac{A^n.R/K_B}{R + A^n.R/K_B}$$

Eliminating R and multiplying by K_B

$$Y = \frac{A^n}{A^n + K_B} \qquad\qquad (2)$$

As it is known that the muscarinic receptor binds to a single agonist n =1 was

used for curve fitting.

Schild equation

Consider agonist A competing with antagonist B for receptor binding site R with dissociation constants K_A and K_B respectively.

The receptor can be in one of three states, either free R, Agonist bound AR or antagonist bound BR. The total available receptor concentration $R_T = R + AR + BR$.

Assuming fractional response Y is equal to fractional binding with the agonist A then

$$Y = \quad AR/ R + AR + BR \tag{3}$$

Assuming that A can only bind to R and not to BR and B can only bind to R and not AR then from the law of mass action, then from (1)

$$K_A = A^n.R/AR \tag{4}$$

And

$$K_B = B^n.R/BR \tag{5}$$

Inverting (3)

$$1/Y \quad = \quad \frac{R + AR + BR}{AR}$$

Substituting for AR and BR utilising (4) and (5)

$$1/Y \quad = \quad \frac{R + A^n.R/ K_A + B^n.R /K_B}{A^n.R / K_A} \tag{6}$$

Dividing by R and inverting the equation

$$Y \quad = \quad \frac{A^n / K_A}{1 + A^n / K_A + B^n/K_B}$$

Multiplying by K_A

$$Y \quad = \quad \frac{A^n}{A^n + K_A (1 + B^n/K_B)} \tag{7}$$

It can be seen from this equation that if there is no antagonist B then the equation is the same as the Hill equation 2.

The complex $K_A (1+ B^n/K_B)$ is the apparent dissociation constant derived from a curve fit in the presence of the antagonist which is a larger value than K_A in the absence of the antagonist and when expressed as a ratio of K_A it is known a the dose ratio (DR). A dose ratio of 2 is derived when $1+ B^n/K_B =2$ ie when $B^n = K_B.$

In a schild plot the $\log_{10}$ of the dose ratio- 1 is plotted against the $\log_{10}$ of the antagonist concentration.

$$DR =1 + B^n/K_B \tag{8}$$

$$DR-1 = B^n/K_B \tag{9}$$

Taking the $\log_{10}$ of both sides

$$\log_{10} (DR-1) = n.\log_{10}B - \log_{10}K_B \tag{10}$$

$- \log_{10}K_B$ is also known as the pK_B then

$$\log_{10} (DR-1) = n.\log_{10}B + pK_B \tag{11}$$

In a Schild plot with $\log_{10} (DR-1)$ on the y axis and $\log_{10}B$ on the x axis a linear graph is expected where the x intercept derives pK_B the experimentally derived binding constant which is also known as the pA_2 as it occurs when the dose ratio is 2 and $\log_{10} (DR-1) = 0$. The slope of the plot is n is the hill number which is typically 1 for one to one binding but can be higher if more than one agonist needs to bind to the receptor to form an activated complex. It can be less than one if there is more than one receptor population to which the agonist may bind and elicit a response. The schild plot and the slope were set to one where there was significant deviation from unity. The shortened value of the Schild equation, which derives the pK_B by applying, a slope of 1

to the point representing the lowest concentration of antagonist and

extrapolating this line to the x axis.

Chapter 7

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