THE MODULATORY ROLE OF ADENOSINE IN DETRUSOR SMOOTH MUSCLE

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DOCTOR OF PHILOSOPHY

By

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Abstract

The contractile process of the mammalian detrusor smooth muscle is mediated through the co-release of acetylcholine (Ach) and adenosine triphosphate (ATP) from the embedded motor nerves. The transmitters are rapidly degraded in the synaptic cleft by extracellular enzymes and the breakdown products are taken back up into the synapse for re-synthesis. Adenosine is the final breakdown product of ATP, and can act through adenosine-specific P1-receptors to modulate the contractile process in detrusor smooth muscle. The nature of modulation by adenosine has not been fully investigated in human detrusor smooth muscle and the possible changes to this signalling system in disease states and the contribution to bladder overactivity.

In this study, *in vitro* muscle strip experiments with guinea-pig and human detrusor showed that adenosine reduces the force of contraction, with both electric-field stimulation and direct-muscle stimulation by the muscarinic-agonist, carbachol. A greater effect of adenosine was found on stable human bladder samples as compared to overactive bladder samples in nerve-mediated contractions. P1-receptor specific agonists indicated that A1-receptors were involved in pre-synaptic modulation of the contractile process, with a possible A2-mediated action on the smooth muscle cell itself. There was also stimulation frequency-dependent differential release of Ach and ATP in guinea-pig detrusor samples, with P1-receptor activity having a preferential effect upon ATP-mediated contractions.

Isolated cell experiments showed that Ca^{2+}-transients evoked by carbachol were significantly reduced by adenosine and $A2_B$-receptor activation in both human and guinea-pig detrusor cells. The use of an adenylate cyclase inhibitor, MDL-12230A, showed that agonist-induced Ca^{2+}-release is influenced by cAMP. However, P1-receptors do not appear to have a modulatory role in this process.

The results from this investigation indicate that adenosine can have a modulatory action upon the contractile process in detrusor smooth muscle, with differential effects of adenosine in stable and overactive human bladders. The implications and further understanding of the modulatory role of adenosine in the neuromuscular transmission in detrusor smooth muscle will be discussed.

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I wish to dedicate this PhD to my parents, Haruyoshi and Winifred, and my sisters, Keiko and Naomi. Also to all my dear friends and colleagues who have supported me throughout.

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List of Abbreviations

Ach	acetylcholine
ATP	adenosine tri-phosphate
IP_3	Inositol trisphosphate
NECA	N-ethylenecycloadenosine
CPA	N6-cyclopentyladenosine
IB-MECA	N^6-(3-iodobenzyl)-5'-(N-methylcarbamoyl)adenosine
MRS-1911	3-ethyl 5-benzyl 2-methyl-6-phenyl-4-phenylethylnyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate
CGS-21680	2-[p-(2-carbonyl-ethyl)-phenylethylamino]-5'-N-ethylcarboxamidoadenosine
Alloxazine	Benzo[g]pteridine-2,4(1H,3H)-dione)
ZM-231385	4-(2-[7-amino-2(2-furyl)[1,2,4]-triazolo[2,3-α][1,3,5]triazin-5-yl amino]ethyl)phenol
MDL-12330A	*cis*-N(2-phenylcyclopentyl)azacyclotridec-1-en-2-amine
TTX	tetrodotoxin
ABMA	α–β-methylene-ATP
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
DMSO	dimethyl sulphoxide
Ca^{2+}	Free ionised calcium
$[Ca^{2+}]$	Concentration of free ionised calcium
$[Ca^{2+}]_i$	Intracellular concentration of free ionised calcium

1.0 Introduction

The urinary bladder has two major functions, to store and void urine completely. The bladder requires an adequate filling capacity (approx. 500 ml in humans) and a low filling pressure, which has to be lower than the filtration pressure of the kidneys (intravesical pressure). Expelling urine from the bladder requires synchronous contraction of the detrusor muscle accompanied by the relaxation of the bladder neck and urethral sphincter, to result in complete expulsion of urine from the bladder.

The regulation of the storage of urine and the process of micturition requires neurogenic and myogenic control. Even though the micturition reflex is an autonomic process, bladder voiding normally involves integration at the spinal level and by the brain stem, as well as overall regulation by the cortex and mid-brain. This complex mixture of mechanisms makes the probability for dysfunction in the lower urinary tract relatively high (Sugaya *et al.*, 2005).

The mechanisms involved in the normal function of the bladder, that facilitate continence and voiding, from the molecular level to neuronal control of the lower urinary tract are incompletely understood. With knowledge of the normal control mechanisms the source of bladder dysfunction can then be investigated.

1.1 Structure and function of the urinary bladder

1.1.1 Different layers of the urinary bladder

The bladder is a highly compliant storage vessel for urine produced by the kidneys. It consists of three major layers; an epithelial layer, a muscular layer and an adventitial layer. The structure of the lower urinary tract is shown in Figure 1.1.

The adventitial layer that surrounds the bladder is involved in attachment to surrounding support structures to keep the bladder correctly positioned, and is composed predominantly of connective tissue.

The smooth muscle layer makes up the majority of the thickness of the bladder. It consists of relatively large smooth muscle bundles that do not have a specific orientation. There is generally very little connective tissue present in this layer. The triangular region between the ureteric orifices and the internal urinary meatus is referred to as the trigone and has been found to have distinct differences in its musculature from the rest of the bladder. The trigone has smaller muscle bundles, a greater amount of connective tissue and a dense adrenergic innervation. It is believed that during voiding the trigone may relax and cause a 'funneling' effect of the base of the bladder to facilitate voiding.

The epithelial layer is referred to as the urothelium, which consists of three cell layers: basal cells, intermediate cells and umbrella cells that line the apical surface. The

urothelium acts as a barrier to prevent contact of urine with the muscle layer. High resistance tight junctions exist between umbrella cells that allow definition of the apical and basolateral surfaces of this layer. The apical side of the umbrella cell layer is lined with uroplakin plaques on the apical surface that reduce the permeability of the urothelium to small molecules such as water and solutes (Birder, 2005).

The urothelium also has a role in transducing sensory information of bladder fullness. It is thought that an increase of vesical pressure causes release of ATP (Ferguson *et al.*, 1997) through an unknown mechanism that acts upon $P2X_3$ receptors of afferent nerves that extend to the basal surface of the urothelium. This causes an increase in afferent nerve firing and information is transmitted through to the central nervous system to assist in the co-ordination of voiding when appropriate. Transient receptor potential vanilloid 1 (TRPV1) receptors have also been shown to be expressed in human and rat urothelium and they are thought to have an important role in the sensory mechanism of the urothelium (Lazzeri *et al.*, 2004;Birder *et al.*, 2001), possibly through modulation of the purinergic pathway.

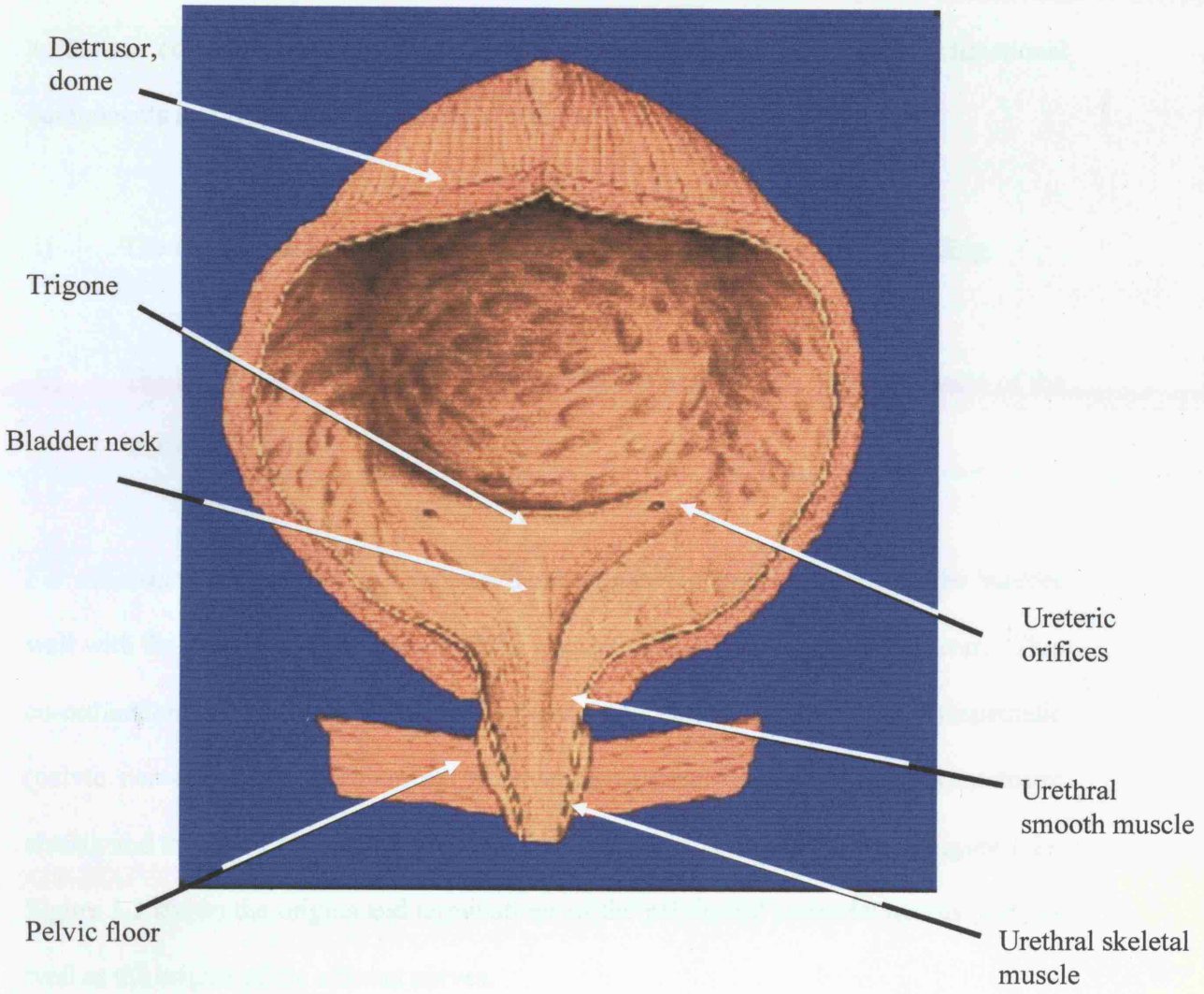


Figure 1.1 The structural components of the cross-section of the lower urinary tract (courtesy of Professor C.Fry)

1.1.2 Innervation of the urinary bladder

In humans, the storage and voiding processes in the bladder are under voluntary and autonomic control, and there is also regulation by learned behaviour. The two functional components involved in the expulsion of urine are:

i) The urinary bladder that acts as the reservoir and pressure head for voiding

ii) The outlet composed of the bladder neck, urethra, and the striated muscle of the urethral sphincter

For efficient voiding there must be co-ordination between the contraction of the bladder wall with the synchronous relaxation of the bladder neck and the urethral sphincter. This co-ordination is mediated by three sets of peripheral nerves, the sacral parasympathetic (pelvic nerves), the thoracolumbar sympathetic (hypogastric nerves and sympathetic chain), and the sacral somatic nerves from Onuf's nucleus (pudendal nerves) (Figure 1.2). Figure 1.2 shows the origins and terminations of the pelvic and pudendal nerves (left) as well as the origins of the afferent nerves.

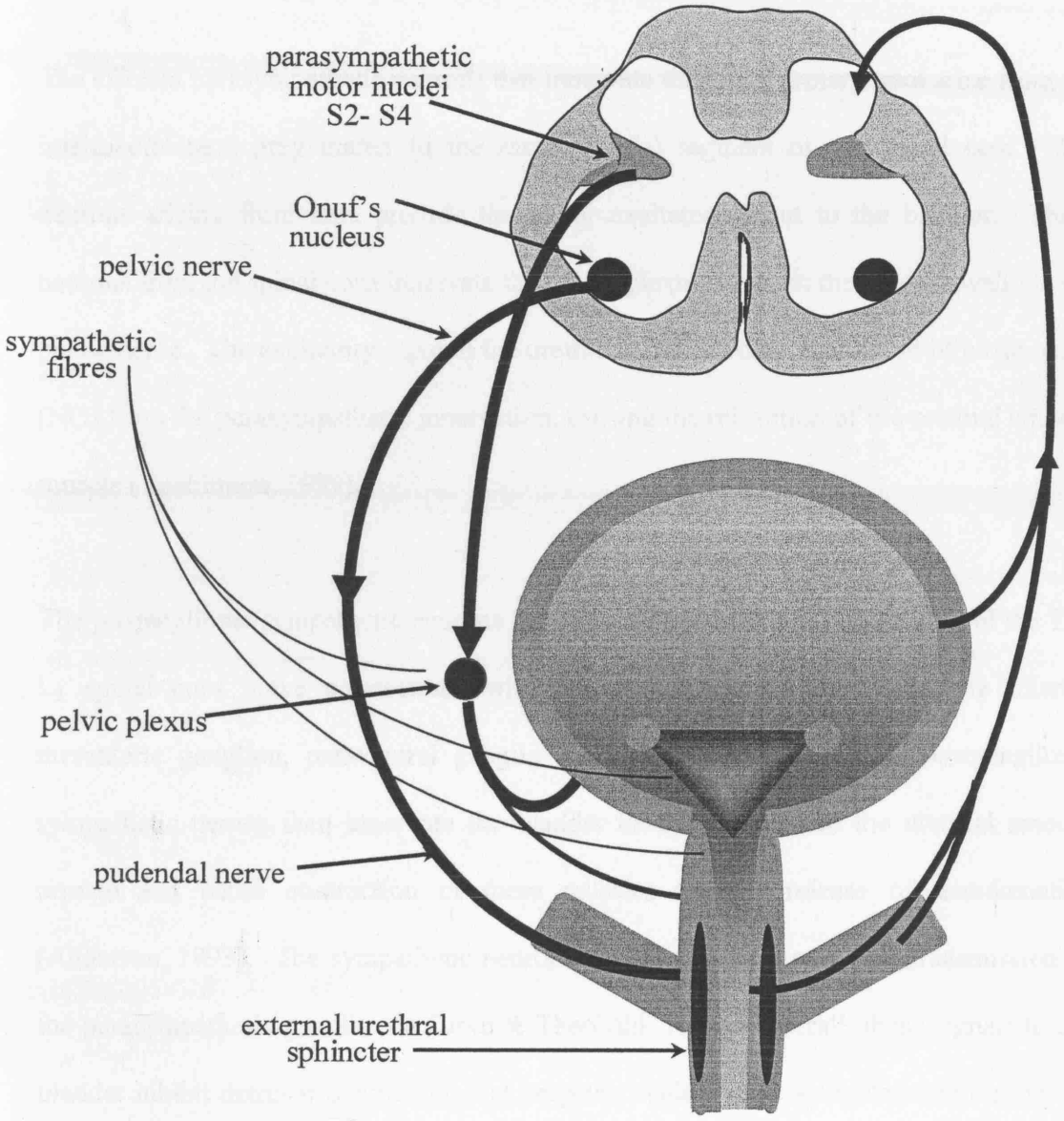


Figure 1.2 Schematic of the local innervation of the bladder and external sphincter, showing efferent (left) and afferent (right) pathways

1.1.3 Efferent pathways

The efferent parasympathetic neurons that innervate the lower urinary tract arise from the intermediolateral grey matter in the sacral (S_2-S_4) segment of the spinal cord. The neurons arising from here provide the major excitatory input to the bladder. These neurons from the spinal cord innervate the pelvic plexus and then the bladder wall via the pelvic nerve. The excitatory input to the urethra is mediated by the release of nitric oxide (NO) from the parasympathetic innervation, causing the relaxation of the urethral smooth muscle (Yoshimura, 1999).

The preganglionic sympathetic neurons from the intermediolateral cell column of the T_{11}-L_2 spinal cord make connections with the postganglionic neurons in the inferior mesenteric ganglion, paraventral ganglia and the pelvic ganglia. The postganglionic sympathetic nerves then innervate the bladder base (trigone) and the urethral smooth muscle and cause contraction of these muscles through release of noradrenaline (Anderson, 1993). The sympathetic neurons also inhibit the ganglionic transmission of the parasympathetic ganglia (de Groat & Theobald, 1976). Overall, these signals to the bladder inhibit detrusor contraction and keep the bladder neck sphincter shut during the filling phase.

The somatic efferents originate from Onuf's nucleus located in the anterior horn of the S_2-S_4 sacral spinal cord to innervate the external striated muscle of the urethral sphincter

(the rhabdosphincter) and the pelvic floor muscles. These neurons release Ach that act through nicotinic receptors to induce contractions (Figure 1.2).

Overall the parasympathetic and somatic efferents are regulated by descending fibres from the pons (the pontine micturition centre, figure 1.3) through a co-ordinated response considered below.

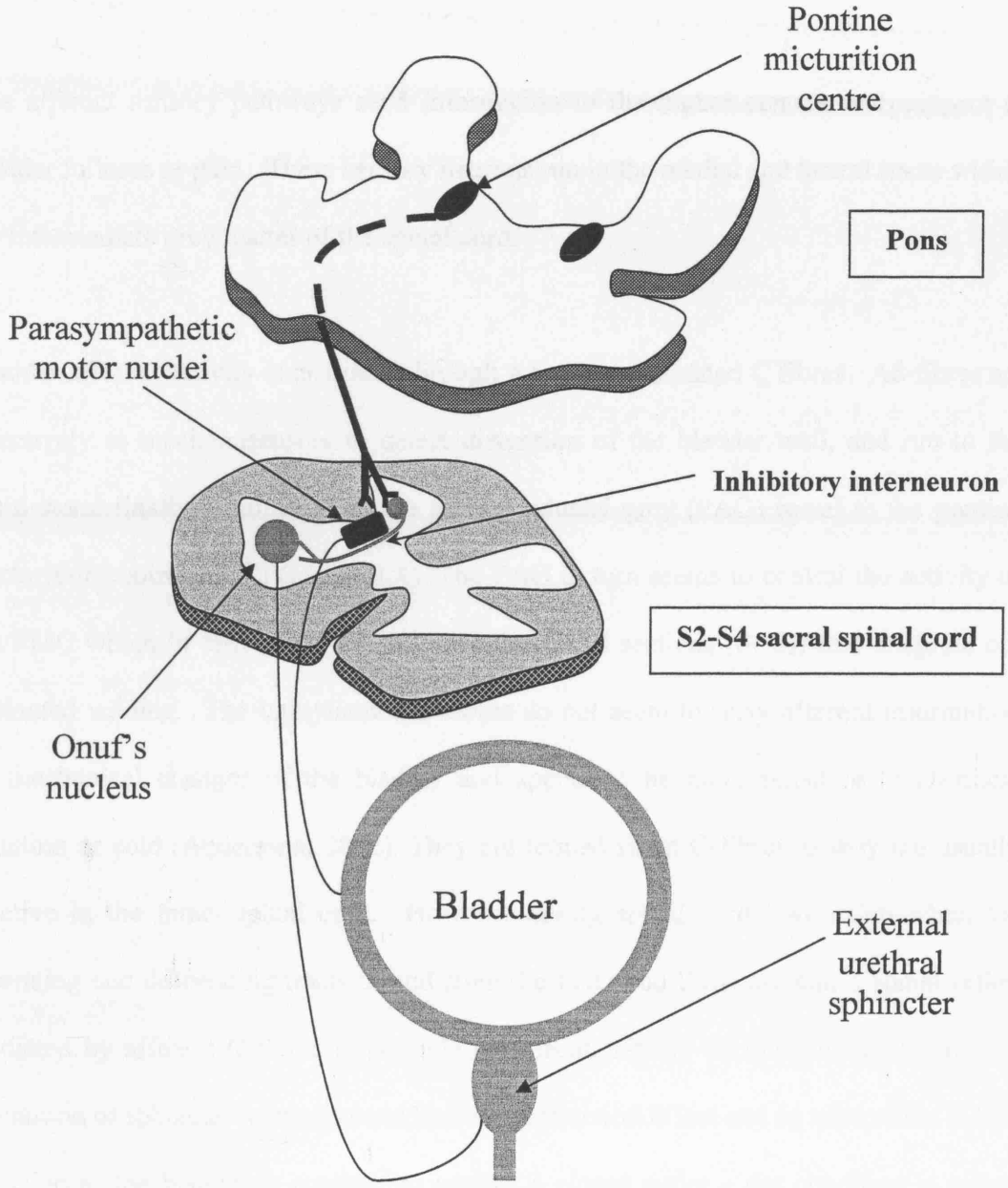


Figure 1.3 Schematic of efferent pathways to the bladder. Signals to void are sent from the PMC to inhibit the activity of the somatic pathway to relax the urethral muscle. There is also release of the inhibition on the parasympathetic motor nuclei to increase the contractile activity of the bladder

1.1.4 Afferent pathways

The afferent sensory pathways send information to the higher centres with respect to bladder fullness or pain. These sensory neurons run in the medial and lateral tracts within the intermediate grey matter of the spinal cord.

Bladder afferent activity is mediated through Aδ or unmyelinated C fibres. Aδ-fibres act effectively as mechanosensors to detect distension of the bladder wall, and run to the brain stem, finally terminating in the peri-aqueductal grey (PAG) rostal to the pontine micturition centre (PMC) (Figure 1.4). The PAG in turn seems to control the activity of the PMC which in turn exerts control over the spinal sections (S_2-S_4) that integrate co-ordinated voiding. The unmylinated C-fibres do not seem to relay afferent information on mechanical changes of the bladder and appear to be more sensitive to chemical irritation or cold (Andersson, 2002). They are termed silent C-fibres as they are usually inactive in the intact spinal cord. However during spinal cord transection when the ascending and descending tracts to and from the PAG and PMC are cut, a spinal reflex mediated by afferent C-fibres is possible (de Groat, 1997). In this case however, co-ordination of sphincter relaxation and bladder contraction is lost and so micturition is less effective as the bladder is contracting against a closed outlet – the condition is called detrusor sphincter dys-synergia. Figure 1.5 summarises the overall control of the lower urinary tract.

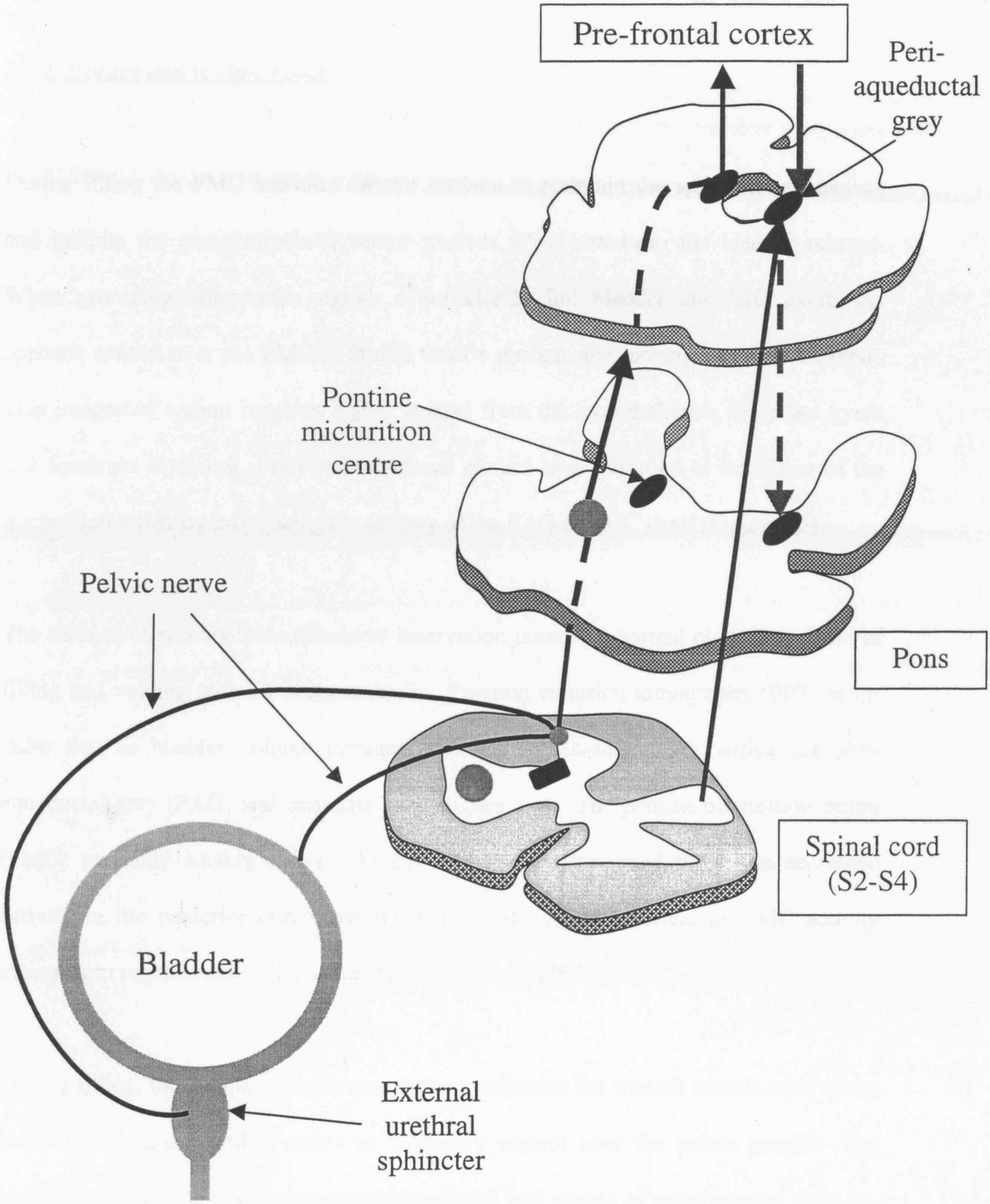


Figure 1.4 Schematic of afferent pathways from the bladder. Afferent fibres run from bladder to the spinal cord, sending the signal to the peri-aqueductal grey and higher centres of the brain

1.1.5 Storage and voiding reflex

During filling the PMC activates Onuf's nucleus to maintain the sphincter mechanism and inhibits the parasympathetic motor nucleus (PMT) to keep the bladder relaxed. When ascending information signals a sufficiently full bladder the PAG exerts the opposite control over the PMC to inhibit Onuf's nucleus and increases the PMT output. This integrated system receives higher control from the hypothalamus, cingulate gyrus and forebrain to ensure social and emotional control over initiation or inhibition of the micturition reflex by influencing the activity of the PAG or PMC itself (Figure 1.5).

The balance of activity from the above innervation maintains normal bladder function of filling and voiding with an intact neuraxis. Positron emission tomography (PET) scans show that as bladder volume increases there is increased activity within the peri-aqueductal grey (PAG) and cingulate area (Figure 1.6) The pontine micturition centre (PMC) was only weakly active. As the urge to void increased there was decreased activity in the posterior cortex hypothalamus and cingulate cortex, as PMC activity increases to regulate controlled voiding (Athwal *et al.*, 2001).

During filling, the sympathetic nervous system maintains the smooth muscle tone of the bladder neck and possibly exerts an inhibitory control over the pelvic ganglia. The bladder neck smooth muscle has α1-adrenoceptors, and release of noradrenaline from the sympathetic innervation keeps the bladder outlet closed during the filling phase: this may also be modulated by conscious control in humans (de Groat *et al.*, 1999). There is a

sparse β_3-adrenoceptor population in the dome region, and it has been proposed that this may contribute to relaxation of the detrusor smooth muscle (Fujimura *et al.*, 1999) and so minimise the rise of intravesical pressure.

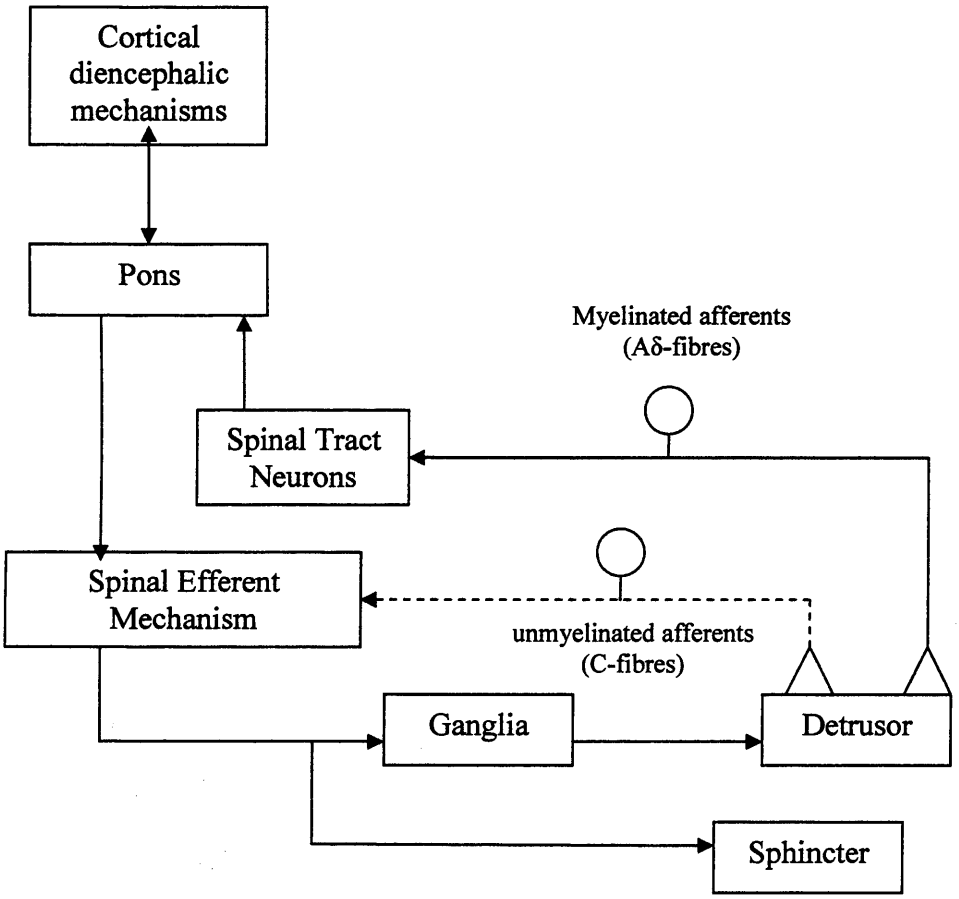


Figure 1.5 Schematic of the micturition reflex pathways that co-ordinate the micturition process, adapted from Yoshimura 1999

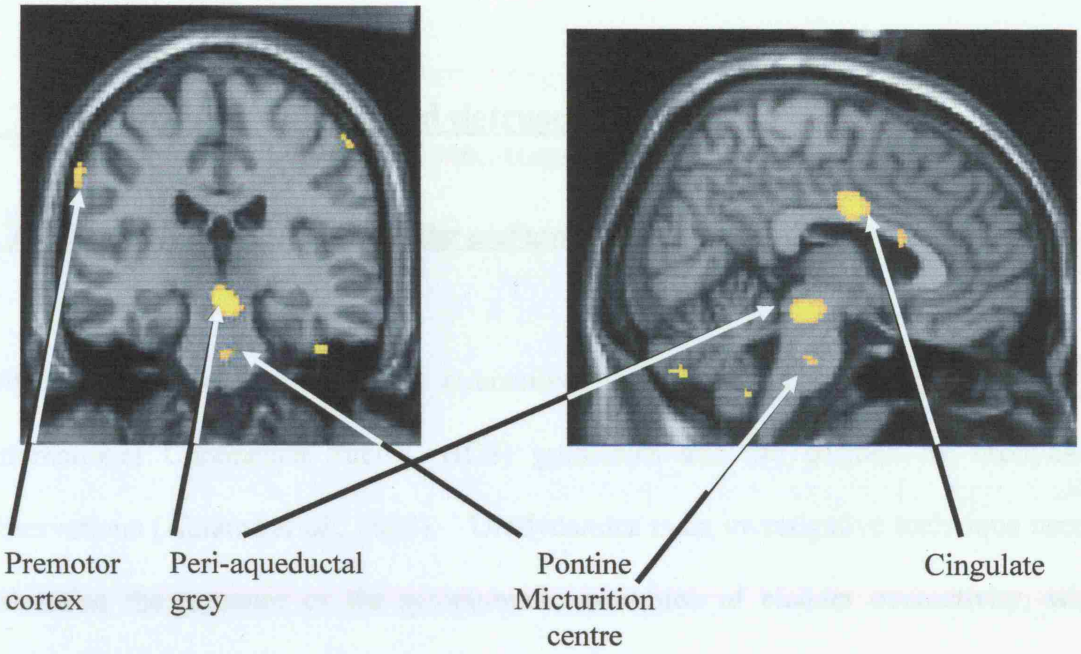


Figure 1.6 PET scan images of a healthy male brain during bladder filling (from Athwal et al., 2001) showing activation of premotor cortex, peri-aquaductal grey, PMC and cingulated gyrus

When the bladder voids, the activity of the parasympathetic system becomes dominant. Acetylcholine released from nerves in the dome region causes contraction of the detrusor smooth muscle. During this time the activity of the external urethral sphincter, especially the rhabdosphincter, diminishes just prior to voiding. The urethra contains both striated and smooth muscles, therefore has a significantly different innervation from that of the bladder dome. The two muscle types together contract to maintain closure of the urethral sphincter and also relax simultaneously to allow for micturition. (de Groat *et al.*, 1993;Lincoln & Burnstock, 1993). There are also muscarinic receptors activated in the urethra that induce nitric oxide production to contribute to relaxation of the muscles. The nitric oxide is produced by nitric oxide synthase of the endothelial cells, which diffuses to

the muscle layer to activate a cGMP-mediated relaxatory pathway. The combined action results in micturition with only relatively small rises of intravesical pressure.

1.2 Urinary incontinence and detrusor overactivity

1.2.1 Diagnosis of overactive bladder and urodynamics

Different symptomologies of the overactive bladder are classified according to the International Continence Society (ICS) guidelines and are defined by urodynamic observations (Abrams *et al.*, 2003). Urodynamics is an investigative technique used to determine the presence of the symptomatic syndrome of bladder overactivity, which refers to the symptoms of urgency, with or without urge incontinence, usually with frequency and nocturia. Observations are obtained from the reaction of the bladder to either natural or artificial filling. This is achieved by obtaining three different pressure measurements;

i) Intravesical – pressure within the bladder, usually measured with a catheter inserted via the urethra.
ii) Abdominal – pressure surrounding the bladder, usually measured with a rectal catheter.
iii) Detrusor – the estimated pressure created by the muscle wall, determined by subtracting abdominal pressure from intravesical pressure.

Urodynamics involves the simultaneous recording of the intravesical and abdominal pressure as the bladder is filled. The filling of the bladder can be physiological or

through artificial filling with either sterile or normal saline through a urethral catheter. The changes in the detrusor pressure can then be monitored as the bladder fills and voids.

There is little increase in the intravesical pressure in the normal bladder as it fills, with no signs of involuntary contractions. Initiation and suppression of voiding should also be under voluntary control. In overactive bladders there may be signs of spontaneous contractions when the patient is trying to suppress micturition and in some instances a decrease of bladder compliance. The ICS has defined that a patient can be diagnosed as having overactivity if the pressure of spontaneous contractions has amplitudes greater than 15cm H_2O during the filling phase of the cystometry.

Lower urinary tract symptoms are divided into three categories; storage, voiding and post-micturition symptoms. These include increased daytime frequency, nocturia, urgency and urinary incontinence.

Urinary incontinence is the involuntary leakage of urine and can be differentiated into certain types. Stress incontinence is leakage caused upon exertion or coughing and sneezing. Urge incontinence is the leakage preceded or accompanied by a strong urge to void. There can also be a combination of urge and stress symptoms to give mixed incontinence symptomology .

There have been many studies looking into the prevalence of urinary incontinence in the general population, however there has been much variation in the defining criteria.

Overactive bladder symptoms have been shown to be highly prevalent, with an estimated 19% of the UK population over 40 years of age having symptoms. The incidence of overactivity has also been shown to increase with age (Milsom *et al.*, 2001). Such a proportion of the population affected could have a significant impact upon the health care system.

1.2.2 Aetiology of bladder detrusor overactivity pathologies

There are several pathologies of bladder overactivity generally defined according to the principal cause of the overactivity. The causes for the symptoms can be neurogenic or myogenic in nature, or through a combination of both. In this study, the patient pathologies were separated into four groups;

1) Stable bladders – no signs of lower urinary tract symptoms associated with neurological damage, outflow obstruction or idiopathic detrusor overactivity.

2) Bladder overactivity due to neurological problems – can occur due to neurogenic damage from stroke, Parkinson's disease, multiple sclerosis or spinal injury.

3) Idiopathic detrusor overactivity – where a specific cause for the overactivity cannot be identified. The pathological processes involved are thought to be increased activity in the sensory afferent mechanism in the bladder wall and the urothelium, which lead to symptoms of urgency. This increase in afferent activity

is thought to trigger release of Ach from non-neuronal as well as post-ganglionic sources (Yoshida *et al.*, 2006), through the increase in afferent activity via reflex-pathways. The release of Ach has also been proposed to contribute to the involuntary contraction of the detrusor muscle (Mostwin *et al.*, 2005).

4) Overactivity associated with outflow obstruction – Bladder overactivity due to outflow obstruction is well established e.g. in those patients with benign prostatic obstruction. The overactivity is thought to be associated with partial denervation which causes the detrusor muscle to become less responsive to intramural nerve stimulation and also to develop super-sensitivity to Ach and ATP (Speakman *et al.*, 1987). There are also changes to the properties of the detrusor smooth muscle (van Koeveringe *et al.*, 1993). These and other causes are thought to contribute to detrusor overactivity.

1.2.3 Treatment of urinary incontinence and overactive bladder

There are a limited number of treatments for the overactive bladder. Generally the only pharmacological intervention is through use of anti-muscarinic drugs, of which there are many in clinical use. Oxybutynin is the most commonly prescribed anti-muscarinic in the UK. However the low selectivity of oxybutynin against muscarinic receptors in other tissues causes a significant incidence of side-effects, such as dry mouth. The severity and frequent side-effects make oxybutynin difficult to tolerate and many do not continue with the medication. Another muscarinic receptor antagonist used for bladder overactivity is

tolterodine, which has been found to have a functional selectivity for bladder muscarinic receptors over those in the salivary gland (Nilvebrant *et al.*, 1997).

It has been shown that multiple injections of botulinum toxin can also be used to treat spontaneous contractions of the overactive bladder. Botulinum toxin produces its effect by causing temporary inhibition of transmitter release from the nerve of the neuromuscular junction. This treatment has shown to be very efficacious from clinical trials with fewer side-effects than using anti-cholinergic drugs (Rajkumar & Conn, 2004).

Another possible avenue for treatment of unstable bladders may be to investigate the apparent increase in purinergic contractions that appears with detrusor overactivity. Atropine-resistant contractions have been observed from detrusor of patients with overactive bladders. The degree of atropine-resistance was found to be greatest in patients with obstructive and idiopathic detrusor overactivity (Bayliss *et al.*, 1999). This increase in ATP-signalling could be due to various reasons, such as changes in neurotransmitter release, receptor profile or drop in activity of extracellular enzymes that hydrolyse ATP (Harvey *et al.*, 2002). This gives support to an investigation of the function of receptors involved in purinergic signalling such as the P1 or P2-receptors described below to explore other avenues to modulate overactive bladder contractions.

1.2.4 Animal models of bladder outlet-obstruction

It has been shown in animal models that there are alterations to neurotransmitter release in obstructed bladders. In a rabbit model of outflow obstruction it was demonstrated that the size of nerve-mediated contractions increased and that the cholinergic component was decreased at the early stages of obstruction (Calvert *et al.*, 2001). An alternative reason for overactivity may be that there are changes to the receptor profile of the overactive bladder. It has been reported that there is loss of $P2X_3$ and $P2X_5$ subtypes in adults diagnosed with urge incontinence (Moore *et al.*, 2001). In obstructed bladders, the $P2X_1$ subtype has been shown to increase in expression possibly as a compensatory mechanism due to alterations in the detrusor smooth muscle content of the bladder. The significance of the observations are however not clear at present.

Bladder outlet obstruction can also cause significant changes to the physiology of the smooth muscle. It has been shown that in rabbits with decompensated bladders that there is a significant reduction in the number of ryanodine receptors and sarcoplasmic reticulum Ca^{2+}-Mg^{2+} ATPase (SERCA2) activity (Stein *et al.*, 2001). These alterations may contribute to the contractile dysfunction associated with obstructions as they are key elements in intracellular Ca^{2+} regulation – see section 1.3.

In summary, there are still many unknown aspects of the myogenic control of detrusor smooth muscle. The cellular mechanisms that regulate the contractile mechanism and relaxation will be examined in the proceeding sections.

1.3 Contractile mechanisms of detrusor smooth muscle

There are three major categories of muscle; skeletal muscle, myocardium and smooth muscle. They all have unique characteristics that allow them to function efficiently in their respective tissues.

As smooth muscle makes up the contractile element of hollow organs such as the intestine or bladder, it requires the ability to shorten over a range of initial resting lengths and maintain tension for long durations. There are two types of smooth muscle based on their contractile behaviour, these are 'tonic' and 'phasic' smooth muscle (Arner *et al.*, 2003). They have been shown to have distinct differences in the kinetics of the regulatory and contractile mechanisms (Arner *et al.*, 2003;Horiuti *et al.*, 1989). Detrusor smooth muscle can be considered as a phasic type as it can develop twitch-type contractions to stimulation by excitatory nerves or rapid application of excitatory transmitter.

1.3.1 Smooth muscle contractile machinery

As with all muscle types, in smooth muscle contractions are elicited through cross-bridge formation with the sliding of a highly organised actin-filament structure over an array of myosin II filaments in an ATP-driven process. Smooth muscle does not have a distinctive organisation of the contractile proteins as in skeletal and myocardial muscle where striations can be easily observed.

There are three filament types within smooth muscle cells: thin, intermediate and thick filaments. Thin filaments consist of polymerised actin monomers, which have tropomyosin molecules arranged across their lengths. Intermediate filaments are composed of desmin and vimentin, and are thought to be involved in structural functions of the smooth muscle cell. Thick filaments consist of a hexameric aggregate of two heavy chains and four myosin light chains, the heavy chain is composed of proteins from the myosin II family and the light chains are regulatory proteins. They are thought to influence the interaction of the myosin heads when the light chain is phosphorylated (Arner *et al.*, 2003).

The process of contraction in smooth muscle is highly dependent upon the intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$. During contractile activation there is a rise of $[Ca^{2+}]_i$ that forms a Ca^{2+}-calmodulin complex. This complex, with myosin light chain kinase, induces smooth muscle contraction by phosphorylation of serine-19 of the regulatory light chain associated with each head of the myosin molecule (Dabrowska *et al.*, 1978;Maita *et al.*, 1981) increasing actin-activated myosin ATPase activity. The increase of Mg^{2+}-ATPase activity allows interaction with the actin filament to produce a contraction.

Contractile activation is countered by the action of myosin light chain phosphatase (MCLP) which continually dephosphorylates the regulatory myosin light chain. There is also a Ca^{2+}-independent pathway to regulate phosphatase activity such as through Rho-associated serine/threonine kinase (Rho-kinase) and protein kinase C/CPI-17 activity

which affects the Ca^{2+} sensitivity of the contractile machinery (Takahashi *et al.*, 2004;Amano *et al.*, 1996).

1.3.2 Intracellular Ca^{2+} rise during smooth muscle contraction

There are two major sources of Ca^{2+} during contractile activation, from the extracellular space and intracellular stores. The pathways of Ca^{2+} entry from extracellular sources include dihyrdropyridine-sensitive and insensitive Ca^{2+}-channels and store-operated non-selective Ca^{2+}-channels. The release from intracellular stores is mediated by ryanodine and IP_3-receptors on the sarcoplasmic reticulum.

The majority of Ca^{2+} entry is through dihydropyridine (DHP)-sensitive Ca^{2+}-channels when the cell membrane is depolarised. There are also non-specific cation channels that can be activated by muscarinic receptors to cause depolarisation through the influx of cations such as Na^+ to open voltage-dependent Ca^{2+}-channels. There are also non-specific cation channels that are activated by mechanical stretch (Sanders, 2001). In many smooth muscles, there also appears to be a coupling of the depletion of the intracellular Ca^{2+} stores and activation of Ca^{2+} entry pathways. The detailed mechanisms involved in the store-operated Ca^{2+} entry has not been fully elucidated, however it is thought that the process is mediated by transient receptor potential (TRP) channels (Albert & Large, 2003).

In detrusor smooth muscle, contractile activation starts from binding of acetylcholine to surface muscarinic M3-receptors or through binding of adenosine triphosphate (ATP) to purinergic $P2X_1$-receptors. The muscarinic receptor activates phospholipase C through a G-protein coupled mechanism, to cause the production of inositol-trisphosphate (IP_3) and diacylglycerol (DAG) (Ferris & Snyder, 1992). IP_3 goes on to bind to specific receptors on the sarcoplasmic reticulum and cause the release of stored Ca^{2+} (Felder, 1995). The rise in the intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$ then goes on to activate the contractile machinery. DAG can also go on to activate protein kinase C (PKC) which will cause the phosphorylation of myosin light chain through a PKC-dependent Ca^{2+}-independent pathway (Harnett & Biancani, 2003). In the case of P2X-receptors, which are ligand-gated non-specific cation channels, these depolarise the cell through the influx of cations, and thus open L-type Ca^{2+} channels to allow Ca^{2+} influx (Isenberg *et al.*, 1992).

1.3.3 Relaxation of smooth muscle contractile machinery

The mechanism of relaxation could in principle involve two processes: a decrease in the intracellular $[Ca^{2+}]$ so that insufficient MLCK is activated to induce the contractile system; and an increase in activity of myosin light chain phosphatase (MLCP).

The majority of the intracellular Ca^{2+} is sequestered back into the sarcoplasmic reticulum (SR) through Ca^{2+}/Mg^{2+}-ATPase driven pumps. These proteins transport two Ca^{2+} ions for every ATP molecule hydrolysed (Haynes, 1983). There are also Ca^{2+}/Mg^{2+}-ATPase driven pumps on the plasma membrane to pump Ca^{2+} out of the cell and lower the $[Ca^{2+}]_i$

after contractile activation (Meiss, 1997). These differ from those on the SR as they have an auto-inhibitory domain that can bind calmodulin and stimulate the activity of this enzyme (Wuytack *et al.*, 1984;Wuytack *et al.*, 1980). In addition Na^{+}-Ca^{2+} exchange can operate in Ca^{2+} efflux mode in detrusor to facilitate Ca^{2+} removal (Wu & Fry, 2001).

Myosin light chain phosphatase (MLCP) consists of three subunits, one catalytic, one regulatory and one of, as yet, unknown function. The activity of MLCP is normally inhibited by G-protein dependent mechanisms (Hartshorne *et al.*, 2004). The inhibition appears to occur through phosphorylation of the regulatory subunit by Rho-associated kinase, which is activated through RhoA, a ras-related monomeric GTP-ase (Somlyo & Somlyo, 2000). MLCP can also be inhibited by the phosphorylation and activation of a smooth muscle specific protein inhibitor CPI-17, occurring through a protein kinase C mediated mechanism (Murthy *et al.*, 2003). These mechanisms contribute to the inhibition of MLCP activity and increase the Ca^{2+} sensitivity of smooth muscle contraction.

There are several signalling pathways that contribute to decreasing Ca^{2+} sensitisation and contractile activity. Protein kinase A (PKA) can phosphorylate MLCK to decrease its affinity to the Ca^{2+}/Calmodulin complex. This leads to more myosin light chain heads being dephosphorylated and a decrease in cross-bridge formation. PKA is activated through an increase of cellular cAMP levels, but this does not appear to be responsible for phosphorylation and activation of PKA (Murthy, 2005). So it is possible that cAMP decreases contractile activity through an as yet, undefined pathway. There is also

relaxatory pathway activated through production of cGMP. Soluable guanylate cyclase is activated by nitric oxide to elevate cGMP levels that activate protein kinase G (PKG). The activity of which has been shown to cause relaxation of the smooth muscle contraction.

1.3.4 Adenylate cyclase signalling and smooth muscle contraction

Cyclic adenosine monophospate (cAMP) has been recognised as an important cellular signalling molecule for many years. The signalling pathway is propagated through the conversion of ATP by adenylate cyclase to cyclic AMP (cAMP). The cAMP signalling pathway is terminated by phosphodiesterases that catalyse the conversion of cAMP to 5'-adenosine monophosphate (5'AMP) (Conti *et al.*, 2003;Robison *et al.*, 1968). Adenylate cyclase is a membrane protein coupled through different heterotrimeric guanine nucleotide binding regulatory proteins (G-proteins) to modulate the enzyme's activity. In mammals there are ten genes that encode for adenylate cyclase (nine membrane bound, one soluble form) which can be divided into five subfamilies based on their amino acid sequence. (Sunahara & Taussig, 2002).

Adenylate cyclase has 12 membrane spanning regions arranged into two distinct domains, and has two large intracellular loops where the catalytic activity is thought to exist. Activity of the enzyme is modulated through the α-subunit of the G_s-protein that stimulates the activity, whilst in other cases activity can be inhibited by coupling to G_i-

proteins. As well as G-proteins, it is thought that alterations in intracellular Ca^{2+} can affect adenylate cyclase activity (Hurley, 1999).

The effector of cAMP is protein kinase A, a tetrameric serine-threonine kinase, containing two regulatory and two catalytic subunits. The regulatory subunits have two cAMP-binding sites that cause the release of the regulatory subunit from the catalytic subunits upon binding of cAMP. (Forman, 1996)

Adenylate cyclase has been shown to have a role in smooth muscle contractions. It is known that cAMP can cause dephosphorylation of the myosin light chain which would inhibit cross-bridge formation. It is also possible to induce contractile relaxation through elevation of cGMP, it is possible that there is cross-talk between the pathway activated by cAMP and cGMP to modulate contractile function. As these cyclic nucleotides can regulate cell surface receptors, G-proteins, phosphlipaseC, IP_3 receptors, the Ca^{2+}- pump on the SR, MLCK, Rho-kinase, Mitogen activated protein (MAP) kinase, myosin phosphatase and plasmalemmal Ca^{2+}-channels (Abdel-Latif, 2001), it is likely that there is cross-talk between these pathways.

There are various mechanisms that could affect the levels of cAMP during contractile activation. For example, the activation of M2-receptors can reduce adenylate cyclase activity which would facilitate the contractile mechanism by reducing the amount of cellular cAMP (Ehlert, 2003).

As cAMP has been shown to have a role in modulating contractions, various phosphodiesterases (PDE) have been identified as possible targets for altering the contractile process. Each PDE subtype has different substrate specificities for either cAMP or cGMP. For example, PDE1 and 5 are cGMP-hydrolysing enzymes, PDE2 and 4 are cAMP-specific, and there are also dual-specificity subtypes such as PDE3 (Rybalkin *et al.*, 2002).

Rabbit and rat detrusor have been shown to express PDE1, 2, 3, 4 and 5. The contractile activity of the muscle was found to be dependent upon a cAMP-mediated system, with experiments using carbachol pre-contracted muscle strips, when addition of PDE inhibitors were shown to cause relaxation shown there was a greater contribution from the cGMP-hydrolysing enzymes in the relaxatory process (Qiu *et al.*, 2002;Qiu *et al.*, 2001).

1.4 Receptors involved in mediating bladder detrusor function

1.4.1 Neurotransmitters involved in contractile activation

Detrusor smooth muscle is innervated extensively by parasympathetic nerves to bring about bladder voiding. In mammals, contractions are mediated through the release of two neurotransmitters from embedded motor nerves, acetylcholine (Ach) that acts through muscarinic receptors, and ATP acting upon P2X receptors. There is also an adrenergic innervation to the bladder neck where the contractions are mediated through α-adrenergic receptors. Adrenergic receptors are also thought to have a major role in modulation of the parasympathetic nerves by the sympathetic neurons – see section 1.1.

When there is contractile activation, Ach and ATP are thought to be co-released from the motor nerves. Ach binds to muscarinic M3-receptors which activate phospholipase C and cause the generation of inositol-trisphosphate (IP_3). IP_3 binds to specific receptors on the sarcoplamic reticulum to cause a rise in intracellular Ca^{2+}. ATP binds to the $P2X_1$-subtype of purinoceptors, causing an influx of cations into the smooth muscle cell and depolarising the cell membrane. This opens L-type Ca^{2+}-channels resulting in further influx of Ca^{2+} into the cells to activate the contractile machinery.

1.4.2 Muscarinic receptors in the bladder

Acetylcholine is one of the contractile neurotransmitters released from the post-ganglionic parasympathetic motor nerves that innervate the detrusor muscle, and act through muscarinic receptors to induce a contraction of the smooth muscle.

There are five distinct muscarinic receptors that have been cloned (m_1-m_5) and most have been pharmacologically characterised. Muscarinic receptors are 7-transmembrane receptors and act as signal transducers through interactions with G-proteins. Each muscarinic subtype interacts with different G-protein subtypes to activate various signalling pathways. The M1, 3 and 5 subtypes are coupled to $G_q/_{11}$ and activate phospholipase C to induce inositol phosphate turnover. The M2 and M4 receptors have been shown to inhibit adenylate cyclase activity through coupling to G_i (Eglen *et al.*, 2001;Chess-Williams, 2002).

It has been found that there are two dominant muscarinic receptor subtypes present in bladder smooth muscle, the M2 and M3-receptors. There are proportionally more M2 than M3-receptors, although it is only the M3-subtype that is directly involved in contractile activation (Wang *et al.*, 1995;Chess-Williams *et al.*, 2001). The activation of the M3-receptor results in the stimulation of phopholipaseC-β with subsequent production of IP_3, causing release of Ca^{2+} from intracellular stores (An *et al.*, 2002).

However, the M2-subtype on the smooth muscle may be involved in modulation of the contractile process through inhibition of adenylate cyclase activity (Hegde *et al.*, 1997). This inhibitory action could be used to oppose sympathetic bladder relaxation caused by β-adrenoceptors and has been proposed to work synergistically with the M3-subtype for efficient emptying of the bladder. (Yamanishi *et al.*, 2002).

There are also possible roles for other subtypes in the regulation of pre-synaptic neurotransmitter release. The M1-receptor has been shown to facilitate the release of noradrenaline and acetylcholine in rat urinary bladder, with the L-type Ca^{2+}-channel playing a major role in its effect (Somogyi *et al.*, 1997;Somogyi *et al.*, 1994). It has also been shown that Ach acts in a negative feedback mechanism via M4-receptors on the motor nerve in human bladder detrusor strips, where nerve-mediated [3H]-Ach release was measured (D'Agostino *et al.*, 2000).

In normal human detrusor muscle, Ach appears to be the sole functional transmitter. This has been shown from the lack of atropine-resistant contractions in healthy human bladder (Kinder & Mundy, 1985). Most other mammalian species show a significant atropine-resistant component to nerve-mediated contractions. This has been identified as due to ATP release from the motor nerve as the contractions are sensitive to ABMA, an agent that initially activates and then desensitises $P2X_1$ receptors (Brown *et al.*, 1979). In animal bladders, the initial phase of the contraction is rapid and is thought to be mediated by ATP release, whilst the second phase is slower and is thought to be governed by Ach

release. It has been suggested that the purinergic mechanism may lead to brief, incomplete voids possibly for the purpose of marking territory in lower mammals.

1.4.3 Adrenergic receptors

Adrenergic receptors are predominantly found in the bladder neck and the trigone region, and are activated by sympathetic innervation. They are involved in the relaxation of the smooth muscle in these regions.

Adrenoceptors are separated into α1, α2 and β-receptors and are seven-transmembrane domain receptors. The α1-receptors are subdivided into $\alpha1_A$, $\alpha1_B$, $\alpha1_D$ and $\alpha1_L$ which all increase the activity of phospholipase C, while α2-receptors are divided into $\alpha2_A$ and $\alpha2_B$, which elicit their action by decreasing the activity of adenylate cyclase. There are 3 different subtypes of β-adrenergic receptors; β1, β2, and β3. The β-adrenergic receptors are all linked to the G_s subtype of G-proteins and are involved in the stimulation of adenylate cyclase activity (Docherty, 1998).

The $\alpha1_A$ and $\alpha1_D$ -subtypes have been shown to be expressed in human detrusor (Malloy *et al.*, 1998). The α1-receptor has been shown to promote the closure of the bladder outlet (de Groat & Yoshimura, 2001) and inhibit the phasic activity of whole bladder preparations (Gillespie, 2004).

All three β-subtypes have also been shown to be expressed in bladder smooth muscle, with the β3-receptor being more highly expressed than other subtypes (Fujimura *et al.*, 1999). The β2 and β3-receptors have been shown to mediate the relaxation of bladder trigone muscle (Yamanishi *et al.*, 2003) and detrusor smooth muscle. The mechanism is thought to be elicited through a rise of cAMP and also modulation of large conductance Ca^{2+}-activated K^{+}-(BK) channels (Uchida *et al.*, 2005).

There have been some studies that have implicated β-adrenergic receptors in modulating the contractile function of detrusor. It has been found that nerve and ATP-mediated contractions were reduced in the presence of β-adrenergic agonists (Hudman *et al.*, 2000a) which is thought to act via activation of a K_{ATP}-channel, as well as by increasing adenylate cyclase and PKA activity (Hudman *et al.*, 2000b).

1.4.4 Purinergic receptors in the bladder

The second neurotransmitter involved in bladder contractile activation has been identified as ATP. In most mammals, this can be demonstrated through atropine-resistant contractions that are abolished by desensitising the P2X-receptor with α,β-methyleneATP (ABMA) (Brown *et al.*, 1979). In healthy human bladder, contractions appear only to be mediated through acetylcholine, as atropine will completely abolish nerve-mediated contractions (Kinder & Mundy, 1985). Humans may have stopped using purinergic signalling as their mictruition behaviour changed through social conditioning. However, it is still possible to cause contractions in human detrusor though exposure to ATP, indicating the presence of functional P2X receptors on the muscle (Hoyle *et al.*, 1989). ATP has also been shown to be released in response to mechanical stretch of the urothelium (Ferguson *et al.*, 1997) and is thought to have a role as a sensory mediator to detect bladder wall distension, possibly by acting through suburothelial myofibroblasts (Wu *et al.*, 2004), and to activate suburothelial afferents (Cockayne *et al.*, 2005).

There are two major classes of purinergic receptors, the P1 and the P2 family. The P1 family is adenosine-specific whilst the P2 family consists of ATP-specific receptors. The families are further subdivided and many different receptor subtypes have been identified. In the case of the P1 receptor family, it is divided into four different types; the A1, $A2_A$, $A2_B$ and A3 receptors. P2 receptors consist of a much larger family; there are 2 major subdivisions, the P2X and P2Y receptors (Ralevic & Burnstock, 1998).

P2X and P2Y receptors also have an affinity for diadenosine polyphosphates (Bo *et al.*, 1994). In rat mesenteric artery, the number of phosphate groups determines the response of the muscle; four or more phosphate groups cause vasocontraction, while three or less cause dilation (Ralevic *et al.*, 1995).

Further purinergic subtypes, such as P3 and P4-receptors, have been described. A P3-like receptor has been described through radio-labelling experiments, this receptor has an affinity for both adenine nucleosides and nucleotides (Yoshioka *et al.*, 2001;Umino *et al.*, 2000). Another subtype has been described which only has affinity for diadenosine polyphosphates, these have been termed P4-receptors (Pintor & Miras-Portugal, 1995;Pintor *et al.*, 2000). It is unclear however what the relationship of these other sub-families is with the P1 and P2 families.

1.4.5 P2-receptors

The P2X receptors are ligand-gated ion channels and have seven distinct receptor subunits described thus far, with the subunit nomenclature $P2X_{1-7}$. P2X receptors form channels by combining two P2X-subunits to form a complete receptor channel. The characteristics of the receptor depend upon the combination of subunit types and homodimeric as well as heterodimeric P2X-receptors have been described. P2Y receptors are G-protein linked receptors and include 11 different members. (Ralevic & Burnstock, 1998)

In the bladder, the $P2X_1$-subtype has been shown to be the receptor involved in contractile activation. A $P2X_1$-deficient mouse model has shown that there is a loss of the purinergic component of nerve-mediated contractions (Vial & Evans, 2000). Most mammals with the exception of humans and old world monkeys show a purinergic contractile component to nerve-mediated contractions in detrusor smooth muscle. This is thought to do with the conditioning of humans and monkeys to micturate only when it is socially acceptable. Activation of P2X-receptors leads to a more rapid increase in intracellular Ca^{2+} as compared to muscarinic receptor activation that relies on second messenger pathways to be activated. Hence this leads to a more rapid contraction of the detrusor and faster expulsion of urine from the bladder. This may be advantageous for smaller animals which need to void more rapidly to reduce the risk from predators.

There is also evidence that demonstrates the role of P2X receptors in mechanosensory transduction in the bladder. $P2X_2$ and $P2X_3$-receptors have been identified as the possible subtypes involved. (Zhong *et al.*, 2003) $P2X_2$ and $P2X_2/P2X_3$ knockout mice showed an increased bladder hyporeflexia and reduced activity of the pelvic afferent fibres in response to bladder filling (Vlaskovska *et al.*, 2001).

It is not only P2X-receptors that are thought to be involved with controlling bladder function. The G-protein coupled P2Y-receptors are also present in detrusor smooth muscle and appear to activate a relaxatory pathway (Boland *et al.*, 1993). In marmoset detrusor muscle strips, a relaxatory pathway through a P2Y-receptor has been described which is thought to be mediated by a cAMP-PKA pathway (McMurray *et al.*, 1998).

This pathway may also help to modulate the effect of the contractile pathway through P2X activation.

The released neurotransmitters are degraded rapidly in the synapse by extracellular enzymes (a mixture of ectonucleotidases for ATP and acetylcholine esterase for Ach). The breakdown products are then rapidly taken back up into the neuron for re-synthesis. The final breakdown product of ATP is adenosine, which itself can act as a modulatory agent to smooth muscle contraction (Kazic & Milosavljevic, 1976).

There are other possible modulatory molecules involved in detrusor contractions. One is vasoactive intestinal protein (VIP), and one study has shown that VIP can reduce spontaneous contractions in human detrusor (Kinder *et al.*, 1985). Also VIP was shown to equally reduce nerve-mediated and carbachol-induced contractions in human and pig detrusor muscle strips (Klarskov *et al.*, 1984). However a detailed mechanism of its action has not been established.

1.5 Ectonucleotidases

The ATP released from the nerve terminal is degraded by a series of nucleotide hydrolysing enzymes in the extracellular space. These ectoenzymes are categorised into two major families, the ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) and the ectonucleotide pyrophosphate/phosphodiesterase (E-NPP) family. The E-NTPDase family can hydrolyse various nucleotide 5'-triphosphates and nucleotide 5'-diphosphates. The E-NPP family has a broader range of substrates it can act upon, having a phosphodiesterase and a nucleotide pyrophosphatase activity. They are able to hydrolyse the phosphodiester bonds of nucleotides and nucleic acids, as well as pyrophosphate bonds of nucleotides and nucleotide sugars. There are several other surface-located enzymes that are capable of hydrolysing extracellular nucleotides, such as ecto-alkaline phosphatase and ecto-5'-nucleotidase. These ectonucleotidases are normally located on the extracellular surface of the cell, however soluble forms have been described, which are thought to arise from proteolytic cleavage (Zimmermann *et al.*, 2000).

At the neuromuscular junction, these ectonucleotidases are located on the surface of nerve and muscle cells and rapidly break down the released ATP to adenosine. The adenosine can be further broken down to inosine or taken back into the cell through the adenosine transporter for nucleotide synthesis. There is also evidence that soluble nucleotidases are co-released with the neurotransmitters in tissues such as vas deferens (Todorov *et al.*, 1997). An ecto-5'-nucleotidase-like and an ecto-nucleoside triphosphate

diphosphohydrolase-like activity have been shown to be present (Mihaylova-Todorova *et al.*, 2002). The localisation of the ecto-nucleotidases has also been described in intestinal smooth muscle using electron microscopy. Ecto-ATPases were found to be located on the axon terminal as well as the smooth muscle surface, and 5'-nucleotidases only on the smooth muscle surface (Nitahara *et al.*, 1995). It is possible that the ecto-ATPase activity breaks down ATP to prevent it from acting through P2-receptors on the nerve terminal or smooth muscle, and the 5'-nucleotidase activity generates adenosine from AMP to act through P1-receptors.

Ecto-nucleotidase activity has also been found at the detrusor neuromuscular junction, where an ecto-ATPase inhibitor, ARL-67156, was shown to enhance the nerve-mediated contraction of guinea-pig detrusor muscle strips (Westfall *et al.*, 1997). In human detrusor, where there are normally no ATP-mediated contractions, the ectonucleotidase activity was shown to decrease in pathological samples, which may account for the increased incidence of atropine-resistant contractions in disease states (Harvey *et al.*, 2002).

1.6 Frequency – dependent co-transmitter release

It has been well established that Ach and ATP can be co-released from the motor nerve terminal (Silinsky, 1975;Silinsky & Redman, 1996), where the release of ATP, and not Ach, has been shown to be dependent on the stimulation frequency. This has also been described in the rat superior cervical ganglion, where there was a greater proportion of ATP released, as compared to Ach, at lower stimulation frequencies (Vizi *et al.*, 1997).

The frequency-dependent nature of ATP release appears to exist also in the nerves that mediate smooth muscle contractions. From studies with the co-transmission of noradrenaline (NA) and ATP in the vas deferens, the release of ATP was frequency-dependent while NA was not (Todorov *et al.*, 1996;Ellis & Burnstock, 1989a) similar to that seen with cholinergic nerves. Also the ATP release was modulated by capsaicin and calcitonin gene-related peptide but NA release was unaffected (Ellis & Burnstock, 1989b), indicating that there is differential control of the co-released neurotransmitters. The sympathetic perivascular nerves innervating the rabbit central ear artery also co-releases ATP and NA, where ATP contributed significantly more to the contractions elicited with short pulse bursts (1s) and low stimulation frequencies (2-5 Hz) (Kennedy *et al.*, 1986).

In circular muscle from guinea-pig vas deferens the amount of ATP released from sympathetic nerves decreased as the age of the animal increased. In adult animals virtually all the sympathetic contractions were mediated by noradrenaline and this was

thought to be due to post-synaptic changes associated with development (Ren *et al.*, 1996).

In bladder, differential release of ATP and Ach has been described using rat detrusor muscle strips. Similar to that previously described in other smooth muscles, it was found that ATP was more readily released at lower compared to higher stimulation frequencies (Brading & Williams, 1990). It has also been shown in guinea-pig urinary bladder that ATP-mediated contractions only occurred in response to single pulse stimulation or a train of pulses at a low frequency (0.5 or 1 Hz), whilst Ach-mediated contractions were associated with a higher stimulation frequency (20 Hz) (Hashitani *et al.*, 2000).

These findings indicate that release of ATP and Ach may have different regulatory pathways in the nerve terminal, such as those described with the co-transmission of NA and ATP.

1.7 Adenosine-receptors (P1-receptors)

1.7.1 Adenosine within cells

Under normal conditions adenosine is produced intracellularly from the precursor, S-adenosylhomocysteine. The reaction is catalysed by S-adenosylhomocysteine hydrolase and the adenosine molecules produced are normally phosphorylated to AMP by adenosine kinase (Thorn & Jarvis, 1996). This ensures that there is a strict control over the concentration of intracellular adenosine. Adenosine is actively transported through nucleoside transporters that also contribute to maintain the concentration balance in tissues such as coronary smooth muscle (Rubin *et al.*, 2000). It is thought that adenosine release and uptake occurs very rapidly, the concentration of extracellular adenosine has been estimated to be 18 nM under control conditions, determined by a cell-column model to measure adenosine outflow from coronary smooth muscle cells (Mattig & Deussen, 2001).

Under adverse conditions, it has been found that there is a change to the adenosine metabolism pathway. For example under ischaemia, there is an increase in the extracellular adenosine concentration from the breakdown of ATP, from both intracellular and extracellular sources. This is apparently a very rapid process as the production of adenosine doubles within five seconds of ischaemia when the cerebral blood flow was altered in the rat brain (Winn *et al.*, 1979).

1.7.2 Characteristics of adenosine-specific P1-receptors

P1-receptors are G-protein linked seven-transmembrane integral proteins, with four distinct subtypes characterised so far; the A1, $A2_A$, $A2_B$ and A3-subtypes. These receptors act primarily by modulating the activity of adenylate cyclase. The A1 and A3-subtypes couple to G_i to inhibit adenylate cyclase activity, while $A2_A$ and $A2_B$ are linked to G_s to stimulate activity (Ralevic & Burnstock, 1998).

Adenosine receptors were characterised in detail during the 1970s by several different laboratories. They demonstrated the existence of two distinct forms of the adenosine receptor, A1 and A2, by their ability to inhibit or activate adenylate cyclase respectively (Londos & Wolff, 1977;van Calker D. *et al.*, 1979). The A2-subtype was further subdivided into the $A2_A$ and $A2_B$-subtypes when it was determined that there was a heterogeneous affinity of adenosine and its analogues with the A2-receptor. Later the A3-subtype was cloned from various tissue types and then characterised pharmacologically as a distinct receptor. The P1-receptor subtypes have different affinities to adenosine, the potency of adenosine to each subtype is summarised in Table 1.1. Within mammals there is a relatively high sequence homology with P1-receptors with the exception of the A3-receptor, which can differ by up to 30% of its amino acid residues between human and rat (Zhou *et al.*, 1992)

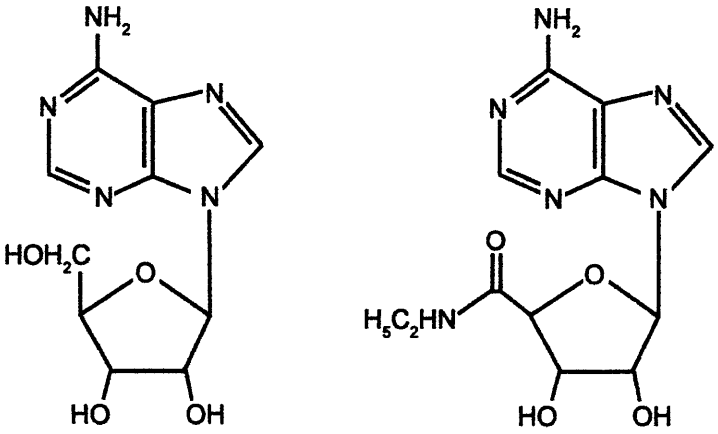


Figure 1.7 The structures of adenosine and general P1-receptor agonist NECA

P1-receptors are widely expressed throughout many different tissues. The expression pattern has been determined by various methods, for example with A1 and $A2_A$-subtypes radioligands have been used to show expression patterns. $A2_B$ and A3-receptors have been investigated to a lesser extent, mostly due to lack of specific ligands, so work has relied more on mRNA expression for targetting.

The A1-receptor is most highly expressed in the brain, dorsal horn of the spinal cord, the eye, adrenal gland and atrium. The $A2_A$-subtype has been found in spleen, thymus, leukocytes, blood platelets and the olfactory bulb. $A2_B$ is expressed in caecum, colon and bladder, while A3-receptors are highly expressed in testis and mast cells. (Fredholm *et al.*, 2001a)

In the bladder, all the P1-subtypes are expressed, with the $A2_B$ subtype more highly expressed than the others (Dixon *et al.*, 1996). There is also evidence for a functional role of adenosine in modulating the contractile properties of detrusor smooth muscle by reducing the force of contractions elicited by agonist and field-stimulation when pre-treated with adenosine (King *et al.*, 1997). Adenosine has also been shown to modulate the contractile activity of other smooth muscle types, such as a reduction of force in ileum (Gustafsson *et al.*, 1978); cat oesophagus, where A1-receptors induce contractions (Shim *et al.*, 2002); taenia coli where there is $A2_B$-mediated relaxation (Prentice & Hourani, 1997); and coronary artery smooth muscle where $A2_A$-mediated relaxation has been demonstrated (Abebe & Mustafa, 2002).

Adenosine receptor expression can differ in specific tissue types between species, such as in vas deferens smooth muscle. By examining the effect of adenosine and its analogues on agonist-induced contractions in the tissue from guinea-pigs there was a facilitatory A1-receptor and no A2-receptor activity, whilst in rabbit tissue an inhibitory post-junctional $A2_A$-receptor was expressed, with no apparent A1-receptor activity (Smith *et al.*, 1999).

Table 1.1 Comparison of the potency of adenosine to human P1-receptor subtypes, data from (Fredholm et al., 2001b)

Subtype	**Potency to human receptors expressed in CHO cells (EC_{50} - μM)**
A1	0.31
$A2_A$	0.73
$A2_B$	23.5
A3	0.29

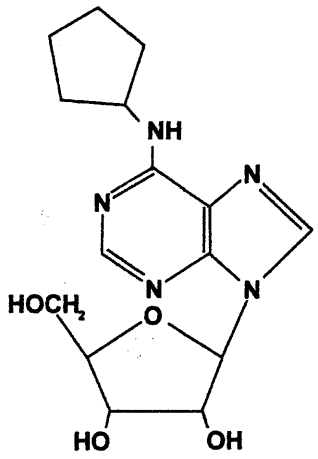
1.7.3 A1-receptors

The A1-receptor has been cloned from various species and shows significant interspecies differences in amino acid sequences. The receptor can interact with various signalling pathways possibly by its ability to couple to different proteins from the $G_{i/o}$ family.

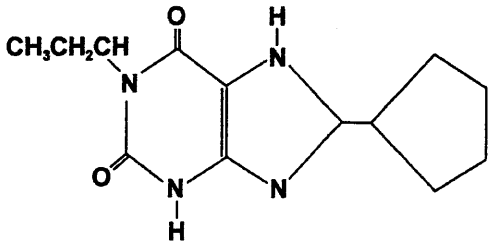
A1-receptors are not exclusively associated with the modulation of adenylate cyclase activity. They have been described to be directly coupled to K^+-channels, particularly in cardiac muscle and are found ubiquitously throughout the central nervous system. A1-subtypes have been linked to ATP-sensitive K^+-channels in chick ventricular myocytes (Liang, 1996) and coupled to K^+-channels via pertussis toxin-sensitive G-proteins (Kirsch *et al.*, 1990). A1-receptors have been shown to hyperpolarise neurons by increasing K^+-conductance which increases neuronal excitability.

A1-receptors can also increase the activity of phosopholipase C, which leads to the production of inositol 1,4,5-trisphosphate (IP_3), diacylglycerol (DAG) and Ca^{2+}-release from intracellular stores (Shim *et al.*, 2002). The release of Ca^{2+} may lead to a further increase in intracellular Ca^{2+} from activation of plasma membrane ion channels. A1-channels can also inhibit Ca^{2+}-influx in chick ciliary ganglion through inhibition of pre-synaptic omega-conotoxin GVIA-sensitive Ca^{2+} channels (Yawo & Chuhma, 1993) and may contribute to the inhibition of neurotransmitter release.

There are several selective ligands for A1-receptors that have been useful in the study of its function. There are a variety of A1-selective agents such as N^6-cyclopentyladenosine (CPA), a selective agonist, or 1,3-dipropyl-8-pentylcycloxanthine (DPCPX), an antagonist, that have helped to characterise this subtype (Figure 1.7). Binding of agonists to A1-receptors can be allosterically modulated by compounds such as PD-81723 (Gao *et al.*, 2005). Binding to the allosteric site is thought to stabilise the interaction with the G-protein and the agonist-A1 complex (Bhattacharya & Linden, 1995).



CPA



DPCPX

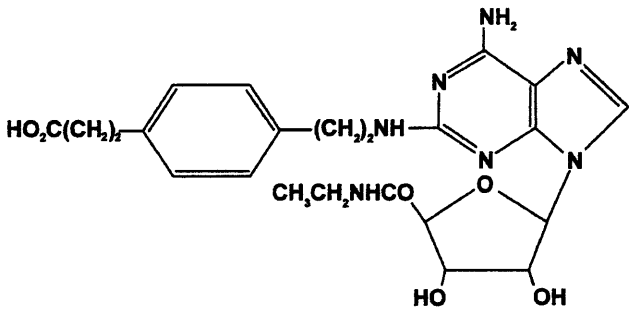
Figure 1.8 The structures of A1-specific compounds

1.7.4 $A2_A$-receptors

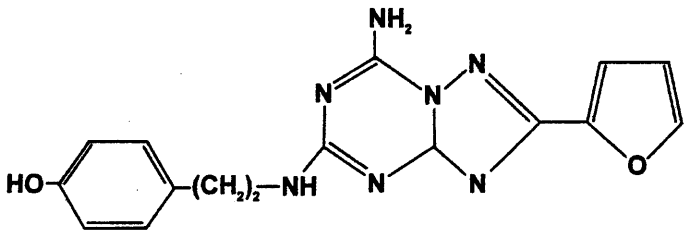
This is the largest of the four subtypes (45kDa) due to its longer intracellular carboxyl terminal. It has been cloned from many different species and was one of the first subtypes to be found. The $A2_A$ subtype stimulates adenylate cyclase activity so is most likely coupled to the G_s protein family, although other G-proteins may be also be involved. $A2_A$-receptors couple very tightly to its G-protein, so there is only slow dissociation of the agonist from the receptor and the receptor-G protein complex is very stable (Ralevic & Burnstock, 1998).

$A2_A$-receptors have been linked to the activation of K_{ATP} channels in arterial smooth muscle (Mutafova-Yambolieva & Keef, 1997) and in guinea-pig bladder (Gopalakrishnan *et al.*, 2002). $A2_A$-receptors have also been implicated in vasodilation of coronary arterial smooth muscle (Conti *et al.*, 1997) and corpus cavernosum where it has been shown to reduce the amount of ATP-mediated relaxation (Filippi *et al.*, 1999)

There have been a number of $A2_A$-specific agonists developed along with a few useful selective antagonists, mostly due to the fact that it is a high affinity receptor. CGS-21680 is a selective agonist, but has been shown to have different affinities depending upon the species from which the receptors are derived (Figure 1.8). Antagonists such as ZM-241385 and SCH-58261 have been shown to be highly selective, although they can have a small amount of additional selectivity towards $A2_B$-receptors (Ongini *et al.*, 1999).



CGS-21680



ZM-241385

Figure 1.9 The structures of $A2_A$-specific compounds

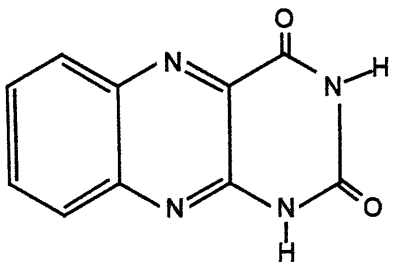
1.7.5 $A2_B$-receptor

The existence of two different A2-receptors came from the heterogeneous nature of the receptor's binding affinity, when it was observed that there was a high and low affinity binding state (Daly *et al.*, 1983). $A2_B$-receptors have been shown to be expressed in a wide range of tissues and in particular is highly expressed in bladder (Dixon *et al.*, 1996).

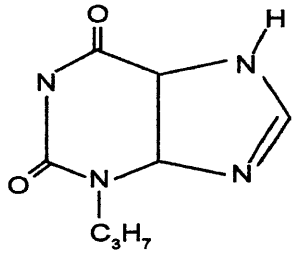
The $A2_B$-subtype is a low affinity receptor and subsequently there are no selective agonists. There are a few selective antagonists, such as alloxazine and enprofylline, however the affinities are in the micro-molar range (Figure 1.9). Thus there has been much work on developing $A2_B$-selective agents (Beukers *et al.*, 2004;Feoktistov & Biaggioni, 1998;Volpini *et al.*, 2003) and the developments of several $A2_B$-selective compounds from the anilide derivatives of an 8-phenylxanthine carboxylic congener have also been described (Kim *et al.*, 2000). The non-xanthine alloxazine has been used as a selective antagonist for the $A2_B$-receptor (Figure 1.9), as it has a nine-fold selectivity over the $A2_A$-subtype. However at concentrations greater than 1 μM the agent also binds to $A2_A$-receptors (Brackett & Daly, 1994). The development of $A2_B$-specific drugs has been driven by the evidence for its role in various disease processes and thus as a possible therapeutic target.

The $A2_B$-subtype is thought to be involved in inflammatory disease such as asthma and chronic obstructive pulmonary disease. $A2_B$-receptors have been found on human lung mast cells and it has been proposed as a potential anti-asthmatic drug target (Feoktistov &

Biaggioni, 1996). Recent studies have found that activation of $A2_B$-receptors increases the transcription of proinflammatory mediators, specifically interleukin-6 and monocyte chemotactic protein-1 (Zhong *et al.*, 2004).



Alloxazine



enprofylline

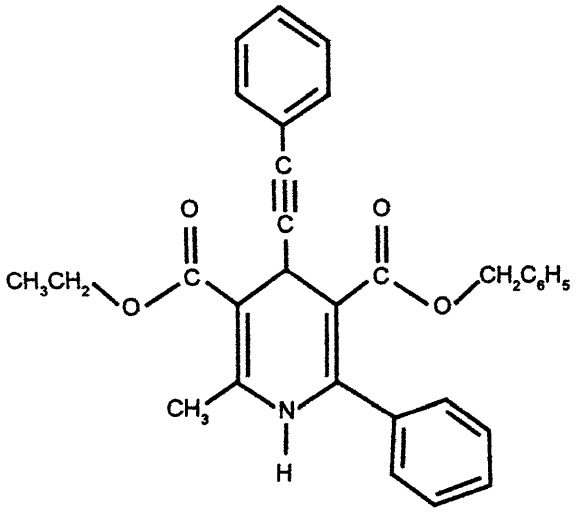
Figure 1.10 The structures of the A2B-selective antagonists

1.7.6 A3-receptors

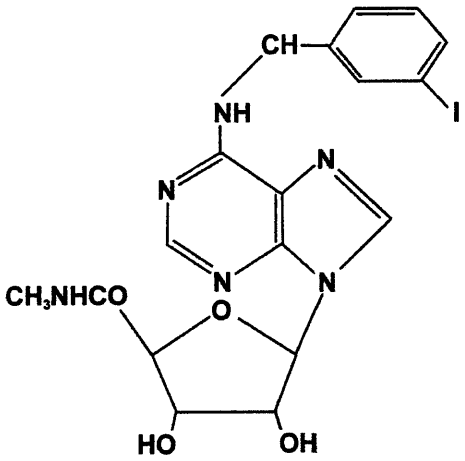
This is the most recently uncovered subtype and was discovered through cloning rather than pharmacological methods. It has a characteristic xanthine-resistant binding site. Thus compounds that have been developed for its study are all non-xanthine structures, such as dihydropyridines, pyridines, isoquinolines, quinazoline derivatives and flavonoids. An example of a specific antagonist for A3-receptors is MDL-1191 and an agonist is IB-MECA (Figure 1.10).

A3-receptors have been characterised in various different tissue types. It has been found to have a role in cell proliferation (Madi *et al.*, 2004;Schulte & Fredholm, 2003) and is also thought to have a role in cardioprotection during ischaemic events (Liang & Jacobson, 1998) and mast cell degranulation (Jin *et al.*, 1997) This is postulated to be due to A3-receptors having a lower affinity for adenosine as compared to A1 and $A2_A$-receptors so may only be activated during times when there is a large amount of adenosine present such as during ischaemic or other adverse conditions. However, this difference does not appear to be evident from the results shown in table 1.1, where A3-receptors showed a similar affinity to A1 and $A2_A$-subtypes.

A summary of the binding affinities of different P1-specific agonists and antagonists is shown in Table 1.1.



MDL-1191



IB-MECA

Figure 1.11 The structures of A3-specific compounds

Table 1.2 Comparison of the binding affinity of various P1-specific agonists

Compound	Subtype selectivity	Action	Binding affinity to human receptors
NECA	All	agonist	A1 – 14 $A2_A$ – 20 $A2_B$ – 330 A3 – 62 (Fredholm *et al.*, 2001a)
Cyclopentyladenosine (CPA)	A1	agonist	A1 - 2.3 $A2_A$ - 790 $A2_B$ – 18,800 A3 – 67 (Klotz, 2000)
CGS-21680	$A2_A$	agonist	A1 - 290 $A2_A$ – 27 $A2_B$ – 88,800 A3 - 67 (Klotz, 2000)
IB-MECA	A3	agonist	A1 – 3.7 $A2_A$ – 2500 $A2_B$ – 11,000 A3 - 1.2 (Klotz, 2000)

EC_{50} values in nM are given for the $A2_B$-subtypes for receptor-activated adenylate cyclase activity in membrane preparations. All other subtypes are Ki values in nM.

Table 1.3 Comparison of the binding affinity of various P1-specific antagonists

Compound	Subtype selectivity	Action	Binding affinity (K_i - nM)
DPCPX	A1	antagonist	A1- 3.9 $A2_A$ – 130 $A2_B$ – 40,000 A3 – 86,000 (Human) (Klotz *et al.*, 1998)
ZM-241385	$A2_A$	antagonist	A1 – 255 $A2_A$ - 0.8 $A2_B$ – 50 A3 - >10000 (Human) (Ongini *et al.*, 1999)
Alloxazine	$A2_B$	antagonist	A1 - 5200 $A2_B$ - 2700 (Rat) (Bruns *et al.*, 1986)
Enprofylline	$A2_B$	antagonist	$A2_B$ - 7000 nM (Human) (Feoktistov & Biaggioni, 1997)
MDL-1191	A3	antagonist	A1 – 39.2 A3 -1.4 (Rat) (Jiang *et al.*, 1997)

1.7.7 Functional effects of adenosine on smooth muscle pre- and post-synaptic events

It has been estimated that in the striated muscle neuromuscular junction, approximately half of the adenosine is derived from the hydrolysis of ATP released from the nerve terminal, with the other half being released from the muscle cell itself (Smith & Lu, 1991). It is thought that the adenosine is either taken back into the nerve through selective transporters or feeds back onto P1-receptors on the pre and post-synaptic sites to elicit a physiological effect.

The A1-receptor has been largely implicated in modulating the pre-synaptic effects by inhibiting the release of neurotransmitters. This has been described in various smooth muscles such as vas deferens (Driessen *et al.*, 1994), ileum (Lee *et al.*, 2001) and bladder (Acevedo *et al.*, 1992). The inhibition of neurotransmitter release is thought to be mediated by inhibiting cAMP production (Chen *et al.*, 1989) and inhibition of Ca^{2+} channels and activation of K^+-channels..

It has been shown in various animal models, that the A1-subtype is located pre-synaptically, and a possible A2-subtype post-synaptically in bladder smooth muscle (Acevedo *et al.*, 1992). The pre-synaptic A1-receptor has been implicated in the inhibition of neurotransmitter release.

On the post-synaptic side it is thought that P1-receptor activation results in a disruption of the inositol phosphate pathway, which would shorten the duration of calcium release

from the sarcoplasmic reticulum. There is also evidence that $A2_A$-receptors act through ATP-sensitive K^+-channels in the guinea pig detrusor to cause relaxation of muscle contractions post-synaptically (Gopalakrishnan *et al.*, 2002).

The role of P1-receptors have not been fully characterised in detrusor smooth muscle, specifically in the human detrusor in its various pathological states. A particular question centres around the presence of an ATP-dependent component of human detrusor nerve-mediated contractions. This represents an additional contractile activation that is associated with bladder overactivity and it remains unclear why it should appear during these bladder pathologies. One possibility is that P1-receptor modulation of neurotransmitter release is modulated with bladder overactivity and if so would represent a potential drug target to manage this condition.

In this study the effect of specific P1-receptor agonist/antagonists in human and guinea pig detrusor smooth muscle was investigated. The study will also try to determine whether there are changes to the P1-receptor function in different pathologies.

1.8 Aims and objectives of thesis

The aim of this project was to investigate the role of adenosine-specific P1-receptors in modulating the contractile activity of guinea-pig and human detrusor smooth muscle. The effects of adenosine and P1-subtype specific agents were investigated on the contractile activity of isolated detrusor strips, which were contracted by electric-field stimulation or through direct-muscle activation with agonists. The intracellular Ca^{2+} signalling pathways affected by P1-receptors were investigated in isolated detrusor myocytes.

The following experimental objectives were addressed through the experiments performed in this study, to determine the role of adenosine on detrusor contractions.

i) To determine the modulatory role of P1-receptors on nerve and agonist-induced contractions and whether this differs between guinea-pig and human preparations.

ii) To determine if there is an altered response to P1-receptors in human detrusor preparations from stable and overactive bladders.

iii) To characterise the effect of P1-receptors on agonist-induced intracellular Ca^{2+} release, and the signalling pathways that it affects.

2.0 Materials and Methods

2.1 SOLUTIONS AND CHEMICALS

2.1.1 Tyrode's solution

A physiological solution of modified Tyrode's solution was used for experiments, and gassed with 95% O_2, 5% CO_2 mixture to maintain a pH of 7.33 ± 0.02. The solution was kept at a constant temperature of 37C° throughout.

Fresh Tyrode's solution was prepared before each experiment using deionised water from a Purite reverse osmosis water filter (Purite Ltd, Thames, Oxon, UK). The composition of the Tyrode's solution is given in Table 2.1. The components were added in either their solid form or from stock solutions of 1M KCl, $MgCl_2$ and NaH_2PO_4, except for $CaCl_2$, which was purchased as a 1M solution (VWR). Stock solutions were made in Analar grade water and stored at 4C°. The solid components of the Tyrode's solution were weighed using a Sartorius balance (Sartorius Ltd. Epsom, Surrey) and liquids were measured using variable volumetric pipettes.

Human tissue samples were collected and transported from the operating theatre in an ungassed, nominally Ca^{2+}-free N-[2-hydroxyethyl]piperazine-N'-[2-ethaneesulfonic acid] (HEPES)-buffered Tyrode's solution with the pH adjusted to 7.6 (composition listed in Table 2.1). This solution was also used for overnight storage of the tissue at 4C°. All chemicals for these solutions were from Sigma, UK.

Table 2.1 Composition of normal Tyrode's solution and Ca^{2+} -free HEPES Tyrode's solution

Chemicals	**Normal Tyrode's solution Concentration (mM)**	**Ca^{2+}-free HEPES solution Concentration (mM)**
NaCl	118	132
$NaHCO_3$	24	--
KCl	4.0	4.0
$MgCl_2.6H_2O$	1.0	--
$NaH_2PO_4.2H_2O$	0.4	0.4
$CaCl_2.6H_2O$	1.8	--
Glucose	6.1	6.1
Sodium pyruvate	5.0	5.0
HEPES	--	10.0

2.1.2 Chemicals and Drugs

Various chemicals and drugs were added to Tyrode's solution to the required concentrations and were used for interventions during the experiments.

Atropine, tetrodotoxin (TTX), carbachol, α–β-methylene-ATP (ABMA), N-ethylenecycloadenosine (NECA), N^6-cyclopentyladenosine (CPA), benzo[g]pteridine-2,4(1H,3H)dione) (alloxazine), N^6-(3-iodobenzyl)-5'-(N-methylcarbamoyl)adenosine (IB-MECA), 3-ethyl 5-benzyl 2-methyl-6-phenyl-4-phenylethylnyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS-1911), 2-[p-(2-carbonyl-ethyl)-phenyl-ethylamino]-5'-N-ethylcarboxamidoadenosine (CGS-21680), 4-(2-[7-amino-2(2-furyl)[1,2,4]-triazolo[2,3-α][1,3,5]triazin-5-yl amino]ethyl)phenol (ZM-241385) and *cis*-N(2-phenylcyclopentyl)azacyclotridec-1-en-2-amine (MDL-12330A) were added from 10 mM stocks to give the required concentrations.

All stocks were made up from the dry solid dissolved in Analar grade water with the exception of NECA, CPA, alloxazine, CGS-21680, IB-MECA, MRS-1911, MDL-12330A and ZM-241385 which were dissolved in dimethyl sulphoxide (DMSO). Stocks were aliquoted, stored at –20C° and thawed for use. All chemicals were purchased from Sigma, with the exception of ZM-241385, which was a gift from Pfizer Ltd.
Adenosine was added from a solid powder to the Tyrode's solution on the day of the experiment to a maximum concentration of 1 mM. Lower concentrations were made through serial dilution.

2.1.3 Tissue collection and patient groups

Detrusor samples were obtained from sections of human tissue excised during open surgery, or from isolated organs of guinea-pigs. Dunkin-Hartley guinea-pigs were humanely sacrificed by schedule 1 methods according to UK Home Office guidelines. The bladder was immediately dissected out and placed into Ca^{2+}-free Tyrode's solution.

Human bladder biopsies were obtained from patients undergoing open surgery with informed written consent from the patient or guardian and local ethical approval from University College London Hospital Trust. The biopsies were collected from surgical theatres, where samples were immediately placed into Ca^{2+}-free Tyrode's solution for transport. Biopsies were dissected from the dome region in a posterior to anterior direction. The human samples were divided into three major groups according to their clinical diagnoses;

(i) 'Control' group of stable bladders. These samples were obtained from patients having a cystectomy or removal of a localised bladder tumour. Whole bladders removed by cystectomy for cancer had the detrusor sample dissected out from regions that were unaffected by tumours.

(ii) Primary overactivity group. These patients were described as having primary overactivity associated with neuropathic disorders or bladder outlet obstruction.

(iii) Idiopathic detrusor overactivity where the cause of the overactivity was unknown.

Overactive samples were obtained from patients with urodynamically proven bladder overactivity. Tissue samples were generally obtained from ileocystoplasty procedures.

2.2 Isometric Tension Measurement

2.2.1 Muscle strip preparation

Bladder samples were placed into a dissection dish with normal Tyrode's solution and immobilised with fine gauge syringe needles. The serosa and mucosa were dissected off full-thickness samples with fine dissecting scissors. The tissue was dissected using a binocular microscope (Nikon AL5, Nikon Corporation, Tokyo, Japan) to identify the orientation of muscle bundles, particularly with human samples.

In human samples the detrusor muscle layer appeared as bundles approximately 0.5 mm in diameter, arranged in a random manner. The guinea pig detrusor layer consisted of densely packed bundles arranged longitudinally as orientated from the apex of the dome towards the bladder outlet. The strips were dissected to get intact bundles. The urothelium was dissected off and strips were cut longitudinally from the dome of the bladder, avoiding the trigone region, as the innervation is different for this area.

Strips were formed from the detursor bundles approximately 1-1.5 mm in diameter and 3-5 mm in length. The strip was tied with surgical suture thread (Persalls sutures, cornea silk, US7/0) and transferred to the superfusion trough of the organ bath

2.2.2 Tissue mounting and equipment set-up

The Tyrode's superfusion solutions were continually gassed with 95% O_2, 5% CO_2 and kept at a constant 37C^{o} in a water bath. The superfusate was delivered to the muscle strip in the superfusion trough with a gravity-fed water-jacketed system via 2 mm diameter tubing. The organ bath volume surrounding the muscle was approximately 0.064 cm^3.

The horizontal micro-perfusion organ bath (Figure 2.1) was mounted on a heavy metal frame standing in tubs of sand to minimise interference of the recording through external vibrations. The muscle strip was mounted at one end to a fixed hook and the other to an isometric force displacement transducer (Model FT.03, Grass instrument Co. USA). The force transducer was adjusted using a micro-manipulator (Prior instruments Ltd., Bishop's Stortford, Hertfordshire, UK) to completely immerse the strip in the superfusate flow and to prevent the strip from touching the sides of the superfusion trough, where the stimulation platinum electrodes were located.

The output from the tension transducer was connected to a bridge amplifier with variable gain and high frequency cut-off at 5 Hz. The output from the amplifier was connected to a chart recorder with a low-pass filter of corner frequency 10 Hz (Multitrace 2; ORMED Ltd, Hertfordshire, UK)

Stimulation pulses were generated by a stimulator (Stimulator Model 200, Palmer Bioscience, Sheerness, Kent) gated by a programmer (Model 150, Palmer Bioscience,

Sheerness, Kent) and delivered to the muscle strip via platinum electrodes in the wall of the superfusion trough. The length of the strip was adjusted to give a maximal amount of tension on electrical stimulation at 20 Hz for human and 8 Hz for guinea pig.

At the end of the experiment the dimensions of the muscle strip were measured in order to normalise the data to force (mN) per mm^2 cross sectional area of muscle.

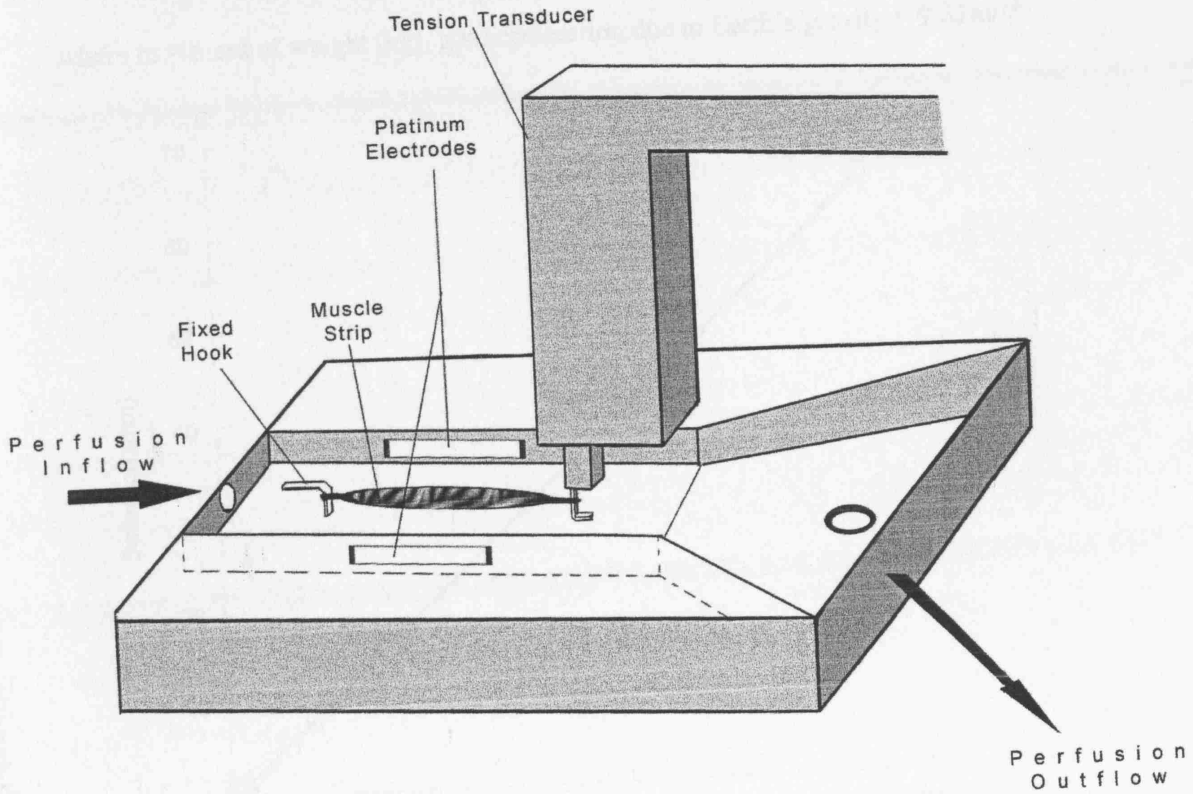


Figure 2.1 Schematic of equipment used to measure isometric tension

2.2.3. Calibration of tension transducer

The tension transducer was calibrated by hanging weights vertically from the force transducer hook. The deflections were plotted as a function of the force exerted by gravity on the weight (see Figure 2.2). The force F in Newtons (N) being:

$$F = m*g \qquad 1)$$

where m = mass of weight (kg), g = acceleration due to Earth's gravity = $9.81 m/s^2$

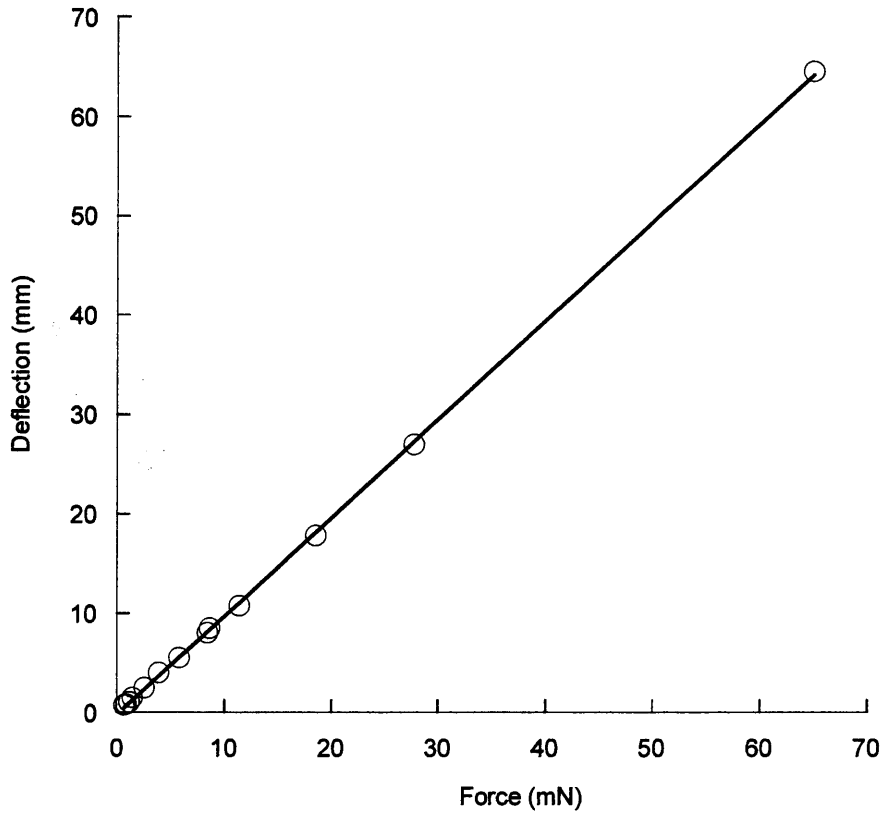


Figure 2.2 Calibration plot of tension transducer. The line was obtained by a linear least-squares fit.

2.2.4. Electric-field stimulation (EFS) experiments

The following protocols were used for both guinea-pig and human detrusor samples. Muscle strips were secured in the organ bath, stretched approx 1.5 times its relaxed state with the micromanipulator to achieve maximum contractions. Phasic contractions were elicited by field-stimulation of the strip from the constant current stimulator via platinum electrodes. The stimulation parameters were 3 second pulse trains (0.1 ms pulse width) every 90 seconds. [The stimulation was chosen to be about 1.5-times the maximum voltage by adjusting the output voltage (25 to 30 V) of the stimulator, 50 V was used in some experiments.]

Muscle strips were stimulated at 8 Hz for guinea pig and 20 Hz for human until a stable response was attained. These frequencies were determined to generate half-maximal ($f_{1/2}$) contractile force for the respective tissues.

A force frequency relationship was determined for each muscle strip by using stimulation frequencies from 1 to 40 Hz incrementally. The effect of P1-agonists on force-frequency relationships was determined by calculating the percentage change of force for each frequency compared to control force-frequency relationships performed pre and post-intervention.

The dose-response inhibition plots for P1-receptor agonists, adenosine, NECA and CPA were generated by superfusing the preparation with the individual agonists while field-stimulating the muscle strip, 8 Hz using guinea-pig tissue. The peak contraction attained in the presence of the agonist was expressed as a percentage of the control contraction in the absence of the agonist. The control contraction was determined from the average of 3-5 contractions before and after exposure to the agonist. The interventions of these P1-agonists were completely reversible after a wash-out of approximately 15 minutes.

2.2.5. Agonist-induced contraction experiments

The acetylcholine analogue, carbachol was used to determine the effects of P1-receptor modulation on post-synaptic contractile mechanisms. The carbachol dose-response was performed non-cumulatively with approximately 20 minutes washout in normal Tyrode's solution between each concentration of carbachol. Each carbachol intervention was for 60 seconds. The carbachol contractions were shown to be stable with repeated applications of the same concentration of carbachol.

To determine the effect of P1-agonists and antagonists on post-synaptic contractions, the preparation was exposed to maximal concentrations of the P1-agonist for 5 minutes before the carbachol intervention. The concentrations of the compounds used for this experiment were estimated from dose-response results or published data. The carbachol solution also contained the same concentration of P1-agonist. The muscle strip was then further exposed to the P1-agonist solution for 2 minutes after the carbachol intervention. The magnitude of carbachol contractions in the presence of P1-receptor modulators was expressed as the proportion of the carbachol contracture before and after the intervention.

2.2.6 Estimation of atropine-resistance

At the end of each experiment using human tissue (stable and overactive), the proportion of the contraction that was atropine-resistant was determined. The preparation was allowed to reach a steady-state contraction using field-stimulated contractions at 20 Hz. The muscle strip was exposed to 1 μM atropine, a muscarinic-receptor antagonist, until the contractions reached a steady state. The extent of direct muscle stimulation was determined by the further addition of a Tyrode's solution containing 1 μM TTX and 1 μM atropine. TTX is a sodium channel blocker and prevents the release of neurotransmitters from the nerve terminal. Therefore any contractions left would be due to direct stimulation of the muscle by EFS.

The atropine-resistant contractions (Figure 2.3) were determined as the contractile component left when the contractions in TTX and atropine were subtracted from the contractions in atropine alone (2 minus 3). This component was then expressed as a percentage of the control contraction minus the TTX-sensitive component (1-3).

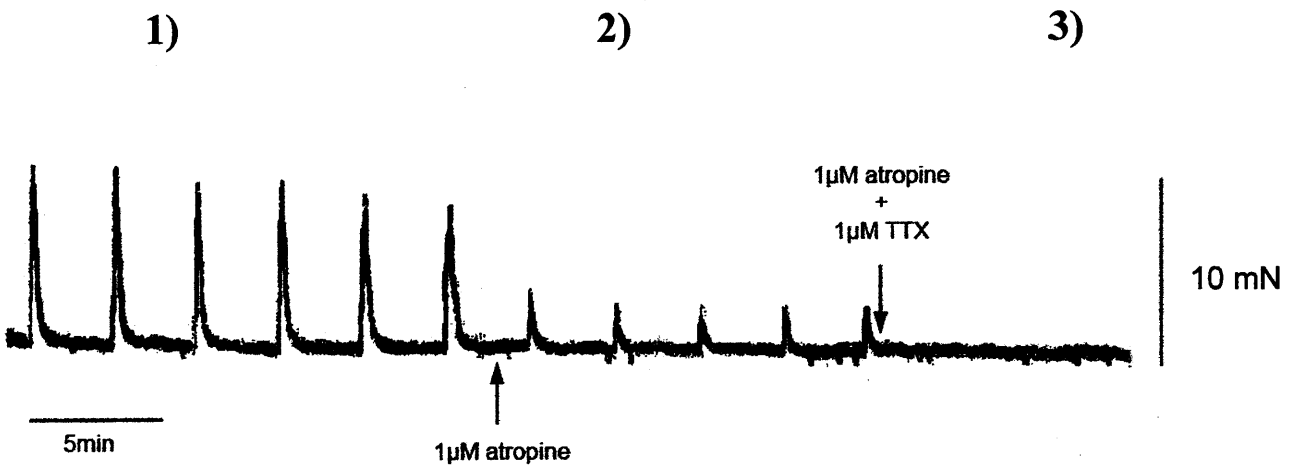


Figure 2.3 Example of atropine-resistant contractions in idiopathic overactive human detrusor muscle strip. 1) nerve-mediated contractions in Tyrode's solution, 2) remaining contractions in 1 µM atropine, 3) elimination of nerve-mediated contractions with addition of 1 µM TTX

2.2.7 Data Analysis for tension experiments

Emperical fits to force-frequency data; $T = \frac{T_{max} \bullet f^n}{f_{1/2}{}^n + f^n}$ 2)

and dose response curves, $T = \frac{T_{max} \bullet [A]^n}{EC_{50}{}^n + [A]^n}$ 3)

were performed by least squares analysis. T_{max} is the estimated maximum effect at the highest frequency or concentration used, $f_{1/2}$ and EC_{50} are the half-maximal frequency or concentration. f and [A] are the frequency of stimulation and agonist concentration and n is a constant. The curve fits were performed in Kaleidagraph (Synergy software).

Frequency-inhibition plots were fitted; by the same method to the function

$$I = 100 - \frac{I_{max} \bullet f^n}{f_{1/2}{}^n + f^n} \qquad 4)$$

2.3 Isolated cell experiments

2.3.1 Detrusor cell isolation

Small pieces of detrusor (1.5-2.0 mm^3) were dissected in the same manner as with the muscle strip protocol. Muscle strips were digested either overnight or on the day of the experiment. For an overnight digestion the enzyme solution (composition given in Table 2.2) was diluted 1 part enzyme solution : 3 parts Ca^{2+}-free HEPES Tyrode's solution for human and 1:6 enzyme dilution for guinea pig and stored for approximately 15 hours at 4°C. The samples were then left at room temperature for 1 hour and then placed in a heating block at 37°C for 30 minutes for human and 15 minutes for guinea-pig samples. The samples were then transferred into 1 ml of Ca^{2+} -free HEPES Tyrode's solution and allowed to rest for 1 hour at 4°C.

When the cells were isolated on the day of the experiment, the tissue was placed in undiluted enzyme solution and left to stand for 45 minutes at room temperature. The samples were then placed on a heating block at 37°C, for 90 minutes for human, and 60 minutes for guinea-pig tissue. The tissue was transferred into 1 ml of Ca^{2+}-free Tyrode's solution and allowed to rest for 1 hour at 4°C. The tissue was then finely chopped and triturated with a blunted glass pipette to generate a cell suspension.

Table 2.2 Composition of dissociation enzyme mixture, all components except collagenase, from Sigma UK.

Components	**Concentration (mg/ml)**
Collagenase, Worthington type I (256 units/mg)	20
Hyaluronidase Type I-S	0.5
Hyaluronidase Type III	0.5
Antitrypsin Type II-S	0.9
Bovine Albumin	5.0

2.3.2 Measurement of intracellular Ca^{2+} by epifluorescence microscopy

Changes of intracellular Ca^{2+} can be measured to show the response of the cell to a stimulus, such as a contractile agonist. Effects of P1-receptors on intracellular Ca^{2+} were measured in guinea-pig and human isolated detrusor myocytes.

The fluorescent indicator used in these experiments was Fura-2 AM (Calbiochem-Novabiochem Corporation, La Jolla, CA, USA) which is an acetoxymethyl (AM) ester form of a Ca^{2+} binding dye. AM esters are lipophilic molecules that freely pass through the cell membrane lipid bilayer. Within the cell, the ester is cleaved by esterases to form the hydrophilic acid and the indicator becomes localised inside the cell.

Two wavelengths were chosen to excite Fura-2 which were 340 and 380 nm and fluorescent emission was collected between 410-510 nm. When Fura-2 binds to Ca^{2+} the emission intensity alters, with an increase when excited at 340 nm and a decrease when excited at 380 nm (Figure 2.4). The ratio of emission at 340 and 380, R, was used as an index of the intracellular $[Ca^{2+}]$, $[Ca^{2+}]_i$. This ratiometric method for estimation of $[Ca^{2+}]_i$ has several advantages including: independence from changes to the absolute magnitude of the signals (e.g. from dye leakage); and an increased efficiency at recording changes to $[Ca^{2+}]_i$.

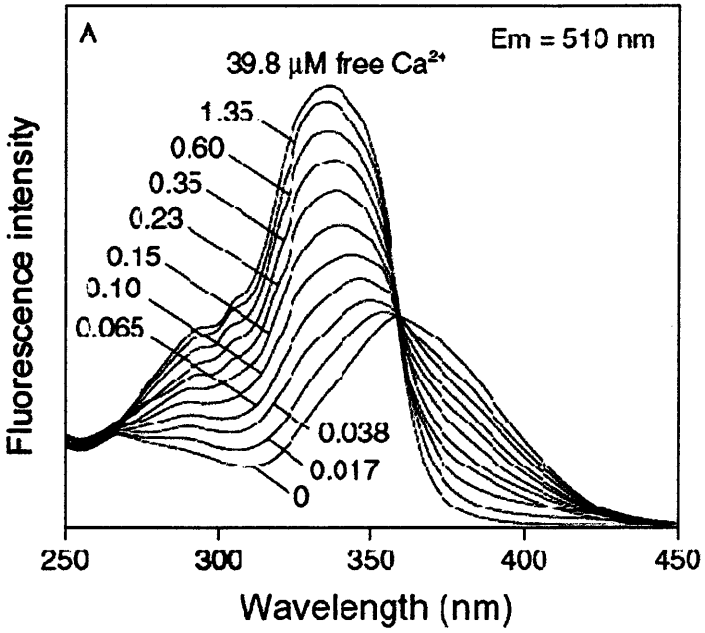


Figure 2.4 Fluorescence emission (detected at 510 nm) spectra of Fura-2 in pH 7.2 buffer as a function of excitation wavelength in solution of various [Ca^{2+}] (diagram from Invitrogen molecular probes handbook)

5 µl of Fura-2 stock solution (1 mM in DMSO) was added to 1 ml of cell suspension to give a final concentration of 5 µM. The suspension was left for at least 20 minutes at 4°C to allow sufficient loading of the cells with the indicator.

Approximately 3-4 drops of the cell suspension were placed in a water-heated Perspex bath with a borosilicate coverslip base (thickness 1 – 0.13-0.17 mm BDH, Poole, Dorset). The borosilicate coverslip base minimised absorption of light and allowed the objective lens to focus closely to the cell of interest. The bath was mounted on the stage of an

inverted microscope and the cells were allowed to adhere to the coverslip by leaving the suspension for 30 minutes at 37°C. The cells were then perfused with Tyrode's solution at a flow rate of 2 ml/min with excess fluid removed by suction.

After perfusion was started and the cells were allowed to settle on the microscope stage, a viable cell was selected for the experiment. Viable cells were distinguished by a bright halo under phase contrast illumination, and a darker colour to the cytoplasm. Damaged cells appeared to have a rough-looking cell outline or appeared transparent.

2.3.3 Experimental set-up for epifluorescence microscopy

A water-jacketed Perspex perfusion bath was placed on an inverted-stage microscope (Diaphot-TMD, Nikon Corporation, Tokyo, Japan). The microscope was mounted on an air table (Ealing Optics Ltd., Watford, UK) and surrounded by a Faraday cage covered in a black blanket.

The epifluroescence system was obtained from Cairn Research Ltd. (Sittingbourne, Kent, UK) and the setup is shown in Figure 2.5. A xenon short-arc light (75 W XBO; Osram Ltd., Berlin, Germany) was used to provide a high intensity and broad bandwidth light source. The light was filtered using a rotating wheel with two radially distributed filters of different bandwidths. (340 ± 10 nm and 380 ± 10 nm) The wheel was spun at 50 rotations/per second, and the filtered light was transmitted to the microscope stage via a quartz fibre optic cable. A dichroic mirror (410 nm) was situated in the sub-stage of the microscope and was used to direct the filtered excitation light beam through the objective of the microscope (x40 quartz objective, numerical aperture 1.3) focused onto a cell in the perfusion bath.

The emitted light passed back through the objective and through the dichroic mirror. The emitted light was reflected by a microscope mirror and focused in a light tube prior to a variable rectangular diaphragm, used to adjust the extracellular light and minimise interfering background emissions. A red screening filter, a K^+ filter from an atomic

absorption spectroscope (>580nm)] was placed in front of the microscope light source to provide illumination but not contaminate the fluorescence emission from the cell.

A second dichroic mirror (510 nm) was placed after the adjustable diaphragm to act as a beam splitter. The higher wavelengths were directed to a CCD camera (Heimann CCD; Alrad Instruments Ltd., Newbury, Berks, UK.) The image was then displayed on a monitor and was used to adjust the positioning of the cell and of the adjustable diaphragm. The lower wavelength signal (410-510 nm) was transmitted to a photo-multiplier tube (PMT) for signal collection.

The intensities of the emitted light from the 410-510 nm range at the two excitation wavelengths (340 and 380 nm) were recorded by two sample-and-hold amplifiers incorporated into the spectrophotometer system. The switch frequency between these two amplifiers was synchronised to that of the rotating filter wheel using an internal high frequency time-clock to allow sampling only during the passage of a specific filter.

The magnitude of the PMT output could be adjusted by changing the voltage applied to the PMT (between -520 to -900 V). An analogue division circuit was also incorporated into the spectrophotometer system to produce a simultaneous ratio of the two signals, which was displayed on an oscilloscope (Model DSO 420; Gould Inc., Essex, UK). The emitted light intensities at the two excitation frequencies as well as the ratio, R, of these two signals were recorded on a moving-paper chart recorder (Model TA 420S; Gould, France).

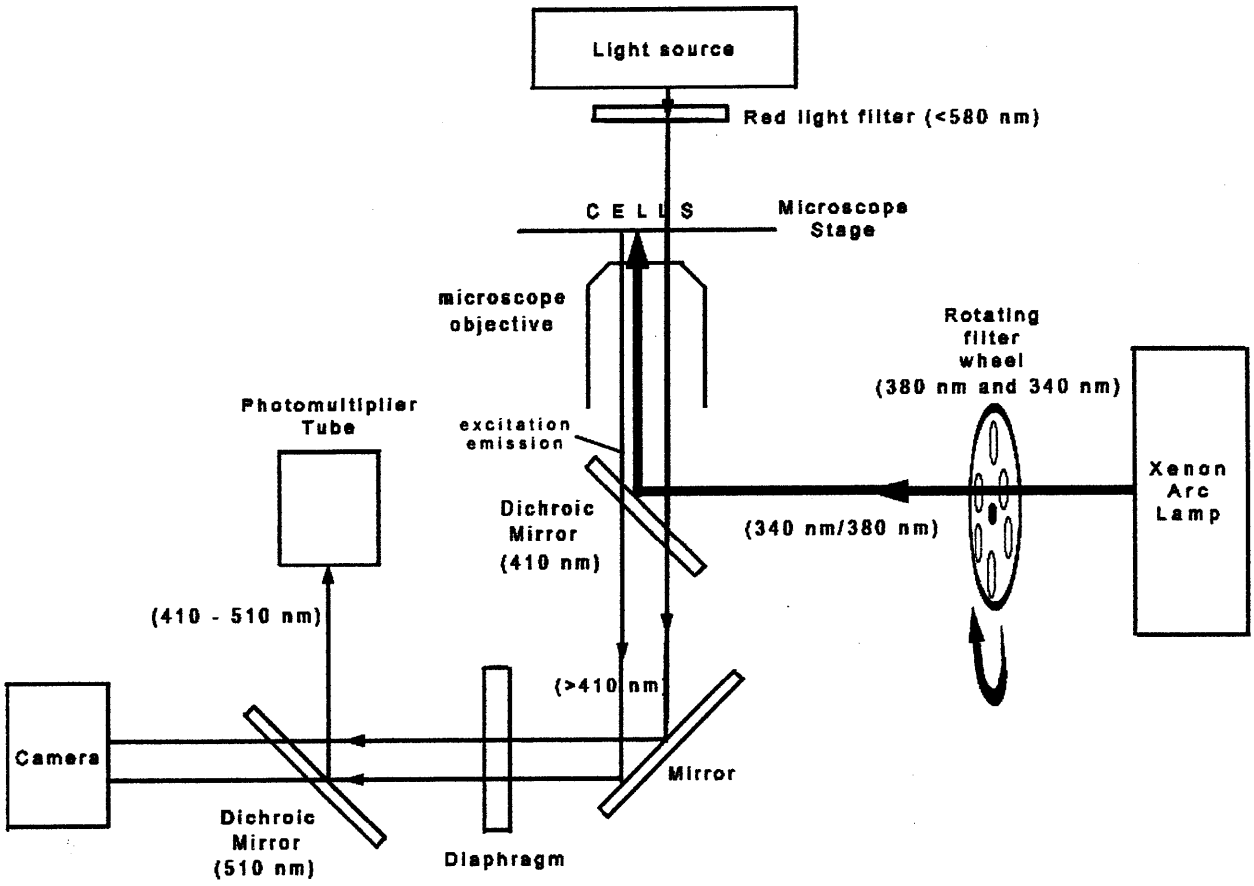


Figure 2.5 Schematic of the epifluoresence microscope to measure the intracellular $[Ca^{2+}]$

2.3.4 Calibration of Fura-2 signal

The objective of the calibration was to calculate the affinity of Fura-2 for Ca^{2+}, K_d, under ionic conditions that mimicked the intracellular environment by measurement of the fluorescence at 340 and 380 nm excitation in solutions of known [Ca^{2+}], and the upper and lower bounds of the R value at very high [Ca^{2+}] (R_{max}) and low [Ca^{2+}] (R_{min}).

The calibration solution contained accurately determined Ca^{2+} concentrations between about 27 nM and about 1 μM. A calibration plot was made by increasing the [Ca^{2+}] and measuring the change in fluorescence of the pentapotassium salt of Fura-2. The ionic form of the flurochrome was used as Fura-2 is only active when it is cleaved from the AM-ester.

Calibration was performed *in vitro* from measurement of the fluorescence signal between 410-510 nm when excited at 340 and 380 nm and calculating the fluorescence ratio (R) as described above. Calibration solutions of known pCa were made up in the base solution shown in Table 2.3.

Table 2.3 Base solution for calibration of Fura-2 signal

Solute	Concentration (mM)
KCl	120
HEPES	10
NaCl	10
$MgCl_2$	1.0
EGTA	5.0 (actual: 4.7)

The base solution had a high $[K^+]$ to mimic the intracellular environment. It is noted that the concentration of EGTA is not exactly 5 mM as the purity is not 100%. The purity of the EGTA was calculated to be 94% which was determined from previous experiments (see method by Fry and Langley, 2001) this made the actual concentration of EGTA to be 4.7 mM.

The solution was titrated using 2 M KOH (VWR) to pH 7.1 and a pH meter (Solex Digital pH meter SE500) and then made up to volume. The solution was divided into 50 ml aliquots and $CaCl_2$ (VWR) added from a 1 M stock solution and titrated back to pH 7.1 with KOH to give a range of free Ca^{2+} concentrations (27 nM – 1.31 μM).

The pCa was calculated by using the following equation;

$$pCa = pK'Ca + \log \frac{[EGTA]}{[CaEGTA]} \qquad 4)$$

The pCa is the negative logarithm (base 10) of the concentration of free Ca^{2+} in the solution; pK'Ca is the negative logarithm of the apparent dissociation constant for Ca^{2+}, K'Ca. The pK'Ca with EGTA has been determined previously using Ca^{2+}-selective electrodes as 6.64 at pH 7.1 (Fry & Langley, 2001), therefore the pCa for each of the solutions could be determined by substituting values of [EGTA] and [CaEGTA].

Table 2.4 Concentration of free Ca^{2+} for each $CaCl_2$ concentration of calibration solution, pK'Ca = 6.64 at pH 7.1

$CaCl_2$ concentration [CaEGTA] (mM)	**EGTA concentration [EGTA] (mM)**	**pCa (6.64 + [EGTA]/[CaEGTA])**	**$[Ca^{2+}]_{free}$ (M)**	**$[Ca^{2+}]_{free}$ (nM)**
0.5	4.2	7.56	2.73×10^{-08}	27.3
1.0	3.7	7.21	6.19×10^{-08}	61.9
2.5	2.2	6.58	2.60×10^{-07}	260
3.85	0.85	5.98	1.06×10^{-06}	1057
4.0	0.7	5.88	1.31×10^{-06}	1309

Two other solutions were also used to give values for R_{min} and R_{max}. The first solution had no added $CaCl_2$; the second had 6 mmol/l $CaCl_2$ added to yield a solution with a free $[Ca^{2+}] > 1mM$

The apparent dissociation constant, K_d, of Fura-2 for Ca^{2+} was determined using the following equation (Grynkiewicz *et al.*, 1985).

$$K_d = [Ca^{2+}]_{free} (R_{max}-R) / \beta(R-R_{min}) \qquad 5)$$

R is the ratio of the 340/380 nm fluorescence signal at a given $[Ca^{2+}]$, R_{min} is the ratio in the solution without Ca^{2+}, R_{max} is the ratio in the solution of excess $CaCl_2$ and β is $F380_{max}/F380_{min}$ [the ratio of the fluorescence signal at 380 nm in the absence of Ca^{2+} ($F380_{min}$) and in the saturating Ca^{2+} ($F380_{max}$)]. K_d is the dissociation constant for Ca^{2+}.

Equation 5 was rearranged to yield a linear relationship for the estimation of K_d

$$\log K_d = \log [Ca^{2+}]_{free} + \log \frac{(R_{max} - R)}{\beta(R - R_{min})}$$

$$pK_d = pCa + \log \beta \frac{(R - R_{min})}{(R_{max} - R)}$$

$$\text{or, } \log \beta \frac{(R - R_{min})}{(R_{max} - R)} = -pCa + pK_d$$

A plot of $\log \beta \frac{(R - R_{min})}{(R_{max} - R)}$ vs –pCa was done and pK_d estimated as the pCa at which

$\log \beta \frac{(R - R_{min})}{(R_{max} - R)} = 0$. (Figure 2.6)

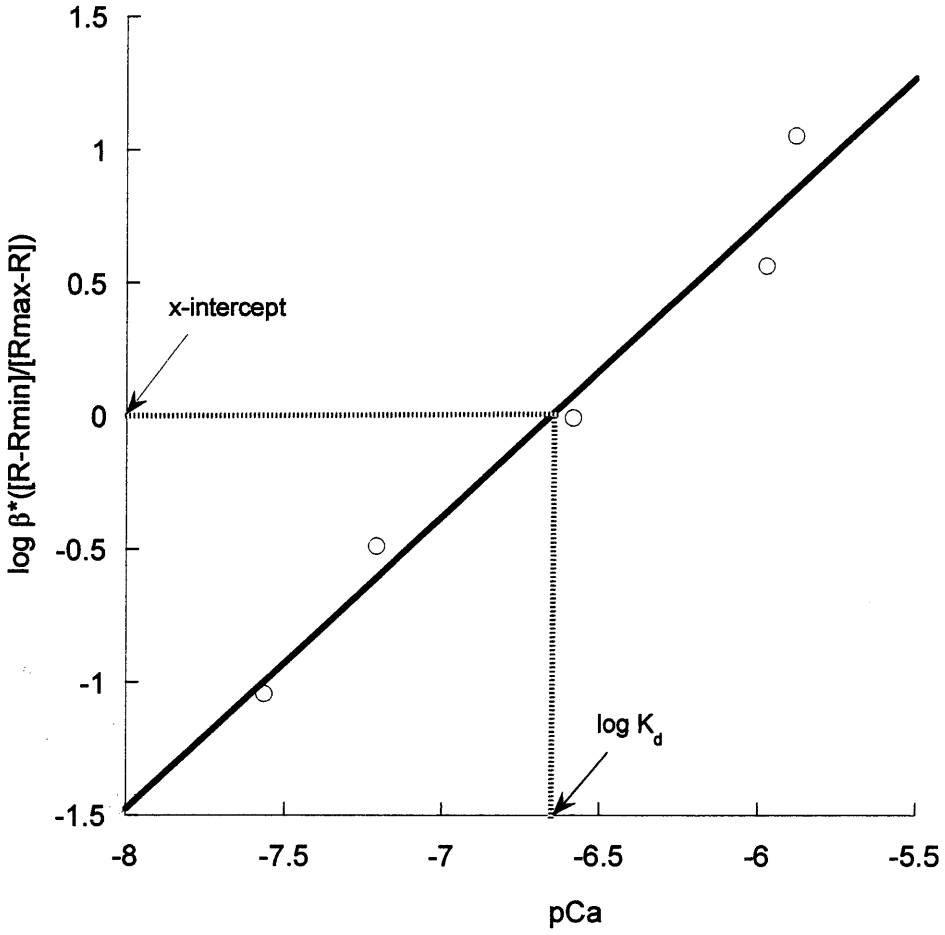


Figure 2.6 Plot of log $\beta^*([R-R_{min}]/[R_{max}-R])$ against log $[Ca^{2+}]_{free}$ to determine K_d of Fura-2 pentapotassium salt. The log $[Ca^{2+}]_{free}$ at the x-intercept corresponds to the log K_d for Fura-2

2.4.5 Effects of P1-specific agents on intracellular Ca^{2+}

Ca^{2+}-transients were elicited by carbachol (1 μM for human and 3 μM for guinea-pig myocytes), 20 mM caffeine or 80 mM KCl-Tyrode's solution. The effects of various P1-specific agents were tested on the Ca^{2+}-transients. Cells were exposed to 5 minutes of maximal concentration of the P1-agonist/antagonist, and then a Ca^{2+}-transient was elicited in the presence of the P1-specific compound by exposure to one of the mentioned agonists for 1 minute. The cell was further exposed to the P1-agonist/anagonist for 2 minutes after the transient and then allowed to wash-out for 5 minutes in normal Tyrode's.

The change in the magnitude of the Ca^{2+}-transient was determined as a percentage of control transients elicited before and after the intervention. The peak height of each Ca^{2+}-transient was measured to determine the $[Ca^{2+}]$.

2.4 Statistical Analysis

Data were analysed by paired Student's *t*-test between pre/post-control and intervention contractions. Comparison of P1-receptor efficacy between human and guinea-pig tissue was made using an unpaired Student's *t*-test. Wilcoxon signed-rank test was used to test for significance of the occurrence of atropine-resistance in human tissue groups. This test was used as the occurrence of atropine-resistance is not normally distributed as there are several zero values.

Chi-squared analysis was used to determine the difference in occurrence of atropine-resistant contractions between different human patient groups. One-way Analysis of Variance (ANOVA) analysis was used for multiple comparisons between sample groups and a Kruskal-Wallis test was used for comparison of non-parametric data. The null hypothesis was rejected at $p < 0.05$. All data was expressed as mean ± standard deviation (s.d) or median values with 25 and 75% quartile values.

3.0 Results

3.1 Contractile experiments

3.1.1 Nerve-mediated contractions of guinea-pig and human detrusor

Prior to interventions, the muscle strip preparations were allowed to equilibrate at a field-stimulation frequency of either 20 Hz for human or 8 Hz for guinea-pig, for approximately 15 to 60 minutes. These frequencies were previously determined as the stimulation frequency for each species that produces a half-maximal contraction.

The mean nerve-mediated tension of guinea-pig at 8 Hz was 29.1 ± 25.4 mN/mm^2 (n=52) and was 53.0 ± 14.4% of the maximal tension generated from the force-frequency curves. Under control conditions, the mean half-maximal stimulation frequency ($f_{1/2}$) of guinea-pig detrusor strips was 9.7 ± 4.4 Hz. (Figure 3.1)

In human preparations, the mean tension generated at 20 Hz was 34.2 ± 28.6 mN/mm^2 (n=33) in stable preparations and 32.1 ± 28.3 mN/mm^2 (n=33) for the collated samples from overactive bladders. The $f_{1/2}$ values were similar between the two groups (Table 3.1) and there was no significant difference in the amount of contractile force generated between stable and overactive samples. However, when the overactive groups were considered according to symptomologies, it was found that the obstructed group had a significantly lower $f_{1/2}$ value compared to stable preparations. The obstructed human bladder $f_{1/2}$ value was also not significantly different from the guinea-pig $f_{1/2}$ value.

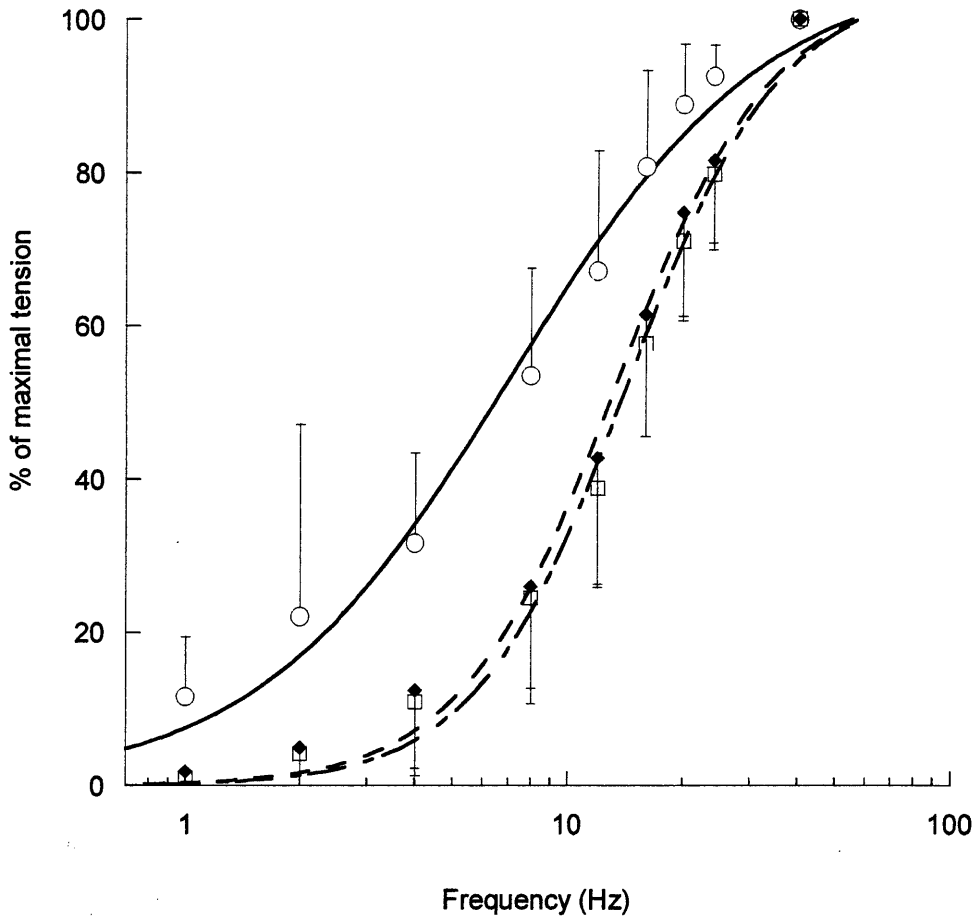


Figure 3.1 Force Frequency plot of guinea-pig and human bladder strips. ***Guinea-pig,*** *Open circles:* ***Stable human,*** *Open squares:* ***Overactive human,*** *diamonds: mean values ± s.d.*

Table 3.1 Summary of force-frequency results from human and guinea-pig samples

Tissue type	**Mean tension (mN/mm^2)**	**Mean Half-Maximal stimulation frequency ($f_{1/2}$) (Hz)**
Guinea Pig (n=52)	29.1 ± 25.4	9.7 ± 4.4
Stable Human (n=33)	34.2 ± 28.6	18.9 ± 7.5
Overactive Human – collated (n=32)	32.1 ± 28.3	15.8 ± 5.5
Human - Idiopathic overactive (n=20)	25.3 ± 18.5	16.7 ± 5.9
Human -Neuropathic overactive (n=8)	32.1 ± 35.2	16.8 ± 3.9
Human -Obstructed overactive (n=4)	65.5 ± 36.8	9.8 ± 2.0*

*$p<0.05$ unpaired Student's *t*-test compared to control

3.1.2 Dose-response to P1-agonists in guinea-pig detrusor strips

Guinea-pig detrusor was used as a model for the concentration-dependent effect of adenosine, NECA (general A1/A2-agonist), and CPA (A1-specific agonist) to be determined. The detrusor strips were exposed to varying concentrations of the agonists for 10 minute intervals at 8 Hz and the percentage change in electric field-stimulation (EFS)-mediated contractions during intervention from pre and post-control periods was determined.

All the compounds tested reduced significantly the EFS contractions in a dose-dependent manner (Figure 3.2). The pEC_{50} (-log EC_{50}) values for the agonists tested were; adenosine: 3.74 ± 0.45 (n=4), NECA: 6.40 ± 0.25 (n=5) and CPA: 5.72 ± 0.33 (n=8). The maximum amount of reduction seen was to 62.3 ± 11% and 61.1 ± 14% of control contractions with adenosine and NECA respectively. CPA caused the least amount of percentage reduction, with contractions to 71 ± 11% of control at the highest CPA concentration. Also the slope of the CPA dose-response was much shallower (Hill coefficient CPA; n= 0.41, NECA; n= 0.66, adenosine; n= 0.58) than the other agonists. It is possible that there is a mixture of receptor subtypes involved as CPA did not reduce the force to the same degree as NECA or adenosine, through comparison of the dose-response curves. The A1-subtype contributes significantly to the reduction of contractions, however there may be an additional A2-component to the reduction as well. In view of the complex response to P1-receptor agonists, a more detailed analysis of the action of P1-agonists and antagonists was carried out

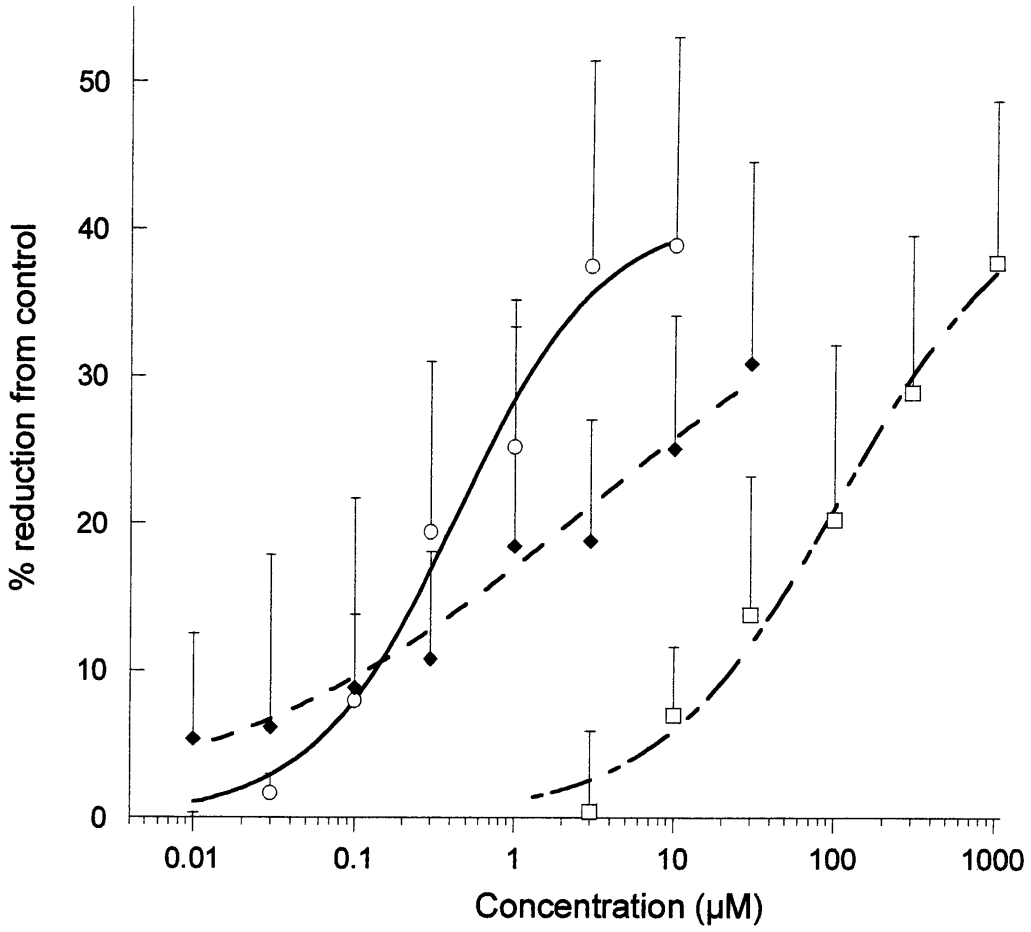


Figure 3.2 Dose-response of EFS-stimulated guinea-pig detrusor strips to adenosine, NECA and CPA at 8Hz. ***1mM Adenosine****, open squares:* ***10 μM NECA****, open circles:* ***10 μM CPA****, filled diamonds; mean ± s.d.*

3.2 Response of P1-specific compounds to nerve-mediated contractions

Human and guinea-pig detrusor strips were treated with selective P1-agonists/antagonists to determine the functional receptor type involved in the modulation of nerve-mediated contractions. The muscle strip was exposed to the agonist/antagonist for five minutes prior to a force frequency (1-40 Hz) curve being generated. The percentage change in the force of contraction was determined from pre and post-intervention control force-frequency relationships. Table 3.2 (p114) gives a summary of the half-maximal frequency and reduction of force for each of the P1-specific agents tested against guinea-pig detrusor.

3.2.1 Effect of P1-specific compounds on the force-frequency relationship in guinea-pig detrusor strips

1 mM adenosine caused a reduction of nerve-mediated contractions, with reduction seen at virtually all the frequencies tested (n=9) (Figure 3.3). The concentration of adenosine was determined from the dose-response experiments to cause maximal effect. The tension was reduced to 67.4 ± 13.5% of control at 8 Hz stimulation and the $f_{1/2}$ value in the presence of adenosine was 15.8 ± 8.7 Hz, the control $f_{1/2}$ was 8.7 ± 4.4 Hz. This showed a significant right-ward shift in the $f_{1/2}$ value as compared to paired-control values (Figure 3.4). In this plot each force-frequency curve was normalised to its own maximum value to illustrate more clearly the shift of $f_{1/2}$ value by adenosine.

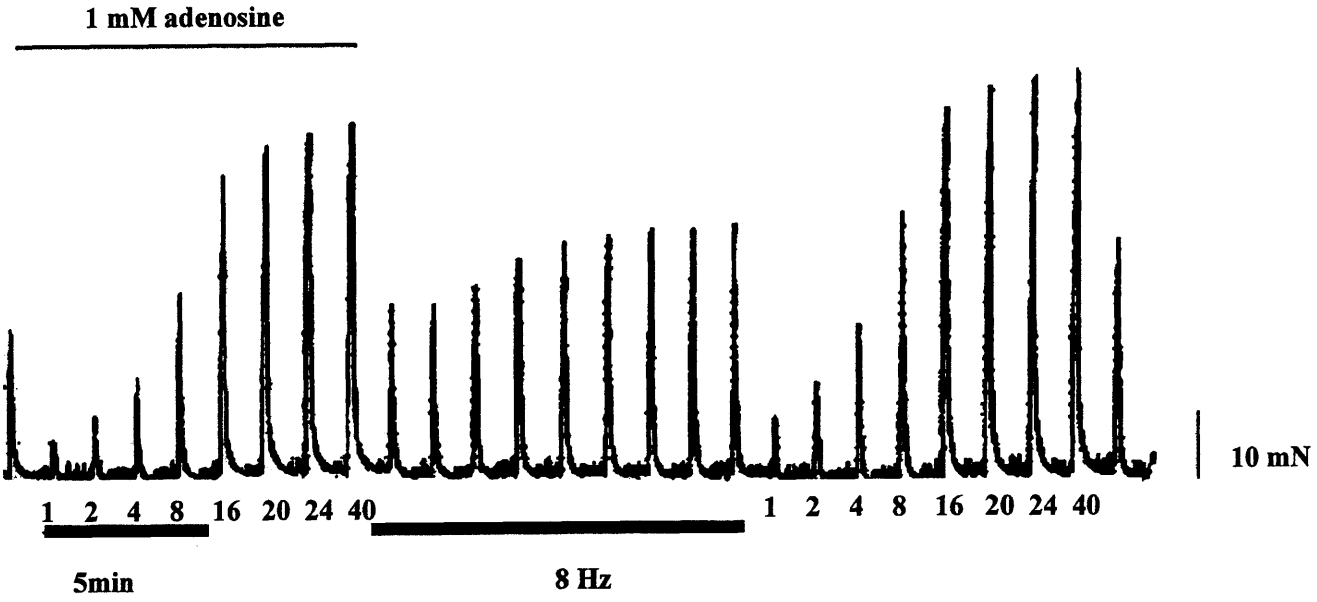


Figure 3.3 Comparison of tension generated from force-frequency relationships in the presence and absence of 1mM adenosine. The stimulation frequency is given under the corresponding contractions and the preparation was in Tyrode's solution unless indicated.

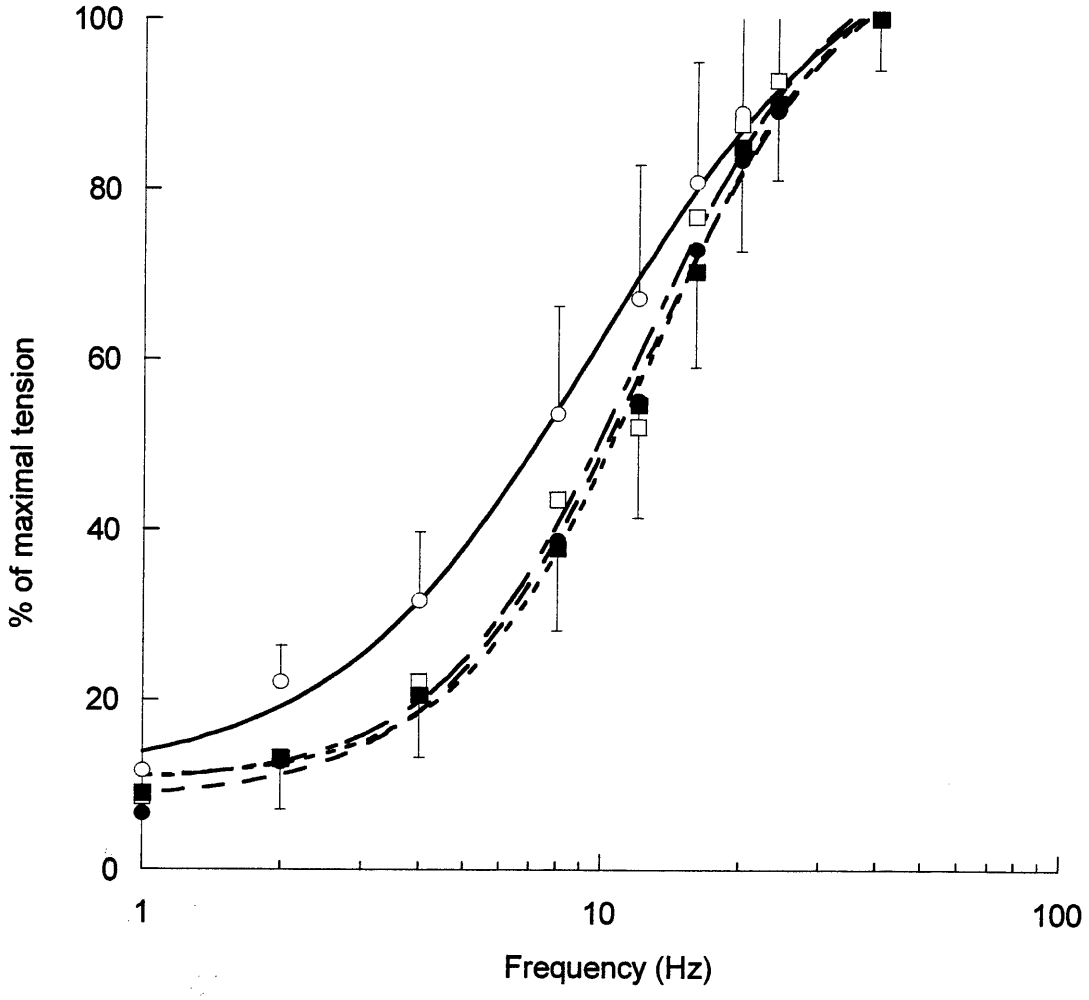


Figure 3.4 Force-frequency of guinea-pig detrusor in the presence of P1-specific compounds. ***Control,*** *open circles:* ***1 mM adenosine,*** *open squares:* ***10 μM NECA,*** *filled circles:* ***10 μM CPA,*** *filled squares. Not all error bars are shown for clarity, mean ± s.d.*

10 μM NECA (A1/A2-agonist) was an equally effective agonist as adenosine in causing reduction in the guinea-pig nerve-mediated contractions. It caused a similar degree of reduction as adenosine at maximum concentrations, but had a smaller EC_{50} value. The percentage reduction of control, at 8 Hz stimulation was 66.4 ± 15.5%. The calculated $f_{1/2}$ was significantly larger (13.8 ± 5.5 Hz, n=30), compared to the control $f_{1/2}$ of 9.5 ± 4.8 Hz; a shift similar to that induced by adenosine.

10 μM CPA, an A1-specific agonist, (determined from previous dose-response experiments to cause maximum reduction) also caused a significant reduction of the nerve-mediated contractions, with a reduction of 71.7 ± 15.6% (n=10) from control at 8 Hz, similar to the maximum reduction seen with the dose-response experiments. The calculated $f_{1/2}$ value was 13.2 ± 6.8 Hz, which however was not significantly different from its respective control force-frequency (10.7 ± 4.6 Hz).

1 μM alloxazine ($A2_B$-antagonist) did not significantly change the force of the nerve-mediated contractions at 8 Hz stimulation; 96.8 ± 9.2% of control (n=10). 1 μM alloxazine was used as it is the highest concentration that would give the most selectivity over the other P1-receptor subtypes (Brackett & Daly, 1994). The calculated $f_{1/2}$ value was 9.7 ± 4.8 Hz, which was not significantly different from control (8.1 ± 2.4 Hz).

1μM alloxazine in combination with 10 μM NECA significantly attenuated nerve-mediated contractions. The reduction in tension was 82.4 ± 10.2% of control at 8Hz, which was, however significantly less than the amount of reduction caused with NECA

alone. The calculated $f_{1/2}$ value was 10.9 ± 3.9 Hz (n=10) and the NECA control $f_{1/2}$ was 12.5 ± 2.9 Hz, these values were not significantly different from one another. The results indicate that alloxazine may reduce the degree of inhibition caused by NECA.

100 nM ZM-241385, an $A2_A$-selective antagonist had no significant effect on nerve-mediated contractions; 96.6 ± 9.4% of control at 8 Hz (n=8). The $f_{1/2}$ value was 12.2 ± 4.6 Hz, which was also not significantly different from control (11.3 ± 2.2 Hz).

100 nM ZM-241385 did not significantly alter the inhibition caused by NECA, with contractions 61.4 ± 16.1 % of control at 8 Hz (n=8). This was not significantly different from the reduction seen with NECA alone. The $f_{1/2}$ value for ZM-241385 with 10 μM NECA was 14.0 ± 2.5 Hz, again similar to the half-maximal frequency seen in the presence of NECA alone, in this series of experiments (control $f_{1/2}$ 16.4 ± 7.2 Hz). Therefore, $A2_A$-receptors do not have a significant role in modulating nerve-mediated contractions in guinea-pig detrusor.

10 μM IB-MECA (n=3), an A3-specific agonist, showed no significant effect on the calculated $f_{1/2}$ value (12.0 ± 2.7 Hz, control $f_{1/2}$: 11.9 ± 3.7 Hz), or on the force generated at 8 Hz (110.6 ± 9.3% of control). Thus there is no A3-receptor activity on modulation of nerve-mediated contractions.

Table 3.2 Summary of force-frequency results for guinea-pig detrusor muscle strips

Compounds	Mean $f_{1/2}$ Half-Maximal stimulation frequency (Hz)	Mean control $f_{1/2}$ Half-Maximal stimulation frequency (Hz)	Mean %-tension of control at 8Hz
Control (n=58)	--	9.6 ± 4.3	--
1 mM adenosine (n=9)	15.8 ± 8.7*	8.8 ± 4.4	67.4 ± 13.5*
10 μM NECA (n=30)	13.8 ± 5.5*	9.5 ± 4.8	66.4 ± 15.5*
10 μM CPA (n=10)	13.2 ± 6.8	10.7 ± 4.6	71.7 ± 15.6*
1 μM alloxazine (n=10)	9.7 ± 4.8	8.1 ± 2.4	96.8 ± 9.2
1 μM alloxazine with 10μM NECA (n=10)	10.9 ± 3.9	8.1 ± 2.4	82.4 ± 10.2*
100 nM ZM-241385 (n=8)	12.2 ± 4.6	11.3 ± 2.2	96.6 ± 9.4
100 nM ZM-241385 and 10 μM NECA (n=8)	14.0 ± 2.5	11.3 ± 2.2	61.4 ± 16.1
10 μM IB-MECA (n=3)	12.0 ± 2.7	11.9 ± 3.7	110.6 ± 9.3

*$p<0.05$ paired Student's *t*-test compared to respective controls

3.2.2 Frequency-dependent inhibition by P1-selective agents in guinea- pig detrusor

In guinea-pig preparations the P1-receptor agonists, adenosine, NECA and CPA caused a reduction of the force of contraction elicited by field-stimulation. The reduction was greater at lower stimulation frequencies (<4 Hz) than at higher frequencies (>20 Hz), where there was little or no reduction of force caused by the agonists (Figure 3.5). For example from figure 3.5, 1 mM adenosine reduced the tension by 41.2 ± 23.4% from control at 2 Hz stimulation while the same concentration of adenosine would only reduce the tension by 5.8 ± 14.0% from control at 20 Hz. A similar pattern was found with 10 μM NECA and CPA. This frequency-dependent inhibition was also manifested as a shift of the force-frequency curve to the right, and an increase in the $f_{1/2}$ value compared to control (see Table 3.2).

This differential reduction at the various stimulation frequencies could be explained by there being a different balance of neurotransmitters released at lower stimulation frequencies compared to those at higher frequency. If P1-receptor activation acts preferentially on the signalling pathways that release specific neurotransmitters, then the effects of the P1-receptor should be more pronounced when there is preferential release of that particular neurotransmitter.

The ratio of the tension at 2 Hz and 20 Hz (T2/T20) using guinea-pig preparations, was compared in the presence of P1-specific compounds (Table 3.3). Of the compounds tested, adenosine, NECA, CPA, and alloxazine in the presence of NECA generated a

significant reduction of the T2/T20 ratio when compared to their respective controls. These results correspond to the observation that adenosine and NECA had a significant effect on the force-frequency relationships and tension. However it is noted that CPA and alloxazine with NECA did not significantly affect the $f_{1/2}$ values, but did reduce the T2/T20 ratio. This latter method may therefore be more effective at measuring frequency-dependent effects of agonists rather than comparison of entire force-frequency relationships.

1 μM alloxazine, 100nM ZM-241385, 100nM ZM-241385 with 10 μM NECA, and 10 μM IB-MECA did not significantly change the T2/T20 ratio compared to their respective controls; nor did they affect $f_{1/2}$ values (Table 3.2).

The results are consistent with the hypothesis that an A1-receptor mediated process is responsible for the frequency-dependent reduction of force by adenosine.

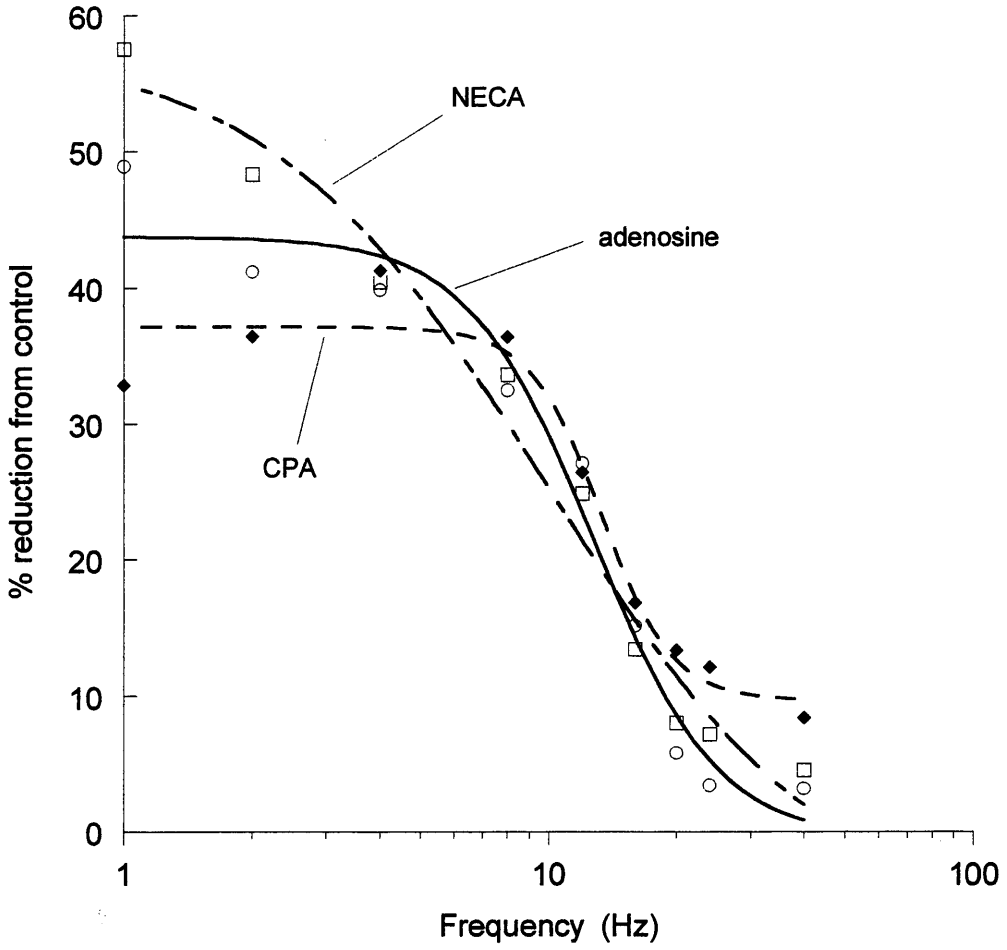


*Figure 3.5 Frequency–dependent inhibition of nerve-mediated contractions in guinea-pig detrusor; **1 mM adenosine**: open circles, **10 μM NECA**: open squares, **10 μM CPA**: filled diamonds, error bars not shown for clarity*

Table 3.3 Comparison of T2/T20 ratios from guinea-pig force-frequency relationships in the presence of P1-specific agents

Compound	T2/T20 ratio	Control T2/T20 ratio
Control (n=58)	--	0.21 ± 0.11
1 mM adenosine (n=9)	0.15 ± 0.08*	0.23 ± 0.08
10 μM NECA (n=29)	0.15 ± 0.09*	0.23 ± 0.11
10 μM CPA (n=9)	0.16 ± 0.06*	0.22 ± 0.10
1 μM alloxazine (n=9)	0.24 ± 0.11	0.26 ± 0.09
1 μM alloxazine + 10 μM NECA (n=8)	0.20 ± 0.10*	0.15 ± 0.08
100 nM ZM-241385 (n=8)	0.16 ± 0.06	0.15 ± 0.06
100 nM ZM-241385 + 10 μM NECA (n=8)	0.10 ± 0.06	0.13 ± 0.08
10 μM IB-MECA (n=3)	0.12 ± 0.03	0.12 ± 0.04

*$p<0.05$ paired Student's *t*-test compared to control

To test further the hypothesis that there is differential release of neurotransmitters at various frequencies of stimulation, three stimulation frequencies were examined; 2 Hz (low frequency), 8 Hz (half-maximal frequency) and 20 Hz (high frequency) in the presence of α-β-methylene-ATP (ABMA) and atropine. These frequencies were chosen to represent low, middle and high frequencies from the curves in figure 3.5. 10 μM ABMA was used to desensitise the P2X receptors, therefore contractions elicited would only result from activation of muscarinic receptors by acetylcholine. 1 μM atropine, a muscarinic receptor antagonist, was used to observe the change in ATP-mediated contractions at the different stimulation frequencies.

ABMA pre-treatment reduced significantly EFS-induced contractions at 2 and 8 Hz, but not at 20 Hz (Figure 3.6). Thus, there was a larger proportion of ATP-mediated contraction at 2 and 8 Hz than at 20 Hz stimulation. When the same stimulation frequencies were tested in the presence of 1 μM atropine, there was a significant reduction at the highest frequency but not at 2 or 8 Hz (Figure 3.7). This does seem to indicate that there is preferential release of ATP at low stimulation frequencies with acetylcholine dominating at higher frequencies.

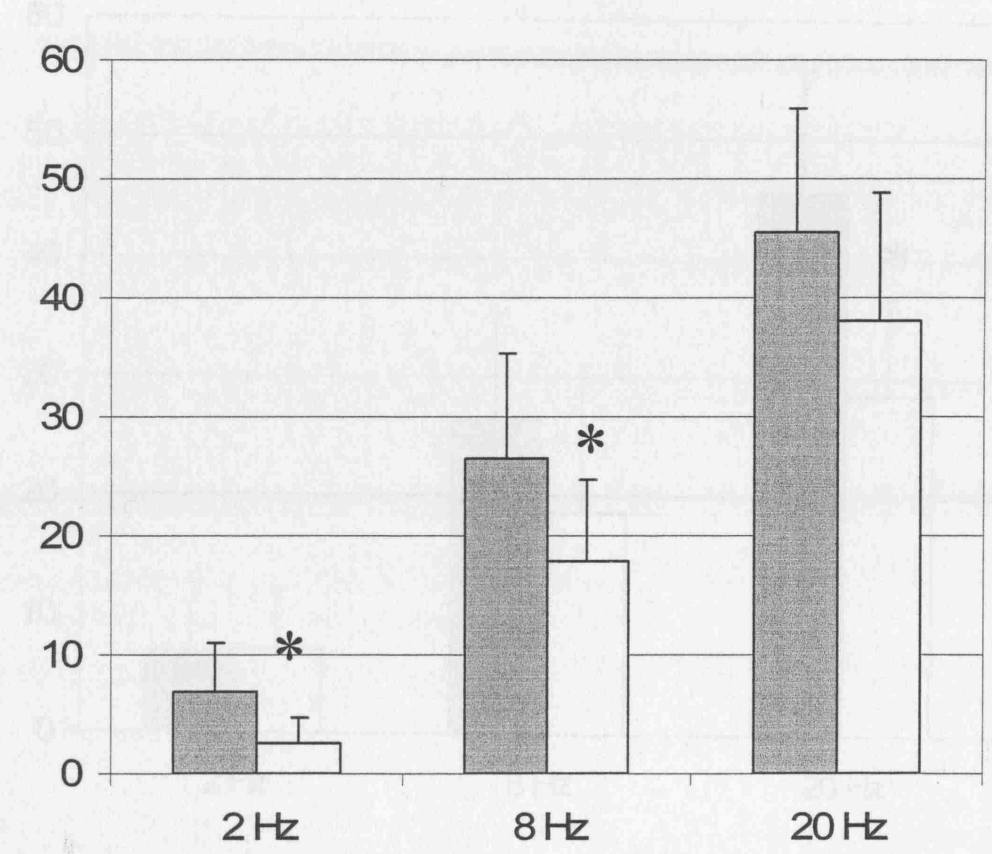


Figure 3.6 Bar chart of tension generated at different field-stimulation frequencies in the presence of 10μM ABMA ***Solid bars:*** *Control tension (Tyrode's solution only),* ***White bars:*** *Tension generated in the presence of 10μM ABMA, (n=8)*

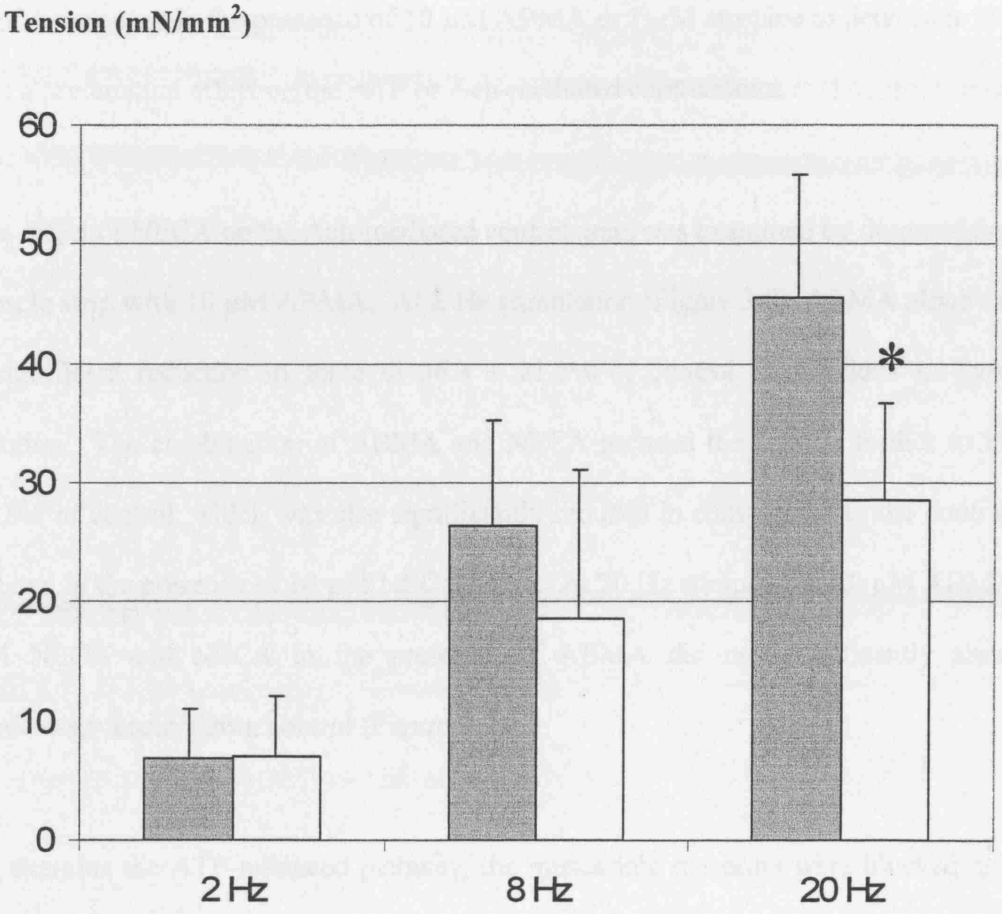


Figure 3.7 Bar chart of tension generated at different field-stimulation frequencies in the presence of 1μM atropine ***Solid bars:*** *Control tension (Tyrode's solution only),* ***White bars:*** *Tension generated in the presence of 1μM atropine, (n=8)*

To examine which neurotransmitter pathway the P1-receptors were acting upon, 10 μM NECA was used in the presence of 10 μM ABMA or 1 μM atropine to determine if there was a preferential effect on the ATP or Ach-mediated contractions.

The effect of NECA on the Ach-mediated contractions was examined by desensitising the muscle strip with 10 μM ABMA. At 2 Hz stimulation (Figure 3.8), ABMA alone caused a significant reduction in force to 36.3 ± 21.2% of control contractions in Tyrode's solution. The combination of ABMA and NECA reduced the tension further to 19.7 ± 15.8% of control, which was also significantly reduced in comparison to the contraction elicited in the presence of 10 μM NECA alone. At 20 Hz stimulation, 10 μM ABMA, 10 μM NECA and NECA in the presence of ABMA did not significantly alter the percentage tension from control (Figure 3.9)

To examine the ATP-mediated pathway, the muscarinic receptors were blocked using 1 μM atropine. At 2 Hz (Figure 3.10), atropine alone did not significantly alter the tension from control (89.9 ± 33.0%). However, the combination of atropine and 10 μM NECA caused a significant reduction of the tension to 34.6 ± 21.9% of control. This reduction was not significantly different from that observed with NECA alone. At 20 Hz stimulation (Figure 3.11), atropine caused a significant reduction in the contractile tension to 62.0 ± 14.6% of control. In combination with NECA there was a further reduction to 48.8 ± 14.3% of control. The reduction seen with the combination of NECA and atropine was also significantly greater than the reduction caused with NECA alone.

The data are consistent with the hypothesis that NECA had a predominant effect on ATP-release (Figure 3.10 and Figure 3.11). A small component of Ach release may also be affected (Figure 3.8), although this was not confirmed by figure 3.9 – see also Discussion (section 4.6, p189).

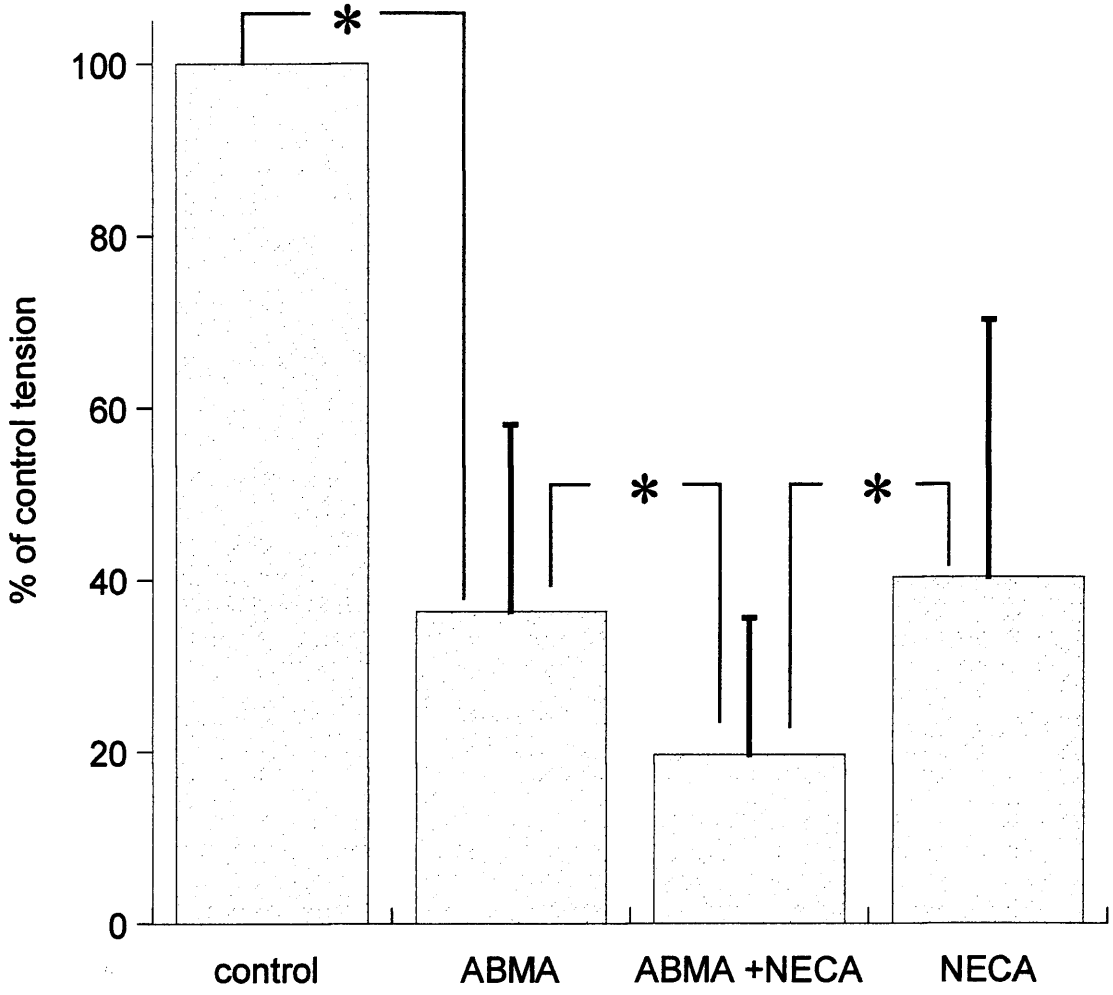


*Figure 3.8 Bar chart of percentage tension of control at 2 Hz in the presence of Tyrode's solution only (control), 10 μM ABMA, 10 μM ABMA and 10 μM NECA, and 10 μM NECA only. Mean ± s.d., *p < 0.05 in paired Student's t-test.*

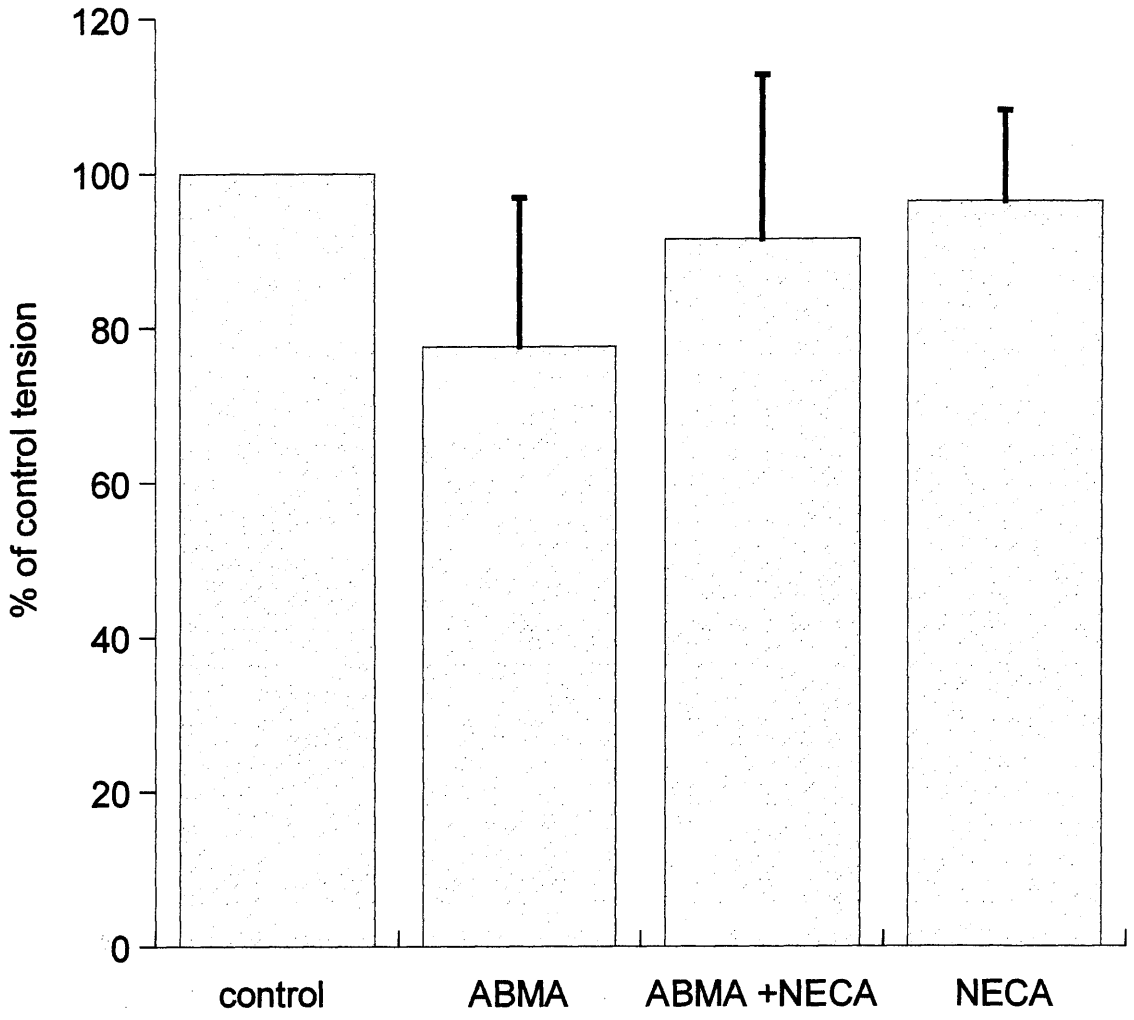


*Figure 3.9 Bar chart of percentage tension of control at 20 Hz in the presence of Tyrode's solution only (control), 10 μM ABMA, 10 μM ABMA and 10 μM NECA, and 10 μM NECA. Mean ± s.d., *p < 0.05 in paired Student's t-test.*

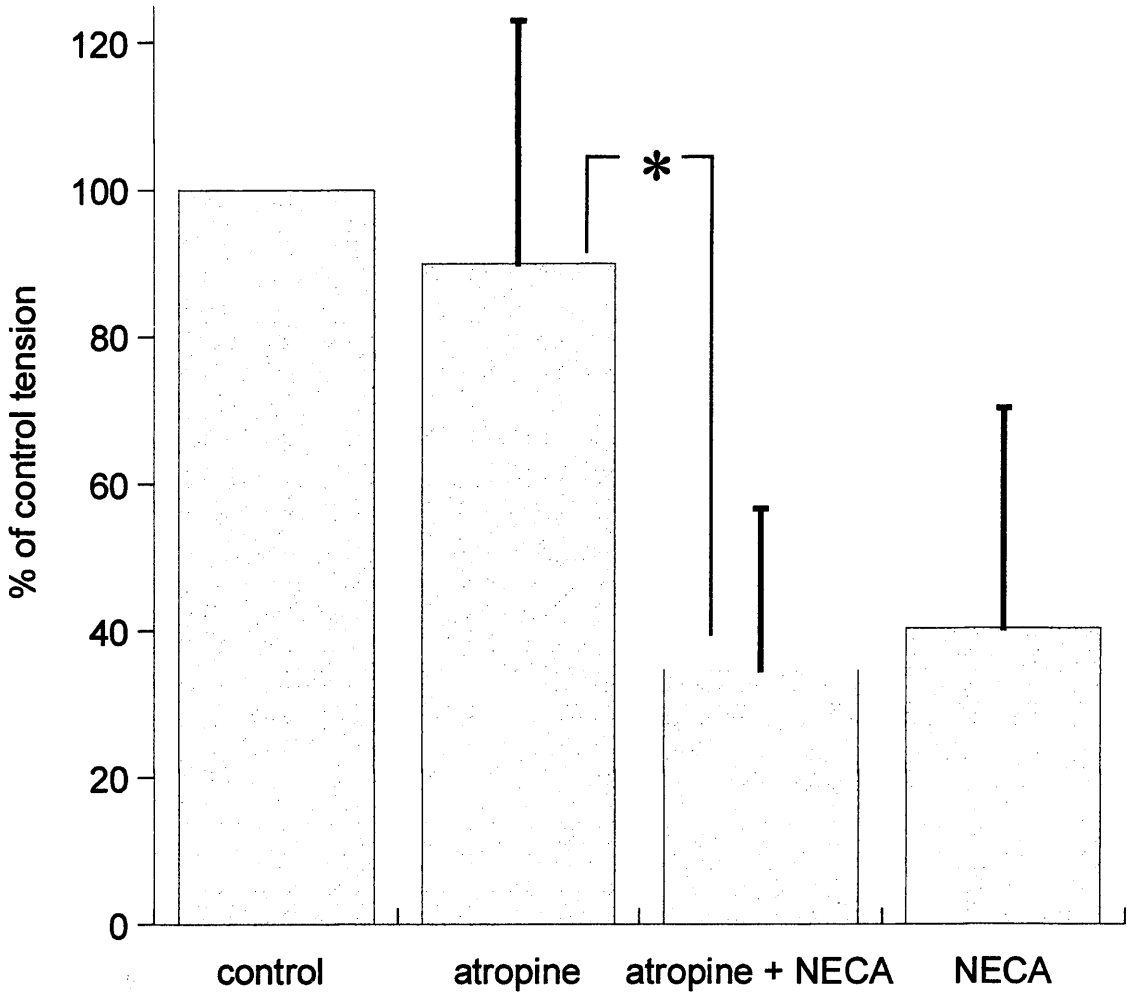


*Figure 3.10 Bar chart of percentage tension of control at 2 Hz in the presence of Tyrode's solution only (control), 1 μM atropine, 1 μM atropine and 10 μM NECA, and 10 μM NECA only. Mean ± s.d., *$p < 0.05$ in paired Student's t-test.*

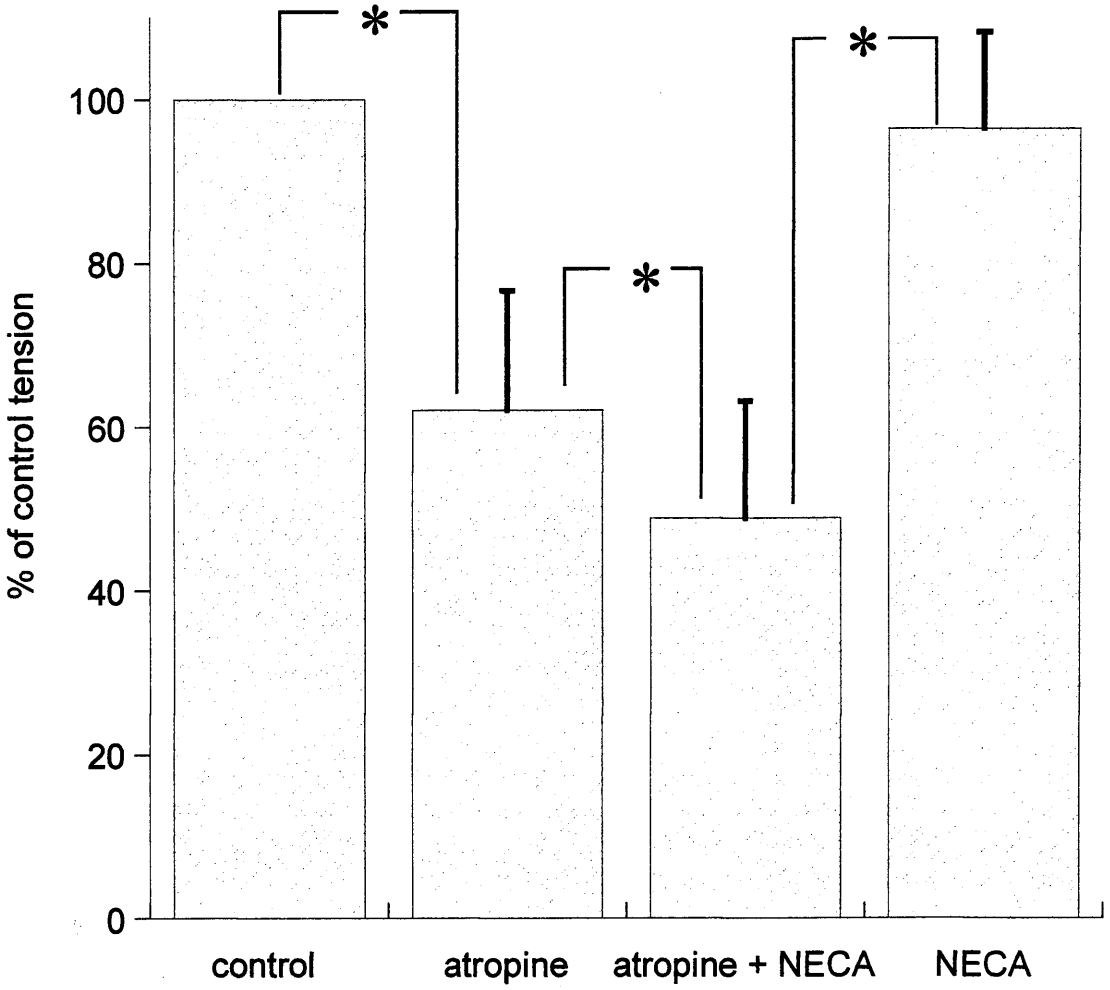


*Figure 3.11 Bar chart of percentage tension of control at 20 Hz in the presence of Tyrode's solution only (control), 1 μM atropine, 1 μM atropine and 10 μM NECA, and 10 μM NECA only. Mean ± s.d., *$p < 0.05$ in paired Student's t-test.*

3.2.3 Effect of P1-specific agents on nerve-mediated contractions in human detrusor

With a similar protocol to guinea-pig strip preparations, human samples were electrically field-stimulated with a series of increasing stimulation frequencies to study the effect of P1-receptors on nerve-mediated contractions. The results obtained for all the P1-specific agents tested are listed in Table 3.4 (p130) for samples from stable bladders and Table 3.5 (p131) for the collated data for samples from overactive bladders.

The control force-frequency relationship in normal Tyrode's solution showed that samples from stable bladders (n=33) had a calculated $f_{1/2}$ value of 18.8 ± 7.5 Hz. With samples from overactive bladders, the idiopathic group (n=20) had an $f_{1/2}$ of 16.7 ± 5.9 Hz, neuropathic (n=8) 16.8 ± 3.9 Hz, and obstructed (n=4) 9.8 ± 2.0 Hz. Only the obstructed group was found to be significantly different from the stable bladder samples. However there was a small 'n'-number for the obstructed group, and so meaningful statistical comparisons were difficult. The collated 'overactive' group had a mean $f_{1/2}$ of 15.8 ± 5.5 Hz (n=32). Force-frequency curves for each sample group is shown in Figure 3.12.

In the presence of 1 mM adenosine, human samples from stable bladders showed a reduction of contractile force to 60.9 ± 17.1% (n=6) of control at 20 Hz (Figure 3.13). This is comparable to the amount of reduction seen with the guinea-pig experiments. Samples from overactive samples (n=6, all idiopathic) showed a reduction of force to 70.0 ± 16.9% of control at 20 Hz; however this value was not significantly different

from control. The $f_{1/2}$ value in the presence of 1mM adenosine was; 22.0 ± 1.5 Hz for samples from stable bladders (Figure 3.13) and 23.1 ± 7.7 Hz for samples from overactive bladders. There was a significant difference in the $f_{1/2}$ values with 1 mM adenosine in stable and overactive bladder samples from their respective controls ($p<0.05$ paired *t*-test).

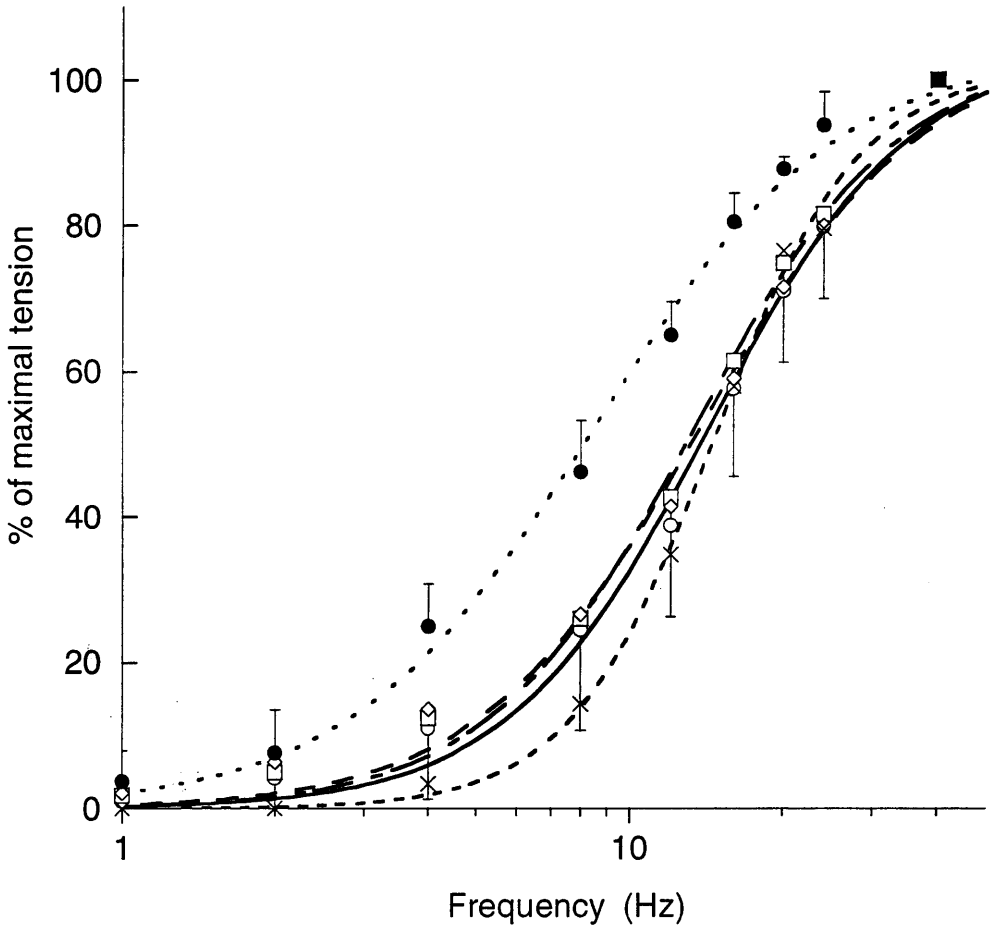


Figure 3.12 Force-frequency curves of human detrusor muscle strip samples. ***Stable bladders****, open circles:* ***grouped 'overactive'****, open squares:* ***idiopathic****, open diamonds:* ***neuropathic****, crosses:* ***obstructed****, filled circles*

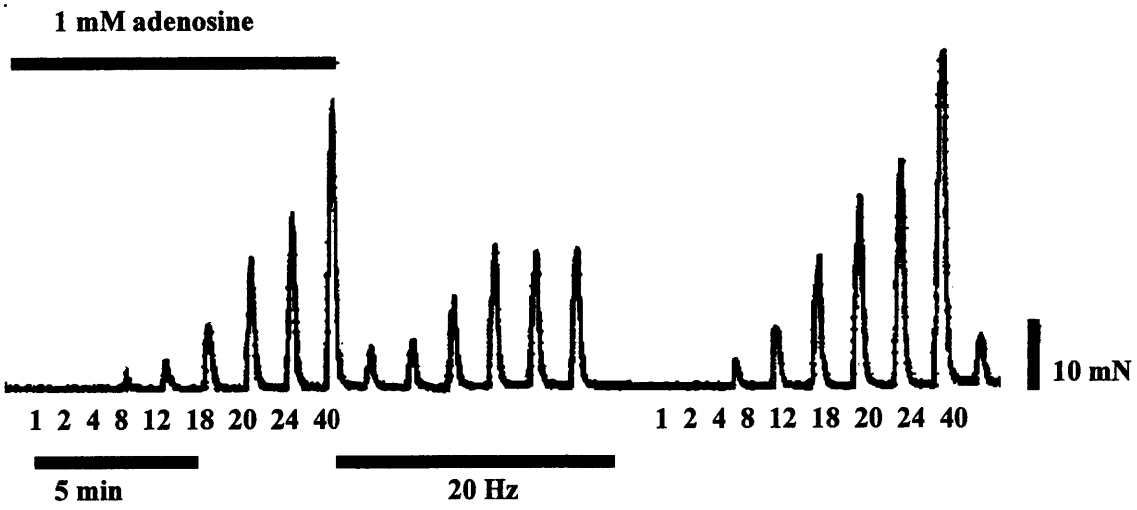


Figure 3.13 Effect of 1 mM adenosine on nerve-mediated contractions of human detrusor from a patient with a stable bladder. Stimulation frequency is given below the corresponding contractions.

10 μM NECA (n=22) produced no significant reduction of nerve-mediated contractions in samples from stable bladders when compared to adenosine. The mean tension was 90.4 ± 19.2 % of control at 20 Hz and the calculated $f_{1/2}$ was 21.1 ± 6.4 Hz. This $f_{1/2}$ value was significantly larger than the control value (18.8 ± 6.5 Hz, $p<0.05$ paired Student's *t*-test).

Samples from overactive bladders also showed little inotropic response to 10 μM NECA. Idiopathic samples (n=17) showed no significant change in the force of contraction data and had a calculated $f_{1/2}$ of 17.7 ± 6.9 Hz which was not significantly different from the control $f_{1/2}$ value (15.6 ± 4.5 Hz). Neuropathic samples in the presence of 10 μM NECA

(n=4) gave contractions 92.8 ± 12.0 % of control at 20 Hz and a calculated $f_{1/2}$ of 23.8 ± 7.7 Hz also no different from control. The mean tension of the collated data from overactive bladders with NECA was 99.3 ± 17.2% of control at 20 Hz. The calculated $f_{1/2}$ was 18.2 ± 7.4 Hz (n=23 including two samples from obstructed bladders) however this was significantly different from the $f_{1/2}$ control value (15.6 ± 4.8 Hz). Overall with samples from overactive bladders, there was no significant effect on the magnitude of nerve-mediated contractions at 20 Hz stimulation, however NECA did cause a significant increase of the $f_{1/2}$ values.

With 10 μM CPA nerve-mediated contractions in samples from stable bladders (n=8) were 89.1 ± 16.9% of control at 20 Hz and from overactive bladders (n=10) were 93.8 ± 29.5% of control. The $f_{1/2}$ value obtained for stable bladder samples was 18.2 ± 4.0 Hz, from samples of idiopathically unstable bladders was 16.5 ± 3.5 Hz and the combined data from overactive bladders (n=10) was 15.7 ± 3.0 Hz. None of these parameters was significantly different from values in Tyrode's solution. The comparison of the force-frequency curves for adenosine, NECA, CPA and Tyrode's solution above is given in Figure 3.14.

1 μM alloxazine did not significantly alter the nerve-mediated contractile force in samples from stable bladders (n=8) with a tension of 105.4 ± 10.7% of control at 20 Hz. Samples from overactive bladders (n=4) had contractions 94.8 ± 17.8% of control in the presence of alloxazine. 1 μM alloxazine in combination with 10 μM NECA had no significant effect on the contractile force of stable samples with tension 89.6 ± 23.1% of

control at 20 Hz (n=8). Samples from overactive bladders generated contractions 95.9 ± 7.7% of control (n=4). The $f_{1/2}$ values were also not significantly altered from the respective controls by alloxazine in samples from both stable and overactive bladders.

100 nM ZM-241385 had no significant effect overall on altering the contractile force of samples from both stable [81.7 ± 29.6% of control (n=6)] and overactive [98.2 ± 7.9% of control (n=7)] bladders. However the $f_{1/2}$ value (23.2 ± 5.1 Hz) from stable bladder samples was significantly larger in than the control value (16.7 ± 3.2 Hz), although there was no significance change in the $f_{1/2}$ value of samples from overactive bladders as compared to control. ZM-241385 in combination with 10 μM NECA also did not significantly alter the force of the contractions in samples from stable bladders (n=5) where contractions were 75.0 ± 28.6% of control. Overactive samples (n=5) had contractions of 96.8 ± 16.4% of control. The $f_{1/2}$ values of the stable bladder samples with were not significantly different from the $f_{1/2}$ values with NECA alone. There was also no significant change in the $f_{1/2}$ value with 100 nM ZM-241385 and 10μM NECA in samples from overactive bladders as compared to the $f_{1/2}$ value from NECA controls. However it is notable that the variability of the effect of ZM-241385 either above as in combination with NECA was large, as evidenced by the large standard deviation values. There was no apparent basis for this variability however in terms of pathological origins of the tissue.

Overall, nerve-mediated contraction of human bladder smooth muscle is less sensitive to P1-specific compounds compared to that from guinea-pig. Only adenosine appeared to have a significant effect on reducing the contractile force in stable, but not overactive, human bladder samples, with all other compounds having no significant effect on the force of nerve-mediated contractions. However NECA caused a significant increase of the $f_{1/2}$ value in samples from stable and overactive bladders, which indicates there may be some P1-receptor activity in human detrusor smooth muscle. However, as with the analogous guinea-pig experiments changes may by more evident at lower frequencies and so the same frequency-dependent analysis as with guinea-pig tissue was carried out.

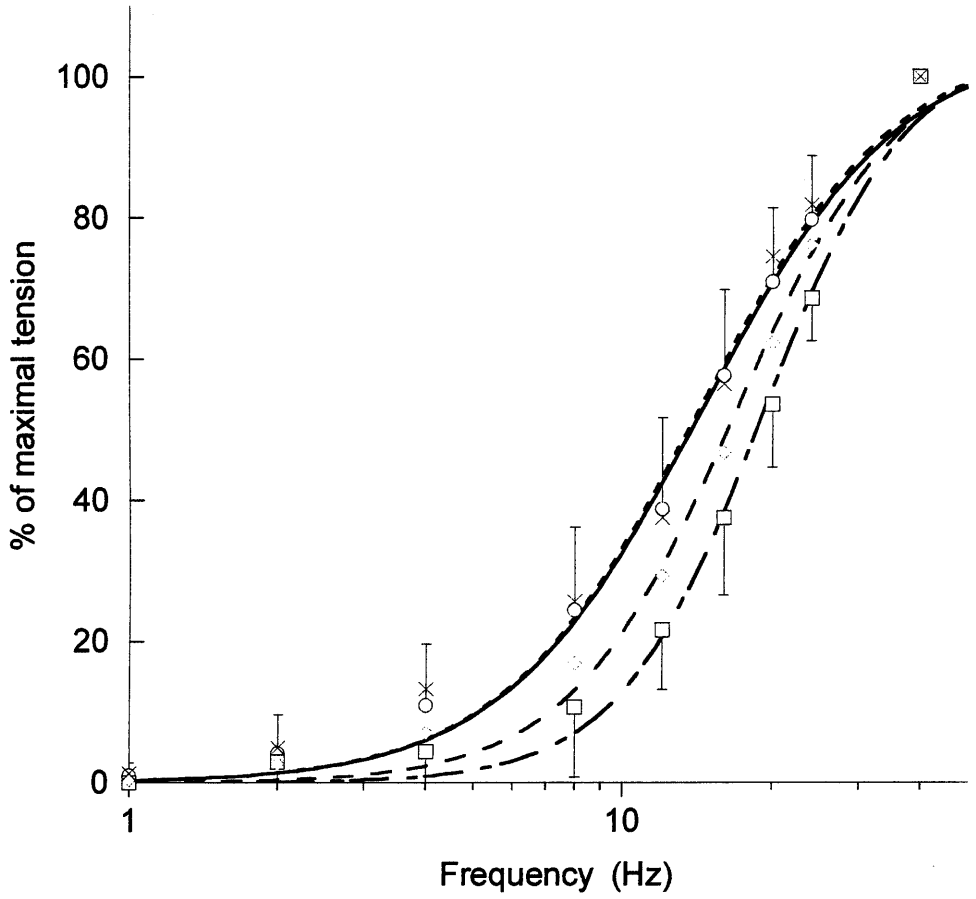


Figure 3.14 Force-frequency curve of stable human detrusor strip in 1 mM adenosine, 10μM NECA, 10μM CPA and control. ***Tyrode's solution,*** *open circles:* ***adenosine,*** *open squares:,* ***NECA,*** *open diamonds:,* ***CPA,*** *crosses*

Table 3.4 Summary of force-frequency results for stable human bladder

Compounds	Mean $f_{1/2}$ half-maximal stimulation frequency (Hz)	Mean control $f_{1/2}$ frequency (Hz)	%-tension of control at 20Hz
1 mM adenosine (n=6)	22.0 ± 1.5*	18.0 ± 0.2	60.9 ± 11.1*
10 μM NECA (n=22)	21.1 ± 6.5*	18.8 ± 6.5	90.4 ± 19.2
10 μM CPA (n=8)	18.2 ± 4.0	18.6 ± 10.2	89.1 ± 16.9
1 μM alloxazine (n=8)	18.5 ± 4.7	22.2 ± 9.9	105.4 ± 10.6
1 μM alloxazine with 10 μM NECA (n=8)	23.2 ± 8.3	20.9 ± 8.3	89.6 ± 23.1
100 nM ZM-241385 (n=6)	23.2 ± 5.1*	16.7 ± 3.2	81.7 ± 29.6
100 nM ZM-241385 and 10 μM NECA (n=6)	22.0 ± 4.5	19.6 ± 4.5	75.0 ± 28.6

*$p<0.05$ as compared to control (paired Student's *t*-test)

Table 3.5 Summary of force-frequency results for collated 'Overactive' human detrusor samples

Compounds	Mean $f_{1/2}$ half-maximal stimulation frequency (Hz)	Mean control $f_{1/2}$ frequency (Hz)	%-tension of control at 20Hz
1 mM adenosine (n=6)	23.1 ± 7.7*	14.9 ± 4.2	70.0 ± 16.9*
10 μM NECA (n=23)	18.0 ± 7.6*	15 ± 5.0	99.3 ± 17.2
10 μM CPA (n=10)	15.8 ± 3.0	16.7 ± 7.1	93.8 ± 29.5
1 μM alloxazine (n=4)	18.7 ± 7.3	15.5 ± 4.0	94.8 ± 17.8
1 μM alloxazine with 10 μM NECA (n=4)	19.5 ± 6.8	17.8 ± 2.7	93.9 ± 7.7
100 nM ZM-241385 (n=7)	25.0 ± 13.7	18.2 ± 5.7	98.2 ± 7.9
100 nM ZM-241385 and 10 μM NECA (n=5)	23.0 ± 11.5	16.5 ± 6.1	96.8 ± 16.4

*$p<0.05$ as compared to control (paired Student's *t*-test)

3.2.4 Frequency-dependent neurotransmitter release in human detrusor muscle strips

A frequency-dependent effect of P1-receptor agonists was apparent in guinea-pig detrusor. Whether this was also true in human samples was tested by examining the force-frequency relationships. A ratio of the tension at 8 Hz (T8 - the lowest frequency where contractions were reliably recorded) and 24 Hz (T24 – a higher frequency, where near-maximal contractions were achieved without much direct muscle stimulation) was calculated. Changes in the T8/T24 ratio in the presence of P1-specific agents would suggest that a frequency-dependent mechanism similar to that seen in guinea-pig detrusor exists also in human tissue.

With samples from stable human bladders only 1 mM adenosine and 10 μM NECA caused a significant reduction of the T8/T24 ratio compared to control values (Table 3.6). Samples from overactive bladders showed a similar reduction of the T8/T24 ratio as compared to control with 10 μM NECA, 10 μM CPA, and 1 μM alloxazine with 10 μM NECA, adenosine however was without effect in the latter samples(Table 3.7).

The T8/T24 ratio may be a more sensitive index for measuring the changes to nerve-mediated contractions of human bladder samples than measuring at the percentage-change of tension at a fixed frequency or calculation of the $f_{1/2}$ values.

Table 3.6 Comparison of T8/T24 ratios of samples from stable human bladder force-frequency relationships in the presence of P1-specific agents

Compound	T8/T24 ratio	Control T8/T24 ratio
Control (n=33)	--	0.30 ± 0.13
1 mM Adenosine (n=6)	0.15 ± 0.13*	0.26 ± 0.14
10 μM NECA (n=30)	0.22 ± 0.14*	0.28 ± 0.13
10 μM CPA (n=8)	0.30 ± 0.18	0.34 ± 0.10
1 μM Alloxazine (n=9)	0.31 ± 0.10	0.30 ± 0.11
1 μM Alloxazine + 10 μM NECA (n=9)	0.27 ± 0.09	0.30 ± 0.11
100 nM ZM-241385 (n=6)	0.16 ± 0.10	0.21 ± 0.09
100 nM ZM-241385 + 10 μM NECA (n=6)	0.14 ± 0.11	0.21 ± 0.09

*$p<0.05$ as compared to control (paired Student's *t*-test)

Table 3.7 Comparison of T8/T24 ratio of collated samples from overactive human detrusor force-frequency relationships in the presence of P1-specific agents

Compound	T8/T24 ratio	Control T8/T24 ratio
Combined control (n=32)	--	0.30 ± 0.17
1 mM Adenosine (n=6)	0.30 ± 0.21	0.32 ± 0.24
10 μM NECA (n=23)	0.26 ± 0.17*	0.31 ± 0.18
10 μM CPA (n=8)	0.27 ± 0.15*	0.34 ± 0.17
1 μM Alloxazine (n=4)	0.21 ± 0.06	0.21 ± 0.09
1 μM Alloxazine + 10 μM NECA (n=4)	0.16 ± 0.06*	0.21 ± 0.09
100 nM ZM-241385 (n=7)	0.26 ± 0.16	0.25 ± 0.16
100 nM ZM-241385 + 10 μM NECA (n=5)	0.25± 0.16	0.29 ± 0.19

*p<0.05 as compared to control (paired Student's *t*-test)

3.3 Atropine-resistance in human detrusor strips

At the end of all human tissue strip experiments, the tissue was stimulated at 20 Hz in the presence of 1 μM atropine (a muscarinic-receptor antagonist), followed by 1 μM tetrodotoxin (TTX) in the presence of 1 μM atropine to determine the amount of atropine-resistant contraction. TTX inhibits the release of neurotransmitters from the nerve terminal by blocking the nerve impulse and was used to estimate the small amount of direct muscle stimulation. The magnitude of the nerve-mediated contraction was calculated as the TTX-sensitive component. The atropine-resistant contraction was the nerve-mediated contraction resistant to atropine.

In stable human bladders, acetylcholine is the sole functional contractile mediator. This has been shown by the fact that atropine will abolish completely nerve-mediated contractions in isolated preparations (Kinder & Mundy, 1985). Atropine-resistant, nerve-mediated contractions have been observed in human tissue obtained from certain pathologies. These atropine-resistant contractions have been shown to be due to activation of P2X-receptors as they are abolished when purinoceptors were desensitised with ABMA (Brown *et al.*, 1979).

With tissue samples from overactive bladders (n=32), 13 samples showed atropine-resistance (four from obstructed bladders, six with neuropathic overactivity, three with idiopathic detrusor overactivity). Of the samples from stable bladders (n=33) four samples also displayed atropine-resistant contractions. The samples that showed

atropine-resistance in the latter group were categorised as those from stable bladders due to lack of urodynamic evidence to prove conclusively the presence of overactivity.

Samples from patients diagnosed with an outlet obstruction (n=4) or with neuropathically overactive bladders (n=6) showed the greatest amount of atropine-resistant contractions, with a median value of 20.7% (quartiles 14.5 and 30.2%) and 21.4% (quartiles 11.6 and 31.0%) of control respectively. The samples categorised as stable (n=4) or idiopathically overactive bladders (n=3) had similar degrees of atropine-resistance to one another. The median values were 8.6% (quartiles 6.2 and 11.3%) and 12.0% (quartiles 7.4 and 13.4%) of control respectively (Figure 3.15).

The results indicate that ATP-mediated contractions can be present in human detrusor, with the incidence being greater in samples from overactive human bladders than stable bladders. Thirteen out of 32 unstable samples showed atropine-resistance and only four out of 33 stable samples showed this phenomenon. This difference in the occurrences of atropine-resistance between samples from overactive and stable bladders was shown to be significant ($p<0.05$) with a χ^2-test of association.

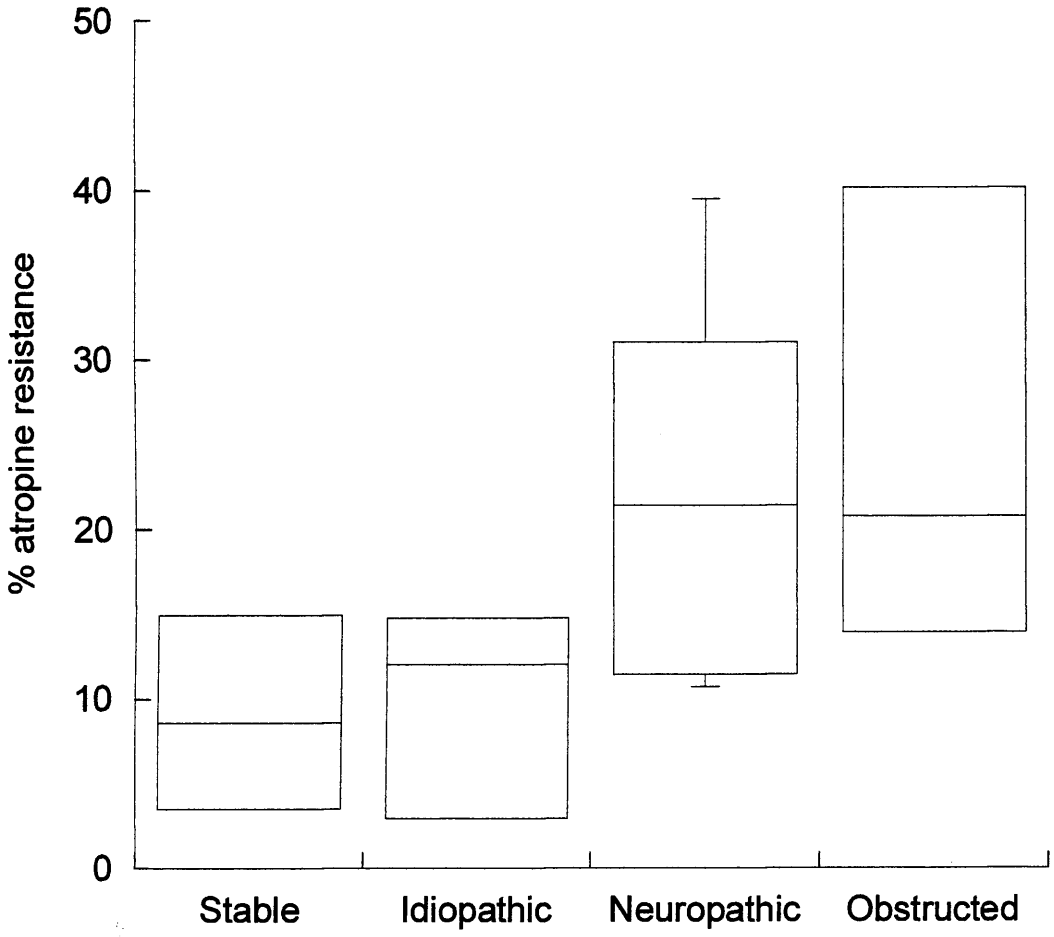


Figure 3.15 Boxplot of atropine-resistance in different pathologies, the median, 25% and 75% percentiles have been plotted. The error bars indicate outlying data points.

3.4 Post-synaptic P1-receptors

3.4.1 Carbachol-dose response in human and guinea-pig detrusor strips

The effects of P1-receptors on modulating post-synaptic contractions were tested using an acetylcholine analogue, carbachol. Carbachol activates the contractile signalling pathway through the M3-receptor and allowed the effects of P1-receptors to be observed on the muscle itself without interference from nerve-stimulation.

Carbachol dose-responses (0.1-10 μM) were used to determine if there was a stimulation-dependent modulation by the P1-receptors as seen with the nerve-mediated contractions. Tissue strips were exposed to maximal concentrations of P1-receptor agonists/antagonists with varying concentrations of carbachol. The effect of the P1-specific compounds was determined as a percentage change from pre and post-intervention control contractions.

3.4.2 Guinea-pig detrusor carbachol dose-response

Guinea-pig control carbachol dose-response curves (n=23) gave an EC_{50} of 1.2 ± 0.8 μM (Figure 3.16). The effect of P1-specific agents on the carbachol dose-response in guinea-pig preparations is given in Table 3.8 (p147).

The presence of 1 mM adenosine resulted in a significant reduction of the contractions, to 75.9 ± 19.3% (n=7) of control with 0.3 μM carbachol. The EC_{50} value in the presence of adenosine (0.8 ± 0.3 μM) was not significantly different from the control carbachol dose-response value. With 10 μM NECA (n=11) the contractile force generated by 0.3 μM carbachol was significantly reduced to 69.7 ± 14.7% of control. The EC_{50} value with NECA was also not significantly different from control results (1.6 ± 1.2 μM).

In the presence of 10 μM CPA (n=6), there was no alteration in the force of contraction with 1 μM carbachol (104.1 ± 26.2% of control). The carbachol dose-response EC_{50} was 0.7 ± 0.1 μM and there was no significant difference with the control EC_{50} value, although it is noted that there is a large scatter in the control data value (Table 3.8). Therefore the A1-dependent depression of contractions elicited by electric field stimulation is most likely due to A1-receptors located not on the muscle but probably on the motor nerve.

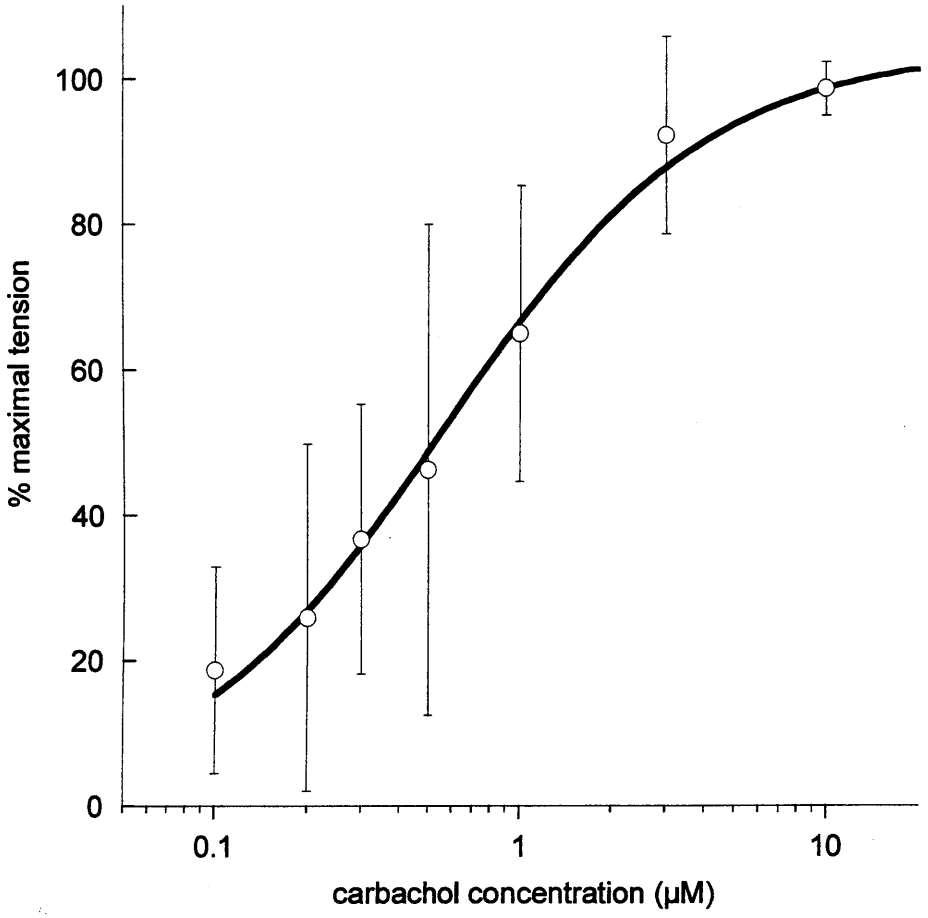


Figure 3.16 Plot of carbachol dose-response of guinea-pig detrusor muscle strip

The EC_{50} values and contractile tension with 1 μM carbachol in the presence of 1 μM alloxazine (n=5), alloxazine with 10 μM NECA (n=5), and 10 μM IB-MECA (n=3) were not significantly different from respective controls. The latter observation correlates with the previous study where A3-receptor activity was absent.

Adenosine, NECA and CPA (all n=4) did not alter the contraction generated by 80 mM KCl-Tyrode's solution, which causes a depolarisation of the cell membrane and opening of L-type calcium channels..

From these results, there was no significant effect on the EC_{50} of the carbachol dose-response by any of the P1-specific agents tested in guinea-pig detrusor muscle strips when used to mimic the effect of adenosine itself. However NECA did produce a reduction in the force of the carbachol contraction.

Table 3.8 Summary of guinea-pig carbachol dose-response results

Compound	Mean EC_{50} (μM)	Mean control EC_{50} (μM)	%-tension of control with 0.3 μM carbachol
1 mM adenosine (n=7)	0.8 ± 0.3	1.0 ± 0.7	75.9 ± 19.5 *
10 μM NECA (n=11)	1.6 ± 1.2	1.2 ± 1.9	69.7 ± 14.7 *
10 μM CPA (n=6)	0.7 ± 0.1	2.7 ± 4.3	104.1 ± 26.2
1 μM alloxazine (n=5)	0.3 ± 0.1	1.3 ± 1.9	84.1 ± 23.1
1 μM alloxazine and 10 μM NECA (n=5)	0.4 ± 0.3	1.3 ± 1.9	76.0 ± 30.0
10 μM IB-MECA (n=3)	1.8 ± 1.1	1.3 ± 0.7	89.0 ± 13.7

*$p<0.05$ as compared to control (paired Student's *t*-test)

3.4.3 Human detrusor from stable bladders: carbachol dose-response

From control carbachol dose-response experiments, the EC_{50} was determined to be 0.3 ± 0.2 μM (n=26) for the stable group (Figure 3.17). Human tissue was more sensitive to carbachol than that from guinea-pig (unpaired Student's *t*-test, $p<0.05$), indicating there could be a species difference in the sensitivity to muscarinic agonists. The results for the effect of P1-specific agents on the carbachol dose-response for human detrusor muscle strips from stable bladders is summarised in Table 3.9 (p151)

The addition of 1 mM adenosine (n=6) caused a significant reduction in the force to 62.6 ± 16.8% of control (Figure 3.17) with 0.3 μM carbachol and yielded a carbachol dose-response EC_{50} of 0.1 ± 1.1 μM, which however was not significantly different from control. 10 μM NECA (n=24) resulted in contractions 87.8 ± 26.0% of control and an EC_{50} value of 0.4 ± 0.6 μM. The force and EC_{50} values were not significantly different from control values.

In the presence of 10 μM CPA (n=4) the force of the contractures was 89.9 ± 17.7% of control and the EC_{50} was 0.1 ± 0.04 μM, both were not significantly different from their respective controls.

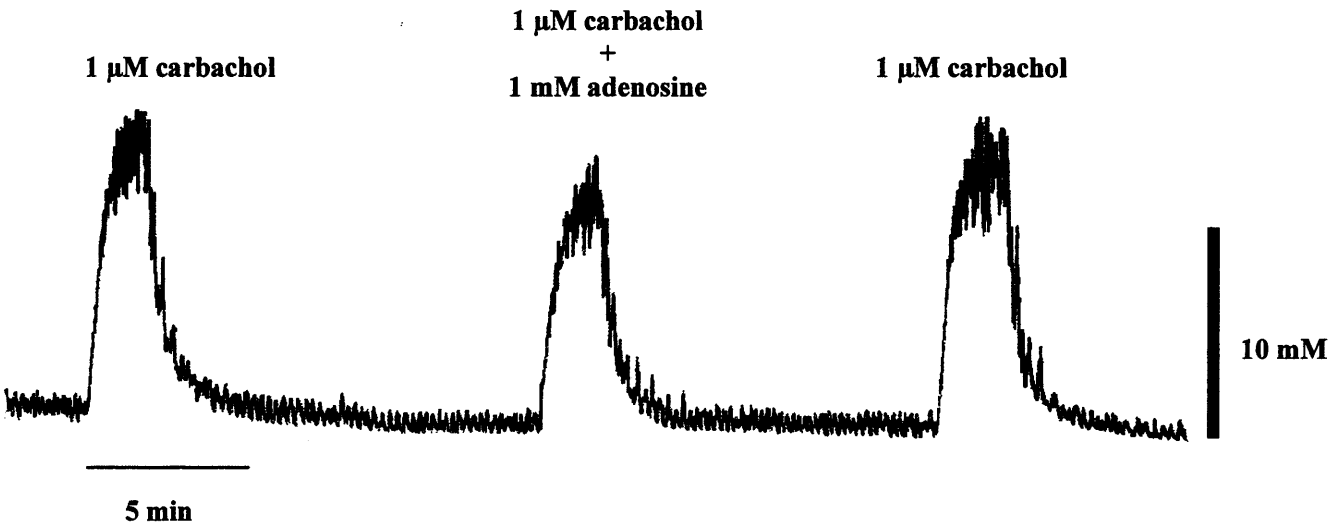


Figure 3.17 Effect of 1mM adenosine on carbachol contractures in a detrusor strip from a stable human bladder.

Addition of 1 µM alloxazine (n=8) resulted in contractions 99.9 ± 13.0% of control and an EC_{50} value of 0.5 ± 0.3 µM. Alloxazine with 10 µM NECA (n=8) resulted in contractions 108.2 ± 14.9% of control and a carbachol EC_{50} of 0.3 ± 0.03 µM. There were no significant difference in the force or EC_{50} values compared to control in both interventions.

In the presence of 100 nM ZM-241385 (n=4) the contractions were 112.3 ± 23.3% of control and the carbachol EC_{50} was 0.3 ± 0.05 µM. The combination of 100 nM ZM-241385 and 10 µM NECA (n=4) resulted in contractions 103.9 ± 13.6% of control and also did not alter the carbachol EC_{50} from control (0.3 ± 0.03 µM).

There appears to be very little response to P1-specific agents in stable human detrusor from stable bladders. Adenosine caused a significant reduction of the carbachol contracture, similar to guinea-pig preparations, and it is possible that adenosine is mediating its effect through a non P1-dependent mechanism.

Table 3.9 Summary of stable human carbachol dose-response results

Compound	Mean EC_{50} (μM)	Mean control EC_{50} (μM)	%-tension of control with 0.3 μM carbachol
1 mM adenosine (n=6)	0.1 ± 1.1	0.3 ± 0.1	62.6 ± 16.8*
10 μM NECA (n=24)	0.4 ± 0.6	0.3 ± 0.2	87.8 ± 26.0
10 μM CPA (n=4)	0.1 ± 0.04	0.2 ± 0.6	89.9 ± 17.7
1 μM alloxazine (n=8)	0.5 ± 0.3	0.4 ± 0.2	99.9 ± 13.0
1 μM alloxazine and 10μM NECA (n=8)	0.3 ± 0.03	0.4 ± 0.2	108.2 ± 14.9
1 μM ZM-241285 (n=4)	0.3 ± 0.05	0.4 ± 0.01	112.3 ± 23.3
1 μM ZM-241385 with 10 μM NECA (n=4)	0.3 ± 0.03	0.4 ± 0.01	103.9 ± 13.6

*$p<0.05$ as compared to control in paired Student's *t*-test

3.4.4 Overactive human detrusor carbachol dose-response

From the collated overactive group (n=26), the control carbachol dose-response resulted in an EC_{50} of 0.4 ± 0.4 μM (Figure 3.18). This was not significantly different from the value obtained with the stable group. Therefore it appears that there is little difference in the muscarinic contractile pathways between the pathological groups. The effect of P1-specific agents on the carbachol dose-response in samples from overactive bladder has been complied in Table 3.10 (p154).

In the presence of 1 mM adenosine (n=4) the carbachol EC_{50} value of 0.8 ± 0.1 μM, which was not significantly different from the control EC_{50} of 0.6 ± 0.1 μM. However the force of the contractions was significantly reduced by adenosine to 61.7 ± 16.6% of control. In the presence of 10 μM NECA (n=16) the EC_{50} was 0.3 ± 0.1 μM, which was not significantly different from the control value. The force of the contraction induced by 0.3μM carbachol was also not affected (90.1 ± 37.2% of control).

Addition of 10 μM CPA (n=10) resulted in a carbachol EC_{50} of 0.6 ± 0.6μM which was not significantly different from the control value (1.2 ± 2.4 μM). The force of contraction was also unaffected, in 0.3μM carbachol the contracture was 102.2 ± 19.8% of control.

In the presence of 1μM alloxazine (n=4) there was no significant change to the force of contraction (107.2 ± 19.8% of control) or the EC_{50} value (0.4 ± 0.2 μM, control: 0.4 ± 0.2 μM) as compared to control. The combination of alloxazine with 10 μM NECA (n=4)

also did not change the force (78.8 ± 18.9% of control) or EC_{50} value (0.5 ± 0.3 μM control: 0.3 ± 0.2 μM).

Addition of 100nM ZM-241385 (n=6) did not alter the force (97.3 ± 16.7%) or the EC_{50} value (0.4 ± 0.5 μM, control: 0.3 ± 0.1 μM). ZM-241385 with 10 μM NECA (n=5) showed no significant change in the EC_{50} (0.6 ± 0.7 μM control: 0.4 ± 0.4 μM) or the force of contractions (84.7 ± 41.8%) from control values.

In human tissue from overactive bladders, the only significant effect observed was the reduction of force by 1mM adenosine. The EC_{50} of the carbachol dose-response was unaffected by all the P1-specific compounds tested, including adenosine. This could indicate the possible presence of a non-P1 adenosine pathway affecting the contractures generated by muscarinic agonists.

Table 3.10 Summary of overactive human carbachol dose-response results

Compound	Mean EC_{50} (μM)	Mean Control EC_{50} (μM)	%-tension of control with 0.3 μM carbachol
1 mM adenosine (n=4)	0.8 ± 0.1	0.6 ± 0.1	61.7 ± 16.6 *
10 μM NECA (n=16)	0.3 ± 0.1	0.4 ± 0.2	90.1 ± 37.2
10 μM CPA (n=10)	0.6 ± 0.6	1.2 ± 2.4	102.2 ± 19.8
1 μM alloxazine (n=4)	0.4 ± 0.2	0.4 ± 0.2	77.1 ± 6.3
1 μM alloxazine and 10 μM NECA (n=4)	0.5 ± 0.3	0.3 ± 0.2	78.8 ± 18.9
1 μM ZM-241285 (n=6)	0.4 ± 0.5	0.3 ± 0.1	97.3 ± 16.7
1 μM ZM-241385 with 10 μM NECA (n=5)	0.6 ± 0.8	0.4 ± 0.4	84.7 ± 41.8

*$p<0.05$ as compared to control, paired Student's t-*test*

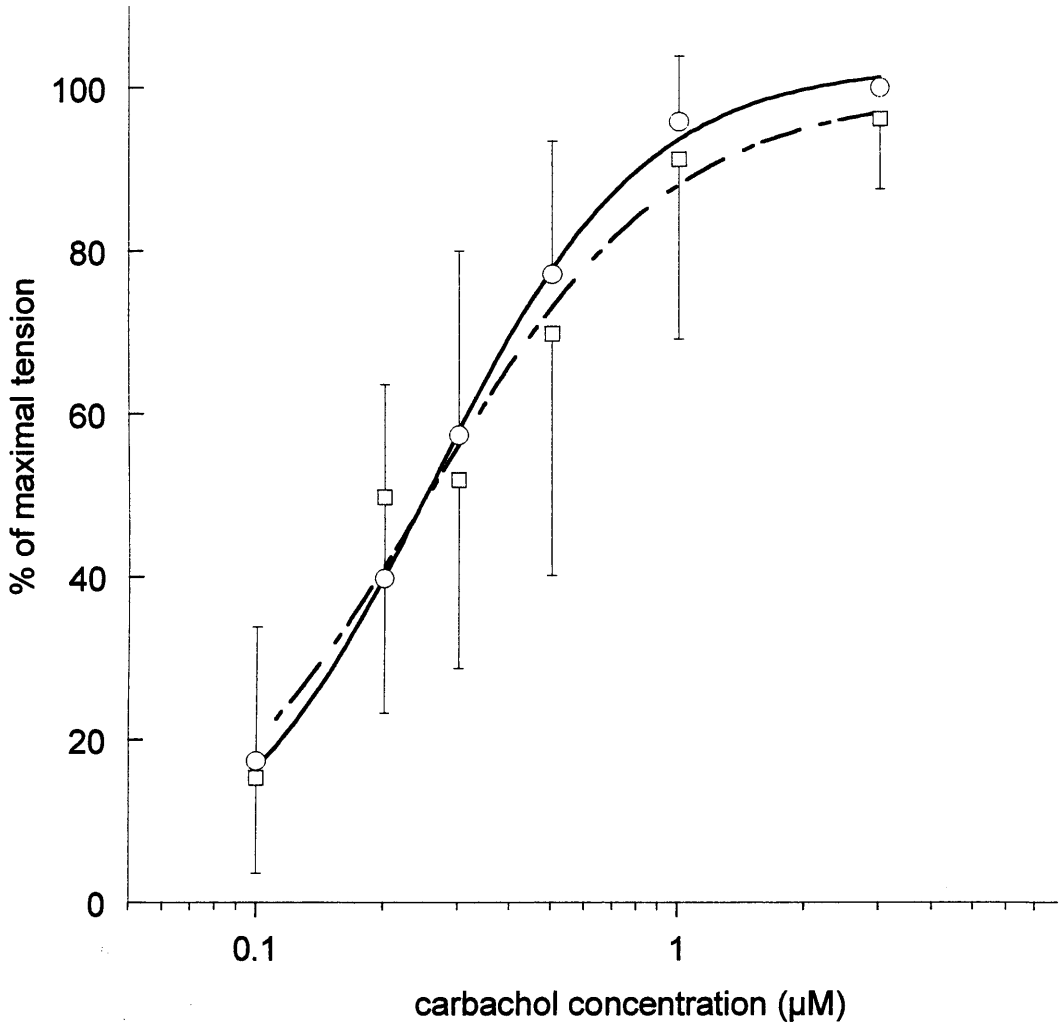


Figure 3.18 Plot of control carbachol dose-response of stable and overactive human detrusor muscle strips. ***Stable,*** *open circles:* ***Overactive,*** *open squares*

3.5 Single-cell epifluorescence microscopy

3.5.1 Isolation of detrusor smooth muscle cells

Detrusor myocytes were isolated by enzymatic digestion of guinea-pig and human bladder samples, either by overnight incubation or digested on the day. The response of these cells to test agents, such as carbachol, in the presence of adenosine receptor agonists and antagonists were investigated. Responses were measured by the change of intracellular [Ca^{2+}] in response to the test agents.

Detrusor samples from both guinea-pig bladders and human (stable and overactive) bladders were used for these experiments. The digestion process differed slightly between the human and guinea-pig preparations. Human samples generally required a longer incubation period (90 minutes on the heating block) in the digestive enzyme mixture than guinea-pig tissue (15 to 45 minutes, a shorter time on the heating block if the sample was incubated in diluted enzyme overnight – see Materials and methods). This may reflect the tissue content differences present between the human and guinea-pig bladder smooth muscle layer.

The viability of the cells was determined by whether they would respond to 1 μM carbachol for human cells or 3 μM carbachol for guinea-pig cells, as the muscarinic pathway is the predominant mechanism for normal detrusor muscle contractile activation. Cells from which a Ca^{2+}-transient could be evoked were used for the remainder of the experimental protocol.

3.5.2 Differences between human and guinea-pig isolated detrusor cells

With the isolated detrusor myocytes used in this study it was found that the resting $[Ca^{2+}]$ was not normally distributed (Figure 3.19). The median guinea-pig myocytes resting $[Ca^{2+}]$ (n=49) was 59 nM (quartiles 26 and 119 nM). For human myocytes from stable bladders (n=18) the median value was 64 nM (quartiles 20 and 77 nM); for samples from overactive bladders (n=19) the median was 86 nM (quartiles 27 and 174 nM). There were no significant differences between any of the groups, as shown through ANOVA.

From comparison of the magnitude of the Ca^{2+}-transients elicited by 1 and 3 μM carbachol to Ca^{2+}-transients elicited by 10 μM carbachol in several human and guinea-pig detrusor myocytes, it was found that human cells were more responsive to carbachol than guinea-pig. This was also shown with the dose-response to carbachol in the previous muscle strip experiments. Therefore, 1 μM carbachol was used to generate Ca^{2+} transients from human cells, and 3 μM was used for guinea-pig myocytes. These concentrations were chosen as they were close to the respective carbachol EC_{50} values from the muscle strip experiments.

The Ca^{2+}-transients were either monophasic (see Figure 3.20) or consisted of several oscillations (see Figure 3.21). The monophasic transient generally showed an undershoot of $[Ca^{2+}]$ after the recovery from the peak of the transient before returning to the pre-intervention $[Ca^{2+}]_i$. In each case the maximum elevation of $[Ca^{2+}]_i$ was recorded as the value of the variable in either control or test solution.

It was quite common to observe shortening of the cell on application of carbachol (Plate 3.1), particularly with the human detrusor myocytes.

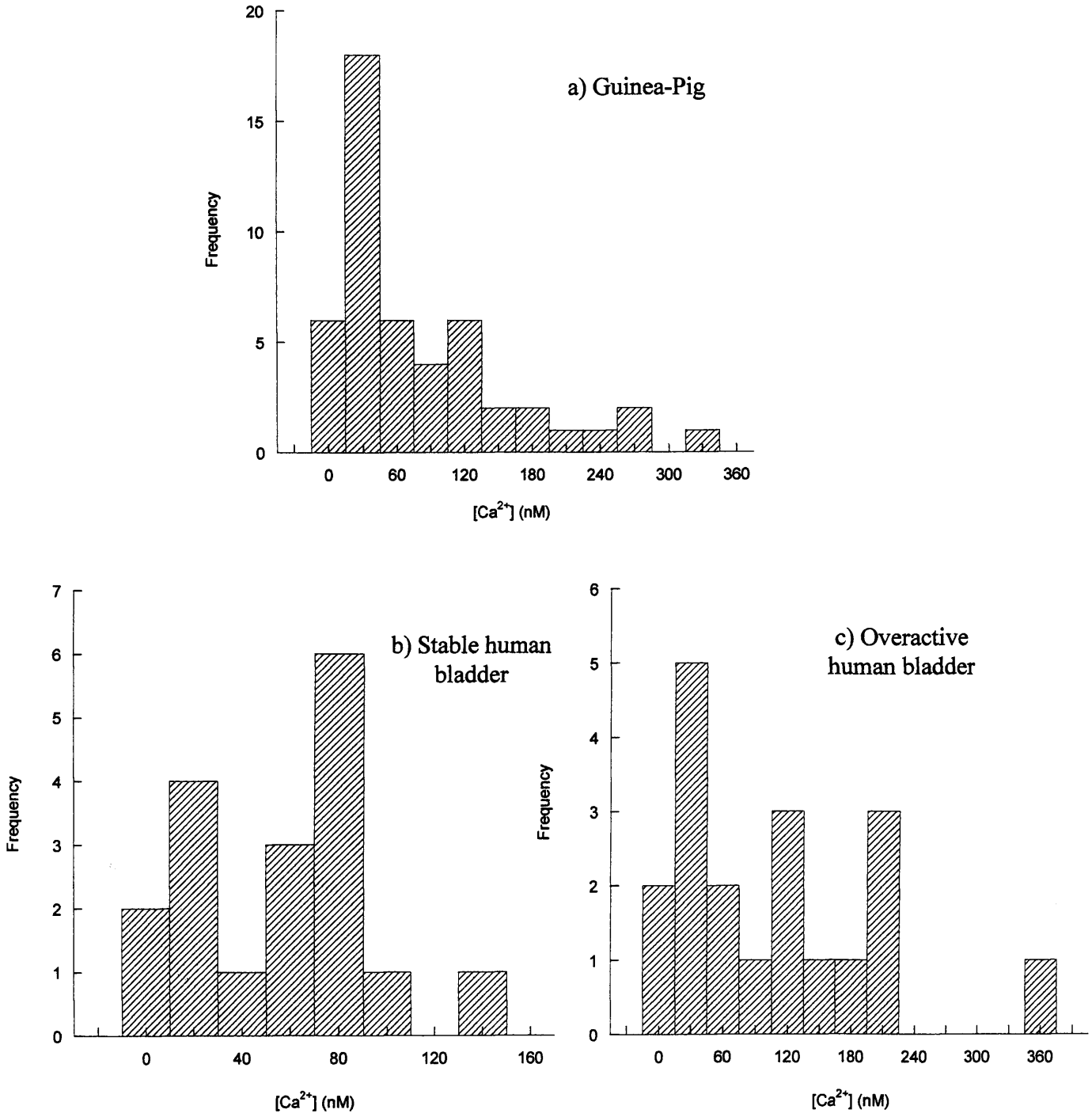
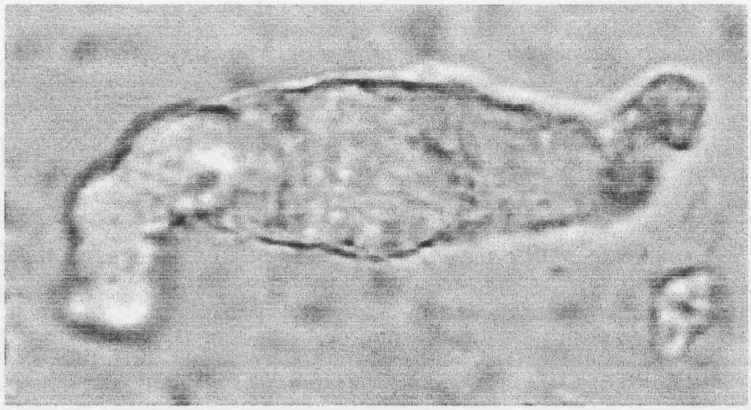
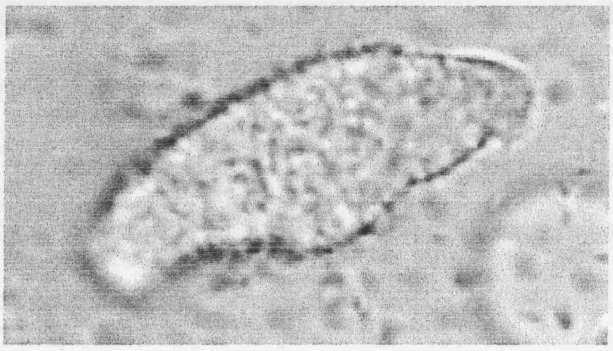


Figure 3.19 Histograms of resting intracellular [Ca^{2+}] of a) guinea-pig, b) human stable and c) overactive bladder myocytes



a) Freshly isolated human detrusor myocyte from stable bladder (magnification x400)



b) The detrusor myocyte from a) after application of 1 μM carbachol for 1 minute (magnification x400)

Plate 3.1. Isolated human detrusor cell before and after application of carbachol

3.5.3 Changes in response to carbachol by adenosine in isolated guinea-pig detrusor cells

With guinea-pig cells, 1 mM adenosine (n=15) caused a significant reduction (paired Student's *t*-test, $p<0.05$) of the carbachol Ca^{2+}-transient to 67.7 ± 22.8% of control (Figure 3.20). Various P1-selective compounds were also tested against the isolated detrusor cells to determine the receptor subtype that caused the adenosine-mediated reduction of the Ca^{2+}-transients.

Addition of 10 μM NECA (n=8) also significantly reduced the carbachol Ca^{2+}-transients to 85.8 ± 10.0% of control. The A1-selective agents, 10 μM CPA (n=3) and 1 μM DPCPX (A1-specific antagonist, n=4) had no significant effect on the Ca^{2+}-transient (92.3 ± 9.6% and 111.1 ± 67.8% of control respectively). 10 μM CGS-21680 (n=6), an $A2_A$-selective agonist, did not cause a significant change to carbachol Ca^{2+}-transients in comparison to controls (79.8 ± 25.0% of control). CGS-21680 was used instead of ZM-241385 as an $A2_A$-specific agent, due to the lack of availability ZM-241385 for the isolated cell experiments.

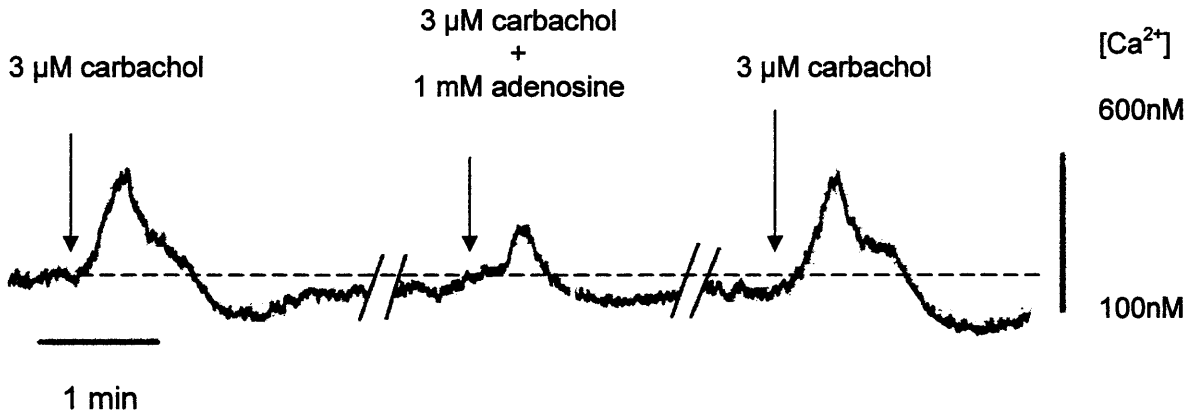


Figure 3.20 Guinea-pig detrusor cells: reduction of carbachol Ca^{2+}-transient by 1 mM adenosine

The $A2_B$-antagonist, alloxazine (1 µM, n=7) itself reduced the carbachol Ca^{2+}-transients significantly to 73.6 ± 20.8% of control. This reduction by alloxazine was in turn attenuated by the additional presence of 1 mM adenosine (80.1 ± 15.4% of control, n=5). This paradoxical effect may be due to the fact that alloxazine has only a slightly higher specificity for the $A2_B$-subtype over the other P1-receptors, and it is also possible that the agent could be exerting a non-specific effect. A different method to investigate the action of $A2_B$-receptors was therefore required to determine if the reduction of the Ca^{2+}-transient by adenosine was mediated by the $A2_B$-subtype.

1 µM DPCPX (A1-antagonist), 1 µM MRS-1191 (A3-antagonist) and 1 µM 8-(3-chlorostyryl) caffeine ($A2_A$-antagonist) in the presence of 1 mM adenosine was used to

isolate the $A2_B$-receptor subtype and study the effect of adenosine. The mixture of these three antagonists without adenosine did not significantly change the magnitude of the carbachol Ca^{2+}-transient (103.0 ± 24.8% of control, n=5, see Table 3.11). With 1 mM adenosine added to the antagonist mixture (n=5), there was a significant reduction in the carbachol Ca^{2+}-transients (62.8 ± 14.5% of control), this was similar to the effect seen with adenosine alone (Figure 3.21).

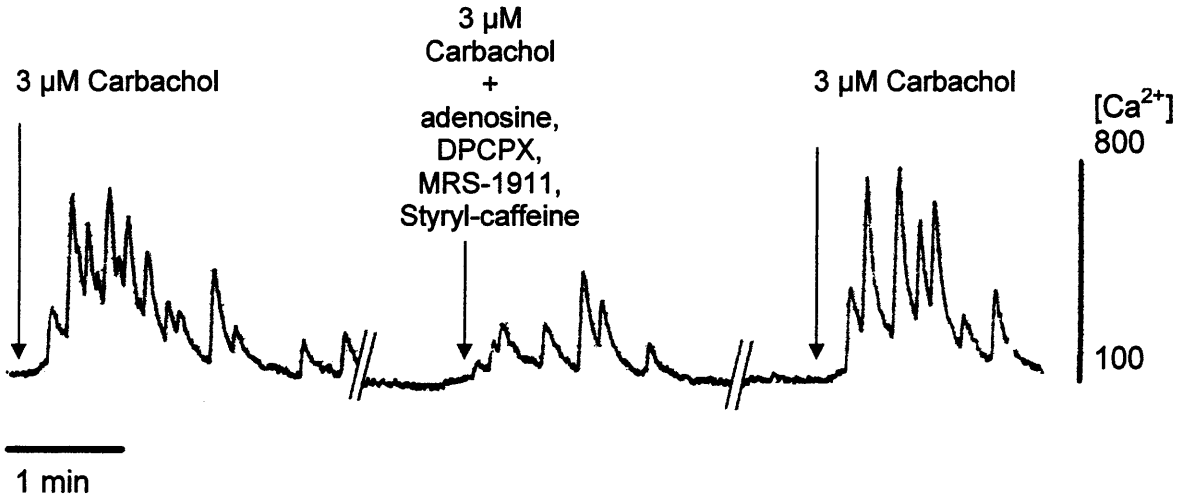


Figure 3.21 Reduction of the carbachol-mediated Ca^{2+}-transient in an isolated guinea-pig detrusor cell by 1 mM adenosine in the presence of 1 µM DPCPX, 1 µM MRS-1911 and 1 µM 8-(3-chlorostyryl)caffeine. The peak height from each intervention was measured to determine the effect of the intervention. There was at least 10 minutes wash-out between each intervention.

Thus, in the guinea-pig detrusor myocytes it may be hypothesised that the $A2_B$-subtype is involved in modulating the Ca^{2+}-transient on activation of muscarinic receptors.

Table 3.11 Effect of P1-specific agents on carbachol-induced Ca^{2+}-transients in isolated guinea-pig cells

Compound	%-reduction of control (transient elicited by 3 µM Carbachol)
1 mM adenosine (n=15)	67.7 ± 22.8 *
10 µM NECA (n=8)	85.8 ± 10.0 *
10 µM CPA (n=3)	90.5 ± 9.3
1µM DPCPX (n=3)	111.1 ± 67.8
1 µM alloxazine (n=7)	73.6 ± 20.8 *
1 µM alloxazine and 1 mM adenosine (n=5)	80.1 ± 15.4*
10 µM CGS-21680 (n=6)	79.8 ± 25.0
1 µM DPCPX, 1 µM 8-(3-chlorostyryl) caffeine, 1 µM MRS-1911 (n=5)	103.0 ± 24.8
1 mM adenosine and 1 µM DPCPX, 1 µM 8-(3-cholorstyryl) caffeine, 1 µM MRS-1911 (n=5)	62.8 ± 14.5 *

*$p<0.05$ compared to control, paired Student's *t*-test

3.5.4 Changes in response to carbachol by P1-receptor specific compounds in isolated human detrusor cells

Isolated human detrusor myocytes were stimulated with 1 μM carbachol in the presence of P1-specific agents to determine the subtypes involved in modulation of carbachol-induced Ca^{2+}-transients. Due to the smaller 'n'-numbers of human cells the results from stable and overactive bladder myocytes were combined. The table of results for the P1-specific agents against human detrusor myocytes is given in Table 3.12 (p167).

The presence of 1 mM adenosine (n=22) caused a significant reduction of the carbachol-induced Ca^{2+}-transients of human detrusor myocytes to 77.8 ± 37.2% of control. Addition of 10 μM NECA (n=15) also reduced Ca^{2+}-transients significantly to 78.6 ± 32.7% of control. Similar to the results seen with guinea-pig myocytes, 1 μM alloxazine also caused a significant reduction of the Ca^{2+}-transients in human detrusor cells (79.0 ± 25.6% of control, n=13).

The addition of 10 μM CPA (n=10) did not significantly alter the carbachol Ca^{2+}-transients (88.5 ± 39.0% of control) as did 1 μM DPCPX (80.5 ± 23.1% of control, n=10). This confirmed that there is no significant A1-receptor activity on the detrusor smooth muscle cell in human bladders.

In the presence of 10 μM CGS-21680, an $A2_A$-specific agonist, (n=5) there was no change to the Ca^{2+}-transients elicited by carbachol (118.1 ± 35.9% of control). The A3-

receptor agonist, 10 μM IB-MECA (n=3) did not significantly alter the carbachol Ca^{2+}-transients in human detrusor cells (89.1 ± 25.3% of control).

To examine if $A2_B$-receptor activity was also apparent in the human detrusor myocytes, therefore the subtraction method with other P1-receptor subtype antagonists, as used with the guinea-pig detrusor myocytes was also examined. 1 μM DPCPX, 1 μM 8-(3-chlorostyryl) caffeine and 1 μM MRS-1911(n=4) did not themselves significantly alter the carbachol Ca^{2+}-transients (101.1 ± 9.1% of control). When 1 mM adenosine was combined with thsee antagonists (n=4) the Ca^{2+}-transients were significantly reduced to 62.7 ± 14.5% of control.

Table 3.12 Effect of P1-specific agents on carbachol-transients in isolated human detrusor cells

Compound	%-reduction of control (transient elicited by 1μM Carbachol)
1 mM adenosine (n=22)	77.8 ± 37.2 *
10 μM NECA (n=15)	78.6 ± 32.7 *
10 μM CPA (n=10)	88.5 ± 39.0
1 μM DPCPX (n=10)	80.5 ± 23.1
10 μM CGS-21680 (n=5)	118.1 ± 35.9
1 μM alloxazine (n=13)	79.0 ± 25.6 *
1 μM alloxazine and 1 mM adenosine (n=9)	86.7 ± 30.2
1 μM DPCPX, 1 μM 8-(3-chlorostyryl) caffeine, 1 μM MRS-1911 (n=4)	101.1 ± 9.1
1 mM adenosine and 1 μM DPCPX, 1 μM 8-(3-cholorstyryl) caffeine, 1 μM MRS-1911 (n=4)	62.7 ± 14.5 *
10 μM IB-MECA (n=3)	89.1 ± 25.3

*$p<0.05$ compared to control, paired Student's *t*-test

3.5.5 Effect of P1-receptors on Ca^{2+} release from intracellular stores and membrane-mediated release in isolated guinea-pig cells

It was investigated whether adenosine receptors modulate the release of Ca^{2+} by an effect on the sarcoplasmic reticulum (SR). This was achieved with 20 mM caffeine, which acts on SR ryanodine receptors to release the stored Ca^{2+}.

The effect of various P1-receptor agonists and antagonists were investigated on the caffeine-mediated release of Ca^{2+} in guinea-pig cells (Table 3.13). The addition of 1 mM adenosine (n=6) did not significantly alter the caffeine Ca^{2+}-transient (108.7 ± 52% of control); 10 μM NECA (n=9) also had no significant effect (108.7 ± 53.6% of control). In the presence of 10 μM CPA (n=9) there was equally no significant change to the Ca^{2+}-transient magnitude (106.6 ± 39.3% of control). Neither 10 μM CGS-21680 (99.1 ± 18.4% of control, n=10) or 1 μM alloxazine (102.5 ± 24.1% of control, n=7) exerted any significant effect on the caffeine Ca^{2+}-transient. Thus there was no significant effect by any of the agents tested on caffeine-induced Ca^{2+}-transients. It may be concluded that adenosine does not exert a direct effect on release of caffeine-sensitive intracellular Ca^{2+} stores.

The effect of a depolarisation-induced Ca^{2+} rise was investigated by stimulating the cells with 80 mM KCl-Tyrode's solution. The high concentration of KCl causes a membrane depolarisation and opens the L-type Ca^{2+}-channel, leading to Ca^{2+}-influx, and possibly Ca^{2+}-induced Ca^{2+} release from the SR. It was found that 1 mM adenosine (104.4 ±

41.1% of control, n=3) and 1 μM alloxazine (97.5 ± 27.3% of control, n=3) did not alter the Ca^{2+}-transients caused by high KCl-Tyrode's.

Therefore the effect of adenosine receptor activation is probably mediated by alternative pathways to transmembrane Ca^{2+} influx or activation of a ryanodine-receptor pathway.

Table 3.13 Effect of P1-specific agents on Ca^{2+}-transients elicited by 20 mM caffeine and 80 mM KCl-Tyrode's solution in guinea-pig detrusor myoctyes

Compound	**Ca^{2+}-transients elicited by 20 mM caffeine (Percentage-reduction of control)**	**Ca^{2+}-transients elicited by 80 mM KCl-Tyrode's solution (Percentage-reduction of control)**
1 mM adenosine	108.7 ± 52 (n=6)	104.4 ± 41.1 (n=3)
10 μM NECA	108.7 ± 53.6 (n=10)	--
10 μM CPA	106.6 ± 39.3 (n=9)	--
10 μM CGS-21680	99.1 ± 18.4 (n=10)	--
1 μM alloxazine	102.5 ± 24.1 (n=7)	97.5 ± 27.3 (n=3)

3.5.6 Effect of adenylate cyclase inhibition on Ca^{2+}-transients in isolated guinea-pig cells

The next step was to investigate further how P1-receptors activation could modulate the carbachol-induced Ca^{2+}-transient. It is known that all P1-receptors are G-protein linked receptors linked to the activity of adenylate cyclase. From the above results, it may be hypothesised that there is an involvement of $A2_B$-receptors. Therefore activation of this receptor should result in an increase of cAMP levels.

To study the second messenger pathway involved with P1-receptor activation, the effect of the adenylate cyclase inhibitor, MDL-12330A was examined. MDL-12330A was shown to cause a reduction of the carbachol-mediated Ca^{2+}-transients in both human and guinea-pig cells in a dose-dependent manner. Figure 3.22 shows data from guinea-pig cells with a maximal reduction of 55.5 ± 25.3% of control at 10 μM and with a half-maximal concentration of 1.6 μM (n=7).

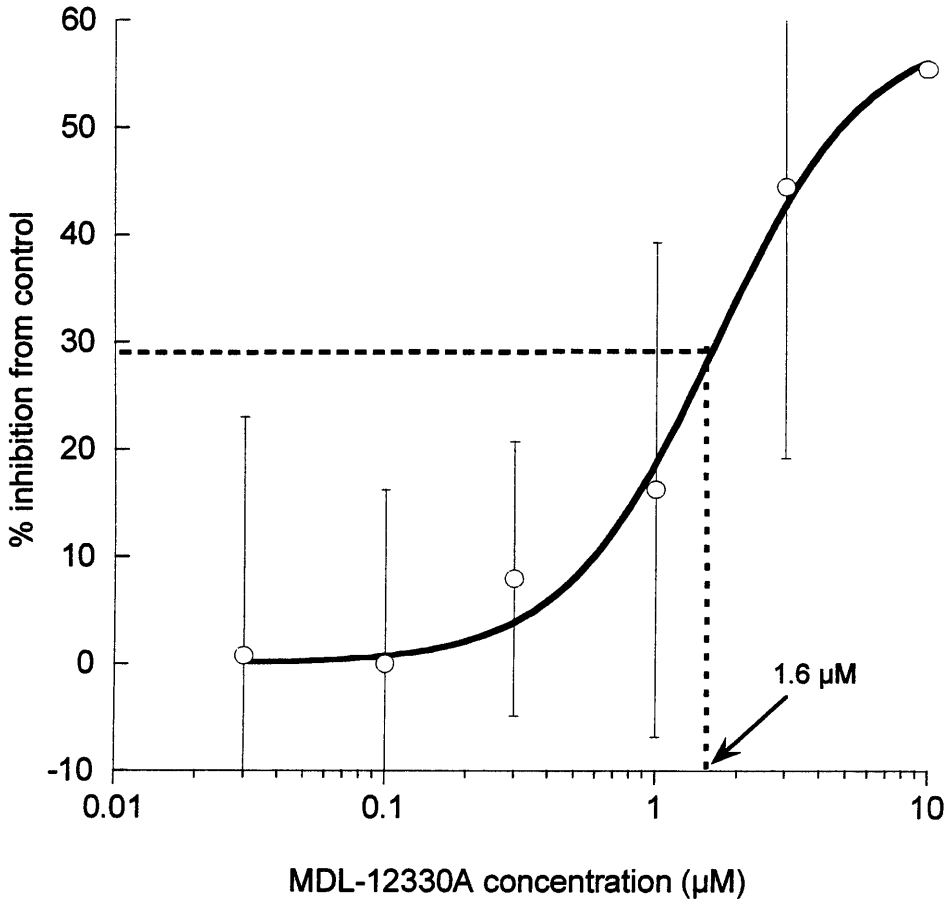


Figure 3.22 Dose-response plot of MDL-12330A inhibition on 3 μM carbachol transients in guinea-pig detrusor myocytes

1 μM MDL-12330A was chosen as the working concentration for further experiments with P1-receptor agonists, although the extent of the reduction was small at this concentration. At 1 μM MDL-12330A, the effects of the inhibition could be still observed and the viability of the cell was maintained. Concentrations of 3 μM MDL-

12330A, or greater caused the cell to stop responding to any further carbachol interventions.

1 μM MDL-12330A had no significant effect on Ca^{2+}-transients elicited by 20 mM caffeine (85.2 ± 20.4% of control, n=6) or 80 mM KCl-Tyrode's (105.9 ± 30.2% of control, n=6). Therefore MDL-12330A appears to affect only second messenger-mediated Ca^{2+}-transients. This indicates that adenylate cyclase may have a role in the contractile signalling pathway of muscarinic receptors.

3.5.7 MDL-12330A and P1-receptor agonists on Ca^{2+}-transients in isolated guinea-pig and stable human detrusor cells

Guinea-pig detrusor cells were exposed to 1 μM MDL-l2330A in the presence of a maximal concentration of a selective P1-agonist, and then a Ca^{2+}-transient was evoked with 3 μM carbachol in the presence of these compounds.

1 μM MDL-12330A with 1 mM adenosine reduced the Ca^{2+}-transients by 44.0 ± 27.4% of control (n=5). However this was not significantly different from the amount of reduction seen with adenosine alone (67.7 ± 22.8% of control, unpaired Student's *t*-test).

From previous experiments with isolated detrusor cells, it is proposed that only the $A2_B$-receptor had any significant effect on carbachol transients. Therefore this was the only

subtype examined with MDL-12330A. The cocktail of other P1-receptor antagonists was again used with adenosine to isolate any potential $A2_B$-action.

Isolated guinea-pig detrusor cells were treated with 1μM MDL-12330A in the presence of 1 mM adenosine, 1 μM DPCPX, 1 μM 8-(3-cholorstyryl) caffeine and 1 μM MRS-1911 (n=5). This allowed the effect of $A2_B$-receptor on adenylate cyclase activity to be observed without interference from the other P1-subtypes. It was found that these carbachol Ca^{2+}-transients were reduced by 71.4 ± 29.4% of control (n=4), however this was not significantly altered from the Ca^{2+}-transients in the presence of the cocktail of agonists and adenosine only (62.7 ± 14.5% of control).

The effect of adenylate cyclase inhibition was also tested in isolated stable human detrusor myocytes. Addition of 1 μM MDL-12330A (n=6) did not significantly change the carbachol-induced Ca^{2+}-transients in human detrusor myocytes (85.8 ± 30.2% of control). The effects of P1-specific agonist/antagonists and MDL-12330A were not examined with human detrusor myocytes due to time constraints.

4.0 Discussion

4.1 Experimental limitations

In this thesis two techniques were used to describe the effect of P1-receptors on the contractile function of detrusor smooth muscle; measurement of isometric tension experiments with dissected muscle strips and measurement of intracellular Ca^{2+} changes in isolated detrusor myocytes.

The tension experiments used small preparations (1mm in width and 3-5mm in length) to allow for adequate perfusion of the tissue by solutions, and the fluid exchange time through the organ bath was sufficient to prevent hypoxia. It is possible however that the contractile response observed may have been distorted by the connective tissue content in the muscle strip. In particular, the amount of connective tissue was found to be greater in human bladder samples than in guinea-pig. In some preparations from overactive human bladders it was necessary to increase the voltage output as there was poor response to electric field stimulation. This may have been due to denervation in these samples and this may have inceased the degree of direct muscle stimulation. However, it was found that 1 μM TTX abolished contractions in all samples from human bladders.

The intracellular Ca^{2+} changes were measured using Fura-2AM which is a dual-excitation dye where the intracellular Ca^{2+} concentration was determined as the ratio of emission intensities on the excitation emission at 340 and 380nm. The Ca^{2+} concentration was

determined relative to an *in vitro* calibration using the pentapotassium salt of Fura-2 so absolute values of [Ca^{2+}] could be determined. As Fura-2 is a ratiometic dye, it has the advantage of being able to correct for loss of fluorescence through photobleaching, dye leakage or compartmentalisation in cell organelles. When using 380 and 340nm excitation it is also possible that is caused autofluorescence from molecules such as NAD(P)H, which can result in oscillations of the emission signal. However in this study it as found that there was very little autofluorescence from detrusor myocytes and this was confirmed by the lack of an emission signal from unloaded detrusor cells.

The AM-ester has another advantage of being able to load in a non-invasive manner and continally accumulates inside the cell as it is converted into Fura-2 though cleavage by intracellular esterases. However, Fura-2AM can load into any membrane bound organelle, therefore Ca^{2+}-changes could be a combination of many different systems that are not activated under physiological conditions.

4.2 Contractile properties of human and guinea pig detrusor to electric-field stimulation

The nature of the nerve-mediated contractions of human and guinea-pig detrusor muscle were assessed from force-frequency relationships and the effect of P1-receptors on these contractions was then investigated.

The amount of force normalised to unit cross-sectional area generated from nerve-mediated contractions of human (stable and overactive bladders) and guinea-pig detrusor were similar at their respective half-maximum ($f_{1/2}$) stimulation frequencies. Thus, the contractility of the detrusor muscle using this method of stimulation was similar between the two species.

There was a difference in the force-frequency relationship between the two species; with human samples requiring a higher stimulation frequency than that for guinea-pigs. The $f_{1/2}$ for human bladder samples was 18.9 ± 7.5 Hz for stable bladders and 15.8 ± 5.5 Hz for overactive bladders, whilst an $f_{1/2}$ value of 9.7 ± 4.4 Hz was found with guinea-pig bladders. This could reflect a difference in the neurotransmitters released with the two species.

ATP is released at lower frequencies of stimulation compared to Ach (see Introduction – section 1.6). This was confirmed in the experiments shown in the thesis, where desensitisation of purinergic $P2X_1$ receptors with ABMA was more effective at blocking contractions at low stimulation frequencies. Guinea-pig detrusor has a greater dependence on ATP in contributing to the contraction than human tissue as evidenced by the significant size of atropine-resistant contractions. This greater release on an ATP component to nerve-mediated contractions in guinea-pig detrusor could account for the smaller $f_{1/2}$ value compared to human detrusor.

With tension experiments using detrusor samples from various species, it has been shown there are two phases to nerve-mediated contractions, (Zhao *et al.*, 1993). The initial phase

is dependent on ATP, compared to the later component which is dependent on acetylcholine. In several species including human and guinea-pig these two phases are often difficult to distinguish as the waveform is dampened by the visco-elastic properties of the extracellular matrix. This bi-component characteristic is attributed to the initial rapid opening of P2X-receptors allowing an influx of Ca^{2+}, depolarising the membrane and opening L-type Ca^{2+}-channels. The second phase is due to muscarinic receptors which rely on the inositol phosphate pathway to cause a rise in intracellular Ca^{2+} through store release (Tammela *et al.*, 1992).

There was little difference in the maximum force, or that of their $f_{1/2}$ frequencies, of nerve-mediated contractions comparing data from stable and the collated overactive human samples. This indicates that the contractile function of the smooth muscle machinery is significantly different between the normal and disease states, and this has also been shown previously (Bayliss *et al.*, 1999).

As there is little change in the contractile function of the detrusor between stable and the collated overactive human bladder sample groups, then it is likely that the cause of overactivity lies elsewhere. It has been suggested that one possible cause of overactivity is due to denervation of the bladder from pathological or neurological damage. It has been found that innervation of the detrusor becomes more scattered in bladders from those with detrusor overactivity and would most likely affect the co-ordination of the contractions and lead to impairment of bladder emptying (Drake *et al.*, 2003)

Detrusor smooth muscle from obstructed bladders has been demonstrated to have increased muscarinic sensitivity. In the pig model of outflow obstruction, there was enhanced response to agonists and a reduction in the sensitivity of intramural electric stimulation. This supersensitivity is thought to be associated with the decrease in neuronal density that occurred as a result of the obstruction (Speakman *et al.*, 1987). From other studies it has been shown that there is an increase in the muscarinic receptor density in denervated, hypertrophic bladders but receptor density decreased below control levels when the bladders underwent a urinary diversion (Nilvebrant *et al.*, 1989). The bladders which underwent the diversion showed enhanced muscarinic sensitivity, thus showing the supersensitivity is not due to a decrease of in the receptor density. It is possible that the increased sensitivity is due to alterations in the regulation of the muscarinic receptors. From the experiments in this thesis, there did not appear to be a significant difference in the carbachol dose-response EC_{50} values from stable and overactive bladders. However, there were no samples from bladders with outlet obstruction used with the carbachol dose-response. The lack of muscarinic supersensitivity has been shown in human samples from overactive bladders including samples from bladders with outlet obstruction (Bayliss *et al.*, 1999).

When the overactive group was separated according to their pathological diagnosis, it was found that idiopathic and neuropathic $f_{1/2}$ values and tension values themselves were not significantly different in samples from each other. However, the $f_{1/2}$ value for samples from the human obstructed group was found to be significantly different from the other two pathologies, and was not significantly different from the guinea-pig tissue

$f_{1/2}$ value. It is difficult to draw any definitive conclusions however as the number of obstructed human bladder samples was small (n=4). It is possible however that outlet obstruction causes significant changes to the contractile properties of the human detrusor muscle compared to the two other disease states investigated. In rabbit models of outlet obstruction, an alteration to the proportion of purinergic and cholinergic-mediated contractions compared to sham animals have been demonstrated. This animal model also showed an increase of atropine-resistant nerve-mediated contractions, indicating an increase in purinergic neurotransmission (Calvert *et al.*, 2001).

There are also alterations in the sensory mechanisms due to outlet obstruction. It has been shown that there is an increase in the expression of TRPV1 vanilloid receptors and nerve-growth factor (NGF) in obstructed rat bladders (Kim *et al.*, 2004). When there is outlet obstruction of the bladder there is a proliferation of afferent nerves in the spinal cord. NGF regulates the expression of TRPV1 receptors in sensory nerves and is thought to contribute to the irritative symptoms.

The changes to the myogenic properties of detrusor, such as due to outlet obstruction, have also been put forward as a possible cause for bladder overactivity. Isolated detrusor muscle usually shows spontaneous contractions when the muscle has been dissected from the urothelium. It has been suggested that these spontaneous contractions are due to the activity of K_{Ca} channels (Imai *et al.*, 2001). These K_{Ca} channels, on both smooth muscle and on the nerve have been suggested to support rhythmic contractile activity and a dysfunction of these receptors could lead to irregular contractions (Herrera *et al.*, 2005).

Adjacent detrusor smooth muscle cells are connected through gap junctions that will permit electrical and possibly chemical signals to travel between cells. These properties are similar to that of myocardium, with the exception that the junctional resistance is higher (Fry *et al.*, 1999). Gap junctions are composed of connexin proteins and there is regulation of contraction within muscle bundles. There is a distinct expression pattern of connexin between the different layers of the bladder. Connexin-43 and 45 appears to be localised specifically to the smooth muscle layer (Sui *et al.*, 2003;Neuhaus *et al.*, 2002). It has been found that connexin-45 is localised to the gap junctions between adjacent muscle cells. Connexin-43 is possibly localized at interstitial cells that surround muscle bundles, as the major site of connexin-43 expression in the bladder wall is in the suburothelial myofibroblast layer (Sui *et al.*, 2002).

Connexin-43 is apparently up-regulated in detrusor of patients with urge symptoms (Haferkamp *et al.*, 2004;Neuhaus *et al.*, 2005). In the urothelium, only connexin-40 was found to be expressed (Neuhaus *et al.*, 2005). As connexin expression alters in pathological states, it could be a contributing factor to the loss of co-ordination of contractions in overactive bladders.

Overall, human detrusor smooth muscle showed little change with stable and overactive samples. Human and guinea-pig detrusor differed significantly with respect to $f_{1/2}$ stimulation frequency and samples from obstructed patients displayed similar nerve-stimulation characteristics to that of guinea-pig detrusor. However this latter conclusion is speculative as there was an insufficient number of obstructed human samples.

4.3 Effect of P1-receptor agonists on nerve-mediated contractions

In guinea-pig detrusor muscle strips, adenosine caused a reduction of nerve-mediated contractions in a dose-dependent manner and 10 μM NECA induced a comparable degree of reduction to that of 1mM adenosine: each of these concentrations produced a maximum effect. This strongly implicates P1-receptors being responsible for this reduction, and agrees with results showing that adenosine receptors have a functional role in modulating detrusor contraction in other species (Nicholls *et al.*, 1992).

From the dose-response results with guinea-pig detrusor muscle strips, the non-selective A1/A2 agonist NECA had a greater potency than adenosine. This is probably because NECA is not subject to breakdown by ectonucleotidases or re-uptake by the adenosine transporter on nerves. The maximum concentration of the A1-receptor agonist CPA caused approximately half the reduction in force to that using adenosine or NECA. This suggests that the A1-receptor plays a significant part in the reduction of nerve-mediated contractions in guinea-pig detrusor smooth muscle but is not solely responsible for the measured attenuation. The remaining component to the reduction could be due to the contribution of the A2 or A3-subtypes. However, A2-specific antagonists did not have any further depressant effect on nerve-mediated contractions in the presence of NECA, and the A3-specific agonist, IB-MECA, also had no significant effect.

It is also possible that the CPA did not show a similar effect to NECA or adenosine due to a more specific agonist-induced desensitisation of A1-receptors. It has been demonstrated that when A1-receptors expressed in cultured cells, were exposed for long periods to a specific agonist or antagonist, the receptor became desensitised and the activity of the receptor was down-regulated (Ciruela *et al.*, 1997). The high affinity state of the A1-receptor has also been shown to require adenosine deaminase activity which co-localises with the A1-receptor on the cell surface after desensitisation and internalisation of the receptor (Saura *et al.*, 1998). From the experiments in this study there was no apparent desensitisation of the response to CPA with nerve-mediated and agonist induced contractions. It is possible that the low slope of the dose-response curve for CPA may point to multiple binding sites. It has been shown that A1-receptors have an allosteric binding site, which enhances the potency of A1-specific agonists but not antagonists, possibly through stabilising coupling to the G-protein (Bhattacharya & Linden, 1995). It is not known whether CPA can also affect this allosteric binding site. From binding studies it has been shown that CPA has the highest affinity for the A1-subtype, with 20-fold selectivity over the A3, 340-fold selectivity over the $A2_A$ and 8000-fold selectivity over the $A2_B$ subtype (Klotz, 2000). As there was no evidence from this study of an A3-receptor activity, it is unlikely CPA would be interacting with the other P1-subtypes.

Adenosine is well known to cause a reduction of ACh release in other smooth muscles such as ileum (Gustafsson *et al.*, 1978) and this has been shown to be mediated through a pre-synaptic A1-receptor. There is also evidence that $A2_A$-receptors have a stimulatory

action on the nerve terminal (Tomaru *et al.*, 1995). Therefore adenosine may not only inhibit, but can also facilitate the release of neurotransmitters. If this was the case, then it would have been expected that CPA would have had a greater effect than NECA. It is possible that the situation in detrusor smooth muscle is different.

Combining these conclusions it may be hypothesised that A1-receptors mediated a pre-synaptic blockade of neurotransmitter release that may account for at least part of the negative inotropic effect of adenosine.

$A2_B$-receptors may cause the remainder of the negative inotropic effect of adenosine on nerve-mediated contractions, most likely at a post-synaptic site. The results from EFS-mediated contractions did not clearly show any $A2_B$-specific activity using the $A2_B$-antagonist, alloxazine. However, alloxazine is not greatly specific to $A2_B$-receptors (approximately 9-fold specificity to $A2_B$-subtype over $A2_A$) and higher concentrations can also bind to other P1-subtypes (Brackett & Daly, 1994). Therefore a more sensitive method of measuring post-synaptic P1-receptor activity was required, which required an examination of P1-receptor activity on Ca^{2+}-transients in isolated detrusor myocytes.

4.4 Mechanisms of adenosine-mediated reduction of nerve-mediated contractions

Other signaling pathways could be involved with modulating smooth muscle contraction. Muscarinic and P2X/Y receptors have also been shown to be involved in pre-junctional modulation of neurotransmitter release. In human detrusor, the M_4-subtype has been implicated in a negative feedback of acetylcholine release from the nerve (D'Agostino *et al.*, 2000). It has also been suggested that there is facilitatory M1-receptor activity as the M1-specific agonist, McN-A 343, caused a significant increase in Ach release (Somogyi *et al.*, 1994). In the rat bladder, M2-receptor antagonists caused an increase of the force of nerve-mediated contractions, and it was hypothesized to be via a pre-junctional inhibitory effect (Braverman *et al.*, 1998a). P1-receptors and M2-receptors are also linked to the activity of adenylate cyclase; adenosine could be contributing to this regulatory mechanism in combination with muscarinic receptors by modulating cAMP-dependent pathways. In rat hemidiaphragm preparations it was demonstrated that the A1-mediated inhibition of Ach release occurs when the facilitatory M1-receptor pathway is functional. However, when the tissue was stimulated with high-frequency bursts, the activity of the $A2_A$-subtype promoted M2-mediated autoinhibition and prevented the M1-receptor facilitation of neurotransmitter release (Oliveira *et al.*, 2002).

P1-receptors are coupled to adenylate cyclase, either by inhibiting or stimulating the activity of the enzyme. The A1-subtype reduces the accumulation of cAMP, and from this study the A1-subtype was definitively characterised as being involved in pre-synaptic

modulation. There is however a multitude of signalling mechanisms besides adenylate cyclase, to which the A1-receptor has been connected. These include phospholipase C (Akbar *et al.*, 1994), K^+-channels (Kirsch *et al.*, 1990) and Ca^{2+}-channels (Wu & Saggau, 1994). A1-receptors can inhibit Ca^{2+}-currents in various neuron types and this could be the mechanism for inhibition of neurotransmitter release in the detrusor. Recently, A1-receptors have been shown to be linked to pre-synaptic L-type Ca^{2+}-channels, preventing excessive neurotransmitter release in mouse neuromuscular junctions (De *et al.*, 2004).

Alternative relaxation pathways could be through the action of nitric oxide (NO). NO activates guanylate cyclase to increase the production of cyclic GMP (cGMP), leading to dephosphorylation of the contractile proteins and subsequent relaxation of the muscle. However, studies have shown that NO and cGMP have very little effect on relaxation in detrusor smooth muscle, but is important in regulating the contractions of the bladder neck and urethra (Ehren *et al.*, 1994).

4.5 Effect of P1-receptor agonists on force-frequency curves of guinea-pig and human detrusor

Force-frequency relationships and their derivative plots were used to determine if the P1-receptor effect was dependent on the degree of contractile stimulation. Three modes of analysis were used to demonstrate any frequency-dependent phenomena: i) calculation of the $f_{1/2}$ value from force frequency curves; ii) calculation of the effect of P1-receptor agonists on force at high and low frequencies (Figure 3.5); iii) caluculation of the ratio of forces at high and low frequencies, $T_{l/h}$ (2 and 20 Hz for guinea-pig, 8 and 24 Hz for human tissue). Table 4.1 summarises the significant effects of P1-receptor agonists on $f_{1/2}$ and $T_{l/h}$ values in experiments from guinea-pig and human tissue.

Table 4.1 A tabulation of a significant increase of $f_{1/2}$ values, or reduction of $T_{l/h}$ ratios by different P1-receptor agonists. Other agonists not shown as there were no significant alterations to either variable in guinea-pig or human tissue (data from Tables 3.2-3.6)

	Guinea-pig		Human, stable bladders		Human, overactive bladders	
	$f_{1/2}$	$T_{l/h}$	$f_{1/2}$	$T_{l/h}$	$f_{1/2}$	$T_{l/h}$
Adenosine	Yes	Yes	Yes	Yes	Yes	
NECA	Yes	Yes	Yes	Yes	Yes	Yes
CPA		Yes				Yes
ZM-241385			Yes			
Alloxazine + NECA		Yes (increase)				Yes

In the presence of adenosine and NECA, $f_{1/2}$ values, from force-frequency plots were significantly increased in samples from both guinea-pig and human bladders. These shifts may be due to inhibition of neurotransmitter release or depression of contractile activity in the smooth muscle itself. Although CPA reduced the force of contraction, there was no significant change to the $f_{1/2}$ values, from any tissue source.

Consistent with the above observations, 1 mM adenosine and 10 μm NECA caused a greater reduction of force at low frequencies (1-8 Hz in guinea-pig tissue) than at higher frequencies (20 Hz) – see figure 3.5. In contrast to the lack of effect of CPA on the force-frequency curve, the A1-agonist also reduced preferentially the force of contraction at lower frequencies. The reason for this discrepancy is not obvious except that the smaller negative inotropic effect of CPA, compared to NECA and adenosine, may have made it more difficult to demonstrate a shift of the entire force-frequency curve.

To confirm further the frequency-dependent nature of the force reduction by P1-agonists, the ratio of tension at low (2 Hz, guinea-pig) and high (20 Hz) frequencies, $T_{l/h}$ (Table 4.1) was compared in the presence and absence of the agonists. A preferential reduction of force at low frequencies would reduce the ratio. In agreement with the data from figure 3.5, adenosine, NECA and CPA all reduced the ratio.

With human tissue the results with $T_{l/h}$ were more variable. NECA consistently reduced the value in guinea-pig and human tissue groups. Adenosine also reduced $T_{l/h}$, but only in tissue from guinea-pigs and stable human bladders, whilst CPA was effective in tissue

from guinea-pig and overactive human bladders. The lack of statistical reduction of $T_{l/h}$ in all human groups, with the various agonists, again may result from the relatively small amount of tension at 8 Hz in the human tissue samples, making evaluation of the ratio prone to significant error.

Other receptor-subtype modulators had less consistent or no effect. The $A2_A$ agonist ZM-241385 increased the $f_{1/2}$ with tissue from stable human bladders, but had no other frequency-dependent effects. The putative $A2_B$ antagonist, alloxazine, had no effect on its own but in the presence of NECA the reduction of $T_{l/h}$, seen with NECA alone, persisted. Interest in this agents stems from the possible involvement of $A2_B$ antagonists on the muscle cells, but there seems to be no additional effect on nerve-mediated contractions.

4.6 Frequency –dependent release of different neurotransmitters

To investigate the nature of the frequency-dependent effects, force-frequency relationships with guinea-pig preparations were generated in the presence of 10 μM ABMA or 1 μM atropine. ABMA was used to desensitise P2X receptors and this allowed any effect of P1-receptor modulators on the cholinergic pathway to be observed. Similarly, in the presence of atropine their effect on purinergic pathways could be measured.

The results showed that there was a greater reduction of force at lower stimulation frequencies in the presence of ABMA (Figure 3.6). Thus, at lower frequencies it may by proposed that a greater proportion of the contraction is mediated via the release of ATP. A greater release of ATP would mean that there would also be a greater proportion of adenosine generated at these low frequencies. By contrast, atropine generated a significant reduction of force at higher frequencies, showing that Ach release was more significant at this range (Figure 3.7).

The effect of 10 μM NECA on the contractile force in the presence and absence of ABMA was examined at 2 Hz (low) stimulation frequency (Figure 3.8). ABMA caused a significant reduction in force from the control contracture. The reduction caused by ABMA was found not to differ from the amount of force generated with NECA alone. This suggests that a majority of the neurotransmitter released at 2 Hz is ATP and that the P1-receptor has a preferential effect on this signalling pathway. However, the

combination of NECA and ABMA caused a further significant reduction when compared to the ABMA contractions, implying NECA may also has an effect on release of cholinergic component. In comparison, at 20 Hz stimulation (Figure 3.9) it was found that ABMA, NECA, and the combination of ABMA with NECA had no significant effect on the contractions compared to control. This seems to support the hypothesis of greater ATP release at lower stimulation frequencies and P1-agonists having a preferential effect on ATP release.

To observe the effect of NECA on the non-cholinergic neurotransmitters at high and low stimulation frequencies, the muscarinic receptors were blocked using 1 μM atropine. At 2 Hz stimulation (Figure 3.10), atropine did not significantly alter the tension compared to control. The addition of NECA in the presence of atropine caused a significant reduction in the tension compared to the force generated with atropine alone. Also the tension generated with NECA and atropine was not significantly different from that in the presence of NECA by itself.

At 20 Hz stimulation (Figure 3.11), atropine caused a significant reduction in force compared to control, indicating there is a greater proportion of Ach released. NECA caused a small but significant reduction in the atropine-mediated contractions, implying there is a P1-dependent effect on puringeric pathways, similar to the conclusion from the ABMA experiments. It was shown that NECA alone has very little effect at 20 Hz stimulation. It is presumed that the contractions in the presence of atropine are due to

ATP and this fits in well with the preferential effect of P1-receptors on the ATP release component.

A frequency-dependent reduction by P1-receptors was also observed with human detrusor samples, as evidenced by the reduction of the $T_{l/h}$ ratio, most consistently by NECA and less so with CPA and adenosine itself – see table 4.1. However, the effect of ABMA and atropine on the action of NECA was not investigated in the same way as in guinea-pig samples because ATP-mediated contractions were normally absent, especially in samples from stable bladders. Of importance however, is that P1-receptor activation, possibly by an A1-dependent action, did produce a low-frequency reduction of nerve-mediated contractions, despite the lack of purinergic contractions. This is consistent with the possibility that adenosine is produced in the neuromuscular junction during stimulation, possibly as a result of ATP release from the nerves themselves, and this action is more dominant at lower frequencies.

The lack of ATP-mediated atropine-resistant contractions is not due to an absence of P2X receptors on the smooth muscle membrane, as addition of ABMA to muscle strips generated a contracture. ATP release from motor nerves is also believed to occur, as attenuation of extracellular ATP breakdown by ectonucleotidases by ARL-67156 increases the magnitude of nerve-mediated contractions (C McCarthy, unpublished data). These nucleotidases are located in the synapse, either membrane-bound or in a soluble form. Moreover, these enzymes are released along with neurotransmitters during nerve stimulation (Todorov *et al.*, 1997). It is postulated that the absence of atropine-resistant,

ATP-dependent contractions in many human detrusor preparations is due to its rapid hydrolysis by ectonucleotidases (Harvey *et al.*, 2002). Reduction of enzyme activity in samples from overactive bladders would therefore limit ATP breakdown and generate residual purinergic contractions (Bayliss *et al.*, 1999). This hypothesis has also been given credence by the fact that addition of the non-specific ATPase, apyrase to human detrusor preparations from overactive bladders reduced the magnitude of nerve-mediated contractions, but is without effect in samples from stable bladders (C McCarthy, unpublished data). Overall, these data suggest that ATP is also released from human detrusor preparations, as with guinea-pig tissue, during nerve-mediated stimulation and that adenosine can influence the magnitude of these contractions. This negative feedback regulation was present in detrusor samples from both stable and overactive bladders, so that a loss of this process does not seem to be responsible for detrusor overactivity, at least in these subsets of bladders that were sampled.

Overall, it may be proposed from these data that there is a differential release of transmitter at various stimulation frequencies to motor nerves in detrusor smooth muscle preparations. At low stimulation frequencies ATP was released in a greater proportion to Ach than at higher frequencies. In addition, adenosine, NECA and CPA reduced the force of nerve-mediated contractions and the effect seemed to be greater at lower stimulation frequencies, i.e. at frequencies less than the $f_{1/2}$ value. If these effects are mediated by altering transmitter release from excitatory nerves, these results are generally consistent with the hypothesis that the action of P1 agonists by A1-receptors in preferentially to reduce ATP release.

These results are broadly consistent with data from other groups using detrusor muscle and other preparations. In guinea-pig detrusor a major proportion of the low-frequency contractions were shown to be mediated by ATP (Todorov *et al.*, 1996;Brading & Williams, 1990), and the co-release of Ach and ATP from bladder motor nerves was shown as long ago as 1975 (Silinsky, 1975). It remains unclear however if ATP and Ach are released from the same or separate vesicles (Starke *et al.*, 1996).

A frequency-dependent effect of adenosine on neurotransmitter release from other tissues has also been observed. In the rat hemidiaphragm, low frequency stimulation resulted in a predominantly A1-inhibitory action, whilst higher frequencies generated a facilitatory effect, apparently mediated by $A2_A$-receptors (Correia-de-Sa *et al.*, 1996). It is possible that in detrusor from overactive bladders there is a similar alteration in the balance of P1-receptor activity as compared to tissue from stable human bladders and guinea-pigs. By contrast, in rabbit ciliary bodies, A1-receptors have been shown to suppress noradrenaline release at stimulation frequencies greater than 20 Hz (Crosson & Gray, 1997). This frequency-dependent release of transmitters could act as a control mechanism to regulate differentially transmitter release on stimulation, effective either at lower or higher frequencies at various neuroeffector junctions. It is possible that such feedback may affect basal transmitter release from quiescent nerves, as is the case at the neuromuscular where adenosine modulates quantal release of Ach (Galkin *et al.*, 2001). A presynaptic site for a modulatory effect of adenosine and ATP on acetylcholine release has also been demonstrated by the fact that the purines have little direct effect on post-synaptic end-plate potentials (Giniatullin & Sokolova, 1998). In the rat hippocampus

adenosine inhibits the release of acetylcholine via an action on A1 receptors (Cunha *et al.*, 1998), and in the same tissue it was found that there was a frequency-dependent release of adenosine and ATP (Cunha *et al.*, 1996), with adenosine preferentially released at lower stimulation frequencies than ATP.

The discussion thus far has concentrated on the potential effects of adenosine at presynaptic sites and it is possible that it may also act on the smooth muscle itself. Adenosine reduces the force of ATP-mediated contractions in detrusor muscle (Sjogren & Andersson, 1979), suggesting a post-synaptic action. It has been shown in thesis also that adenosine may also have direct effects on detrusor muscle itself. Some previous work also suggests such an additional action. In the bladder neck P2Y receptors mediate relaxation of pre-contracted muscle (Tong *et al.*, 1997). In addition, P2Y receptors may also interact with A1 receptors to modulate intracellular Ca^{2+} (Fredholm *et al.*, 2003), and it has also been shown that P2Y receptors mediate an inhibitory effect of detrusor muscle contraction (Hourani *et al.*, 1988;McMurray *et al.*, 1998). Thus, it seems there is an antagonistic effect of adenosine and ATP in mediating intracellular Ca^{2+} during contractions, and this will be considered later here.

4.7 Carbachol-induced contractions and P1-specific compounds

Carbachol was used to induce contractions in detrusor preparations through activation of muscarinic receptors. This allowed the effect of post-synaptic P1-receptors to be studied, without interference from neurotransmitters released from electric field stimulation.

With guinea-pig tissue, adenosine and NECA reduced the carbachol-induced contraction to a similar degree using various carbachol concentrations. CPA had no significant effect on carbachol-contractures, and neither did $A2_A$ and $A2_B$-specific antagonists. The A3-agonist, IB-MECA also had no effect on carbachol contractures. The lack of effect of CPA might indicate that the reduction was not mediated by A1-receptors. However the lack of effect of the A2 and A3-receptor modulators made further conclusion difficult. Moreover, the lack of a specific high affinity $A2_B$-receptor modulator make any conclusion about the involvement or not of this subtype at least equivocal.

Adenosine was the only agent to reduce the magnitude of the carbachol contracture using human detrusor preparations. All other P1-specific compounds and the non-specific agent NECA showed no significant effect on the carbachol dose-response or the absolute magnitude of the carbachol contracture. This might also rule out an effect through P1-specific receptors but with the same caveat as that with data from guinea-pig preparations.

In the guinea-pig bladder $A2_A$-receptors are coupled to ATP-sensitive K^+-channels. It is thought that the activation of adenylate cyclase via $A2_A$-receptors increases cellular

cAMP, which in turn hyperpolarises the smooth muscle by opening K_{ATP} channels (Gopalakrishnan *et al.*, 2002). In our investigation, there was no significant effect of adenosine or P1-specific agonists on high K^+-induced contractions in guinea-pig tissue which does not support this pathway. In addition, ZM-241385, an $A2_A$-specific antagonist, had no effect on nerve-mediated or carbachol-induced contractions. This means that even if $A2_A$-receptors are linked to K_{ATP} channels and thereby modulate membrane potential, they do not have a significant effect on muscarinic-receptor induced contractions.

Overall, the reduction by adenosine of carbachol-mediated contractions was smaller than that of nerve-mediated contractions. Therefore the major action of adenosine in reducing contractile force can be considered to be via P1-receptors on the nerve-terminal. This has also been concluded in bladder tissue from fetal sheep (Thiruchelvam *et al.*, 2003) and other smooth muscle types such as ileum (Lee *et al.*, 2001). However, the observation of some depression of the carbachol-induced contraction does imply some action by adenosine on the muscle cell itself.

The small effect of adenosine on carbachol-induced contractions could be due to activation of M2-receptors as well as M3-receptors on the smooth muscle. M2-receptors are coupled to G_i and G_o proteins that inhibit adenylate cyclase activity in a Ca^{2+}-dependent manner. As A2-receptors activate adenylate cyclase, the conflicting M2-pathway may be dampening the effect of the adenosine.

Another possibility is that the relaxation is mediated through an adenosine-specific non-P1-mechanism. There have been non-P1 adenosine specific receptors described (Lorenzen *et al.*, 1993;Lorenzen *et al.*, 1992) although their physiological role has yet to be described in any great detail. Modulation of L-type Ca^{2+}-channels by P1-receptors is another possible mechanism to modulate smooth muscle contractions (Katsuragi *et al.*, 1990).

It would be of interest to determine if muscarinic receptor mediated contractions or P2X-mediated contractions are preferentially affected more by P1-receptor activity. This could be done by stimulating contractions using either acetylcholine or ABMA, these experiments remain to be done. An experimental consideration is that $P2X_1$ receptors on the muscle cell are easily desensitised making comparison of the effect of the two agonists difficult.

4.8 Atropine-resistance and effect of P1-receptors

Atropine-resistant contractions arise from non-cholinergic neurotransmitters, in the case of detrusor smooth muscle this has been identified as ATP (Burnstock *et al.*, 1978). Atropine-resistance can be observed in detrusor muscle from nearly all mammalian species, with the exception of humans and old-world monkeys where the contractions are mediated solely by acetylcholine (Kinder & Mundy, 1985;Anderson, 1993). Even with functional P2X receptors present in human detrusor, in healthy bladders there are normally no atropine-resistant contractions.

Atropine-resistance was observed in several of the human samples obtained in this study. The majority were found in the samples from overactive bladders, with the largest percentage of atropine-resistance in patients diagnosed with outlet obstruction. This is similar to previous findings where atropine-resistance was found to be greater in samples from overactive bladders and in particular those with outlet obstruction (Bayliss *et al.*, 1999). Pathological states increase the magnitude of ATP-mediated contractions, possibly due to a decrease of ectonucleotidase activity of detrusor tissue from patients with unstable bladders (Harvey *et al.*, 2002). This would mean that the ATP released from the nerve-terminal would not be degraded efficiently and increases the chance of binding to a post-synaptic P2X receptor.

An increase of atropine-resistance could also be due to an alteration in the expression of the purinergic receptors. The bladder of men with outlet obstruction has been found to express a greater amount of various subtypes of P2X receptors compared to control patients (O'Reilly *et al.*, 2001). The expressions of P2X and P2Y receptors also have been shown to change in other pathological conditions, such as in interstitial cystitis (Birder *et al.*, 2004) and patients with urge incontinence (Moore *et al.*, 2001). Atropine-resistance may not however be isolated to pathological states and appears to increase naturally as a consequence of ageing (Yoshida *et al.*, 2001).

4.9 Isolated guinea-pig and human detrusor myocytes

Isolated cells were also used to investigate post-junctional P1-receptors and their modulation by adenosine. There is little interference from other tissue types, and fluid exchange was much faster than the muscle strip set-ups to improve access by test compounds. The intracellular [Ca^{2+}] was the experimental variable. Although such changes mediate the rise of tension the two experiment systems are not entirely analogous because other cellular factors also influence tension development that are independent of changes to intracellular [Ca^{2+}]. Both human and guinea-pig tissues were used to isolate detrusor cells.

There were certain characteristics that differed between human and guinea-pig detrusor cells. The sensitivity of the cells to carbachol was different between human and guinea-pig. Human cells generally required 1 μM carbachol where as guinea-pig required 3 μM to elicit a similar sized Ca^{2+}-transient in proportion to the maximum response. This could be due to different sensitivity of the muscarinic receptors on human and guinea-pig bladder myocytes.

The values of resting Ca^{2+} were not normally distributed within the study population. With cells from guinea-pig and overactive human bladders there was a tendency to have higher resting Ca^{2+} values than stable human detrusor cells. However there was no significant difference between each of these groups.

4.10 Effect of P1-specific compounds on isolated detrusor cells

Adenosine and NECA both caused a reduction of the magnitude of carbachol-induced Ca^{2+}-transients in human and guinea-pig cells, showing that there are functional P1-receptors on the detrusor smooth muscle surface. With the use of P1-specific compounds, it is hypothesised that this reduction was mediated through $A2_B$-receptors. This is consistent with the results from the EFS experiments, where an A2-mediated effect was observed but the subtype could not be elucidated clearly.

With the use of subtype-specific compounds there was an indication that only the $A2_B$-receptor was involved in this modulation of the $[Ca^{2+}]$ rise. However, as $A2_B$-receptors have only a low affinity for adenosine, there are very few selective compounds to test this hypothesis directly. Alloxazine was the most selective antagonist available at the time of these experiments however it can also bind to $A2_A$-receptors at higher concentrations and so definite conclusions were difficult to make. The approach used in this study was to compare the effect of adenosine in the absence and presence of A1, $A2_A$, and A3 receptor antagonists. The lack of differences in the depressant effect of adenosine allowed the suggestion to be proposed that adenosine acts via $A2_B$-receptors. Both alloxazine and the antagonist mixture method gave similar results indicating that the response seen is due to $A2_B$-receptor activity.

$A2_B$-receptors have been identified as having a functional role in other smooth muscles. In guinea-pig coronary artery $A2_B$-receptors were involved in hyperpolarisation of the muscle. The effects were mediated through activation of adenylate cyclase and opening of K_{ATP} channels (Mutafova-Yambolieva & Keef, 1997). Also $A2_B$-receptors have been identified as being responsible in relaxing guinea-pig taenia coli smooth muscle (Prentice & Hourani, 1997).

How much of an effect $A2_B$-receptors exerts on the contractile function of the bladder is still unknown. As $A2_B$-receptors have a lower affinity (refer to Table 1.1) for adenosine compared to the other subtypes, they would only be fully activated in the presence of very high concentrations of adenosine. The concentration of adenosine created from the breakdown of ATP in the bladder is not fully known. The amount of adenosine formed after electrical stimulation of guinea-pig vas deferens has been measured using high-pressure liquid chromatography to be around 0.5 nmol/g of tissue (Levitt *et al.*, 1984). However, it is assumed the local concentration of adenosine would be lowered very quickly through re-uptake by the adenosine transporter on the nerve terminal. Therefore it is not possible at present to estimate the importance of the $A2_B$-receptor in regulating normal contractile function *in vivo*.

If the $A2_B$-subtype is the functional receptor in detrusor smooth muscle, this would make it a difficult to target with therapeutic agents due to its low affinity. There have been several attempts to develop molecules that can specifically target $A2_B$-receptors.

(Beukers *et al.*, 2000;Beukers *et al.*, 2004). However there has yet to be a reliable $A2_B$-selective agent for use in pharmacological studies.

Adenosine is known to be released in high concentrations from tissues under ischaemic conditions, and it is possible that under these circumstances the $A2_B$-receptor has a significant role. $A2_B$-receptors can have a role in transcriptional activation and control of apoptosis (Peyot *et al.*, 2000). Because the $A2_B$-subtype is a very low affinity receptor, it would only be activated when there is a high local concentration of adenosine, such as during ischaemic events. Large quantities of adenosine can be released from arterial smooth muscle under ischaemic conditions (Winn *et al.*, 1979). The activation of $A2_B$-receptors could then induce transcription of proteins to protect against ischaemic damage.

There are different sources of adenosine that arise either from the catabolism of ATP or directly released from the muscle in response to the contraction. In taenia coli upon transmural stimulation, a third of adenosine released was from a neuronal source, with the rest of the release presumed to be from the smooth muscle itself (Rutherford & Burnstock, 1978). It has been shown that the P1-receptors activated are affected by the source of the adenosine. For example, in rat hemidiaphragm excitatory $A2_A$-receptors on the motor nerve endings were preferentially activated by adenosine generated by catabolism of adenine nucleotides (Cunha *et al.*, 1998). It could be possible therefore that the adenosine released by the muscle is involved in modulating the post-synaptic $A2_B$-receptors.

4.11 Effect of adenylate cyclase inhibition on Ca^{2+}-release in isolated detrusor cells

MDL-12330A was used to inhibit adenylate cycalse activity to determine how this would affect carbachol-mediated Ca^{2+}-release in the detrusor smooth muscle cell. MDL-12330A caused a dose-dependent reduction of the carbachol-mediated Ca^{2+}-transient, indicating that adenylate cyclase activity was involved. However, MDL-12330A is an irreversible adenylate cyclase inhibitor at high concentrations and is not specific for the various adenylate cyclase subtypes. This would make it difficult to decipher which cAMP pathway is being affected. It was found that after washout of 1 μM MDL-l2330A, the pre and post-carbachol Ca^{2+}-transients were similar, suggesting that adenylate cyclase activity was not completely lost. This was not true for concentrations greater than 3 μM MDL-12330A, where responses to carbachol were lost after the intervention. There are reversible inhibitors available for adenylate cyclase, such as SQ-22536, and it would be interesting to use these agents to explore here fully the P1-pathways in detrusor smooth muscle.

It is possible also that the activity of adenylate cyclase is mediated through M2-receptors that inhibits the activity of adenylate cyclase. M2-receptors outnumber M3-receptors in expression by 9 to 1 in rat and 3 to 1 in human bladder smooth muscle (Chess-Williams, 2002). The sheer number of M2-receptors indicates that there must be some functional role for them. In normal bladders, Ach-induced contractions are mediated via the M3-

subtype. However in detrusor samples from rats and humans with neurogenic damage, the M2-receptor also regulated contractile activation (Pontari *et al.*, 2004).

From the previous experiments with isolated detrusor cells, it was proposed that adenosine also caused a decrease in the Ca^{2+} transients through the action of $A2_B$-receptors. These were hypothesised to affect Ca^{2+} release by stimulating the activity of adenylate cyclase and increasing the intracellular cAMP concentration. Therefore, it was postulated that if MDL-12330A was added in conjunction with adenosine, it should attenuate the effect of the $A2_B$-receptor and hence the reduction of the carbachol-induced Ca^{2+}-transient. The combination of adenosine and MDL-12330A did not alter the reduction of Ca^{2+} transients in comparison to transients in the presence of adenosine alone. This seems to indicate that the two mechanisms are separate from each other.

It is possible that the P1 and the M2-receptors mediate different subtypes of adenylate cyclase and thus there may be an interaction between the two signalling pathways. M2-receptors have been shown to be responsible for causing relaxation of ATP-mediated contractions in rat detrusor (Giglio *et al.*, 2001). The role of M2-receptors could however become more important in pathological conditions. In the denervated rat model it was found that there was an increase in the expression and activity of M2-receptors in carbachol-mediated contractions (Braverman *et al.*, 1998b).

4.12 Overall Conclusions

Adenosine receptors have a modulatory effect on human and guinea-pig detrusor contractions. A predominant amount of the effect appears to be through prejunctional A1-receptors by reducing the amount of neurotransmitter released in particular ATP. There was also an apparent A2-mediated component to the reduction seen with adenosine. The effect of adenosine itself was not qualitatively different between guinea-pig and human detrusor samples, as well as those from stable and overactive bladders but these were quantitative differences.

Through isolated cell experiments, it is proposed that there was a functional post-junctional $A2_B$-receptor that affected the intracellular rise of Ca^{2+} induced by carbachol. The carbachol Ca^{2+}-transients were significantly reduced by the adenylate cyclase inhibitor, MDL-12330A in both human and guinea-pig detrusor cells. However the reduction of the Ca^{2+}-transients by adenosine was not affected by MDL-12330A, indicating that the reduction seen by $A2_B$-receptors is not mediated by an adenylate cyclase pathway.

These results indicate that cAMP has a role in modulating the detrusor contractile process, possibly via M2-receptors that decrease adenylate cyclase activity and reduce the de-phosphorylation of the myosin light chain and that the $A2_B$-receptor The schematic of the receptors involved are given in Figure 4.1

Even with the presence of P1-receptors on the detrusor muscle itself, it seems unlikely that they play a significant role in modulating the contractile process. $A2_B$-receptors have a low affinity for adenosine and it is unlikely that under normal physiological conditions the extracellular concentration of adenosine would be high enough to activate the receptor.

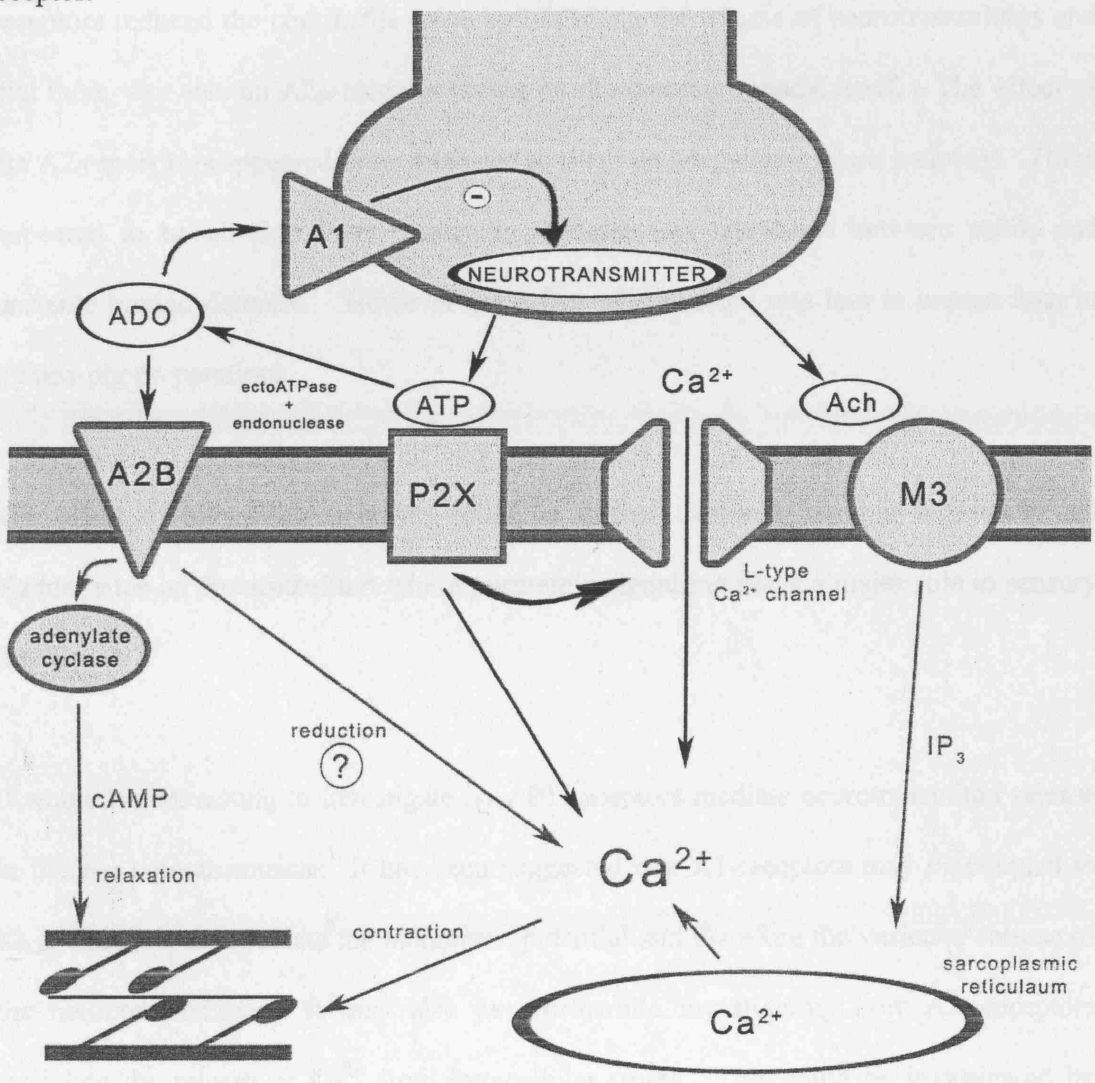


Figure 4.1 Schematic of receptors and intracellular signalling pathways involved in detrusor contraction

4.13 Further research

There are many possible sites of interaction where adenosine could be acting to modulate contractile activity in detrusor smooth muscle. From this study it was found that A1-receptors reduced the contractile force by inhibiting the release of neurotransmitters and that there was also an $A2_B$-receptor action on the detrusor muscle itself. The effect of the $A2_B$-receptors appeared to be mediated through an adenylate cyclase pathway. There appeared to be no significant change in the effect of adenosine between stable and unstable human detrusor. However the effect of adenosine was less in human than in guinea-pig preparations.

The effect of adenosine receptors could be further explored in other regions of the bladder such as the urothelium where purinergic signalling plays a major role in sensory function.

It would be interesting to investigate how P1-receptors mediate neurotransmitter release in bladder smooth muscle. It has been suggested that A1-receptors may be coupled to K_{ATP}-channels to modulate the membrane potential and therefore the vesicular release of the neurotransmitters. It may also be worthwhile investigating how $A2_B$-receptors modulate the release of Ca^{2+} from intracellular stores. This could be investigated by patch-clamp to characterise the effect of adenosine on different ion channels.

It may also be interesting to look into the effect of using adenosine transporter inhibitors to determine how much of an effect the native adenosine produced from ATP has on contractile function. These inhibitors may prove a possible pharmacological target for treatment of the overactive bladder as they should prolong the duration of action of adenosine is present in the synaptic cleft and cause further relaxation of the smooth muscle.

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