THE ROLE OF P1 RECEPTORS IN REGULATING DETRUSOR CONTRACTION: THEIR RELEVANCE IN THE OVERACTIVE BLADDER.

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Statement of originality

I, Mahreen Hussain, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Bladder motor nerves co-release acetylcholine and ATP. ATP is degraded to adenosine at the neuro-muscular junction where it is hypothesized to modulate detrusor contraction by acting on P1 receptors. This thesis aimed to investigate the function, distribution and expression of P1 receptor subtypes in modulating guinea-pig and human detrusor contractility, and whether human bladder pathologies affected any actions.

The contractile effects of adenosine and P1-subtype (A_1, A_{2A}, A_{2B}, A_3) selective agents were investigated using isolated detrusor strips, contracted by nerve-mediated field-stimulation or with agonists. P1-receptor subtype expression in human detrusor samples, from both stable and overactive bladders, was determined; mRNA expression using the polymerase chain reaction and protein expression using Western blotting.

Adenosine depressed both nerve- and agonist-mediated contractions. With human samples the effect was greater in overactive bladders compared to those from stable bladders; and more with neurogenic (NDO) compared to idiopathic (IDO) overactive bladders. ATP was preferentially released at lower stimulation frequencies, particularly from guinea-pig and overactive human samples. Adenosine depressed force more at low frequencies.

The A_1-selective agent CPA had a major depressant effect in guinea-pig and NDO samples, less so in IDO and stable human samples, but less than with adenosine itself. With NDO samples A_{2B}-receptor modulation also altered contraction; A_{2A} and A_3-receptor modulators had no significant effect. A_1-receptor expression and transcription was low in all sample groups. A_{2A}-receptor expression was low in NDO samples, but A_{2B}- and A_3-receptor expression and translation were equivalent in all groups.

The data are consistent with the hypothesis that ATP is released from motor nerves, preferentially at low stimulation frequencies and that adenosine affects selectively ATP release. I hypothesise that pre-junctional A_1-receptors are the major P1-receptor modulators, with a possible additional action of A_{2B}-receptors in NDO bladders, along with possible non-selective effects of adenosine.

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To my baby son, Sirus Zaviar Pakzad, to whom I dedicate this thesis. It was you motivated me to finish it - may be to avoid having to change your dirty nappies!

Finally to my husband, Farrokh; my best friend and partner in all life has to throw at us. You are my number one.

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List of Abbreviations

Ach	acetylcholine
ABMA	α–β-methylene-ATP
Alloxazine	benzo[g]pteridine-2,4(1H,3H)-dione)
ATP	adenosine tri-phosphate
Ca^{2+}	Free ionised calcium
CGS-21680	2-[p-(2-carbonyl-ethyl)-phenylethylamino]-5'-N-ethylcarboxamidoadenosine
CPA	N6-cyclopentyladenosine
DMSO	dimethyl sulphoxide
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonicacid]
IB-MECA	N^6-(3-iodobenzyl)-5'-(N-methylcarbamoyl)adenosine
IDO	Idiopathic detrusor overactivity
IP_3	inositol trisphosphate
NECA	N-ethylenecycloadenosine
NDO	Neurogenic detrusor overactivity
TTX	tetrodotoxin

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CHAPTER ONE

Introduction

1.1 The normal structure and function of the urinary bladder

The urinary bladder, together with the urethra, forms a functional unit to store urine and periodically expel its contents at a socially acceptable time and place in adults. To fulfil these requirements the bladder needs to be compliant (increase in volume without a great increase in intravesical pressure), and contractile to generate a sufficiently high pressure to expel urine from the bladder. Storage and voiding of the bladder are regulated by the internal and external urethral sphincters as well as lower urinary tract support.

1.1.1 Gross anatomy of the bladder

The empty adult bladder lies in the anterior half of the pelvis, bordered anteriorly by the pubic symphysis. As it fills, it rises above the symphisis and out of the pelvis, with normal capacity being 400-500 ml. Superiorly, it is covered by peritoneum which is reflected anteriorly onto the abdominal wall, and which peels upwards as the bladder distends. This phenomenon allows access to the anterior wall of the bladder through the retropubic space without entering the peritoneal cavity.

Posterior to the bladder in the female, separated by a thin layer of pelvic fascia, are the vagina and cervix. In the male, the vasa deferentia and seminal vesicles abut the bladder base. Posterior to them lies the rectum separated by Denonvillier's fascia. This is a tough fascia formed by the fusion of the peritoneal layers of the rectovesical pouch.

Inferiorly in males, the prostate is attached to the bladder base, with the urethra traversing the prostate before piercing the pelvic floor. In females the bladder rests on

the muscles of the pelvic floor (levator ani and coccygeus). The urethra pierces the pelvic floor anteriorly.

The median umbilical ligament (the obliterated urachus) extends from the dome of the bladder to the umbilicus. The left and right ureters enter the bladder postero-inferiorly approximately 5 cm apart, at an oblique angle. This oblique intramural course provides a flutter-valve mechanism which prevents reflux of urine from the bladder to the kidneys. The ureters traverse the detrusor for 2 cm before opening into the bladder base at the extremities of a transverse inter-ureteric ridge, 2.5 cm in length (the inter-ureteric bar), which forms the proximal part of the trigone. The trigone is the triangular area bounded by the ureteric orifices and the internal sphincter (bladder neck). Unlike the rest of the bladder it is relatively fixed and does not change much in size during the filling phase of micturition. The internal sphincter is not a true sphincter in so much as it is not circular. It is a "thickened area" formed by the interlacing of detrusor muscle fibres as they pass distally to become the urethra.

In the male the external sphincter sits in the membranous urethra, which extends from the apex of the prostate through levator ani to the bulb of the penis.

The blood supply to the bladder is from the anterior branch of the internal iliac artery via the superior and inferior vesical arteries. Venous drainage is to the internal iliac veins and lymphatic drainage is along the vesical blood vessels to internal iliac nodes and then onto the para-aortic nodes.

1.1.2 Histology of the bladder

Bladder

The bladder wall consists of a network of smooth muscle bundles (detrusor), interlaced randomly throughout the bladder. The lumen is lined by transitional epithelium, beneath which lies sub-epithelial connective tissue.

Internal sphincter

The internal sphincter or bladder neck differs between the sexes. In the male the detrusor assumes three distinct layers: inner longitudinal, middle circular and outer longitudinal. The innermost fibres pass through the internal meatus and become continuous with the inner longitudinal smooth muscle of the urethra. The middle layer forms the circular preprostatic sphincter which provides continence at the level of the bladder neck. The outer longitudinal fibres fuse at their lateral aspects to form a loop around the bladder neck which also participates in the provision of continence. The anterior fibromuscular stroma of the prostate merges with the bladder wall at the level of the bladder neck. The dense adrenergic innervation of the bladder neck results in its closure upon stimulation.

In the female the bladder neck comprises an inner longitudinal layer of smooth muscle which passes downwards and becomes continuous with the inner longitudinal layer of the urethra. The middle circular layer in the female is certainly not as robust as in the male. Despite this the bladder neck remains closed at rest probably due to the presence of circular elastic fibres and the valvular effect of the vascular sub-epithelial tissue, which contributes to "sealing" the urethra. Conversely, urine enters the proximal urethra during coughing, in half of all continent females (Versi *et al*, 1986).

External sphincter

Smooth muscle within the urethra is arranged in an inner thick longitudinal layer, surrounded by a thin circular layer. In the male this smooth muscle extends from the base of the bladder to the membranous urethra. Striated muscle is found external to the smooth muscle and forms the rhabdosphincter. External to this are the periurethral striated muscles of the pelvic floor. In the male the sphincter is at the level of the prostatic apex. In the female, the striated muscle extends from the proximal urethra, distally. In both the male and the female, the rhabdosphincter is horse-shoe shaped and deficient posteriorly. In the female it is separated from the vagina by a thin fascial layer, however there are additional structures which contribute to the sphincter mechanism: the compressor urethrae and urethrovaginal sphincter. The posterior wall remains rigid if there is adequate support from the pelvic floor musculature.

The peri-urethral sling derived from levator ani closes the sphincter in response to sudden sharp rises in intravesical pressure. It consists of fast twitch fibres which can constrict the urethra rapidly, for short periods. This becomes useful in maintaining continence during times of sudden raised intravesical pressure, such as coughing or straining. However it is the rhabdosphincter which prevents incontinence, under normal circumstances. These slow-twitch fibres are able to sustain prolonged tonic contraction.

1.1.3 Innervation of the lower urinary tract

The lower urinary tract is innervated by three types of peripheral nerves: parasympathetic (cholinergic), sympathetic (noradrenergic) nerves and somatic (cholinergic) nerves.

- *Parasympathetic pathways* – Arise at the level of the sacral spinal cord (segments S_2-S_4). They contract the bladder (including the trigone) and also relax the urethra. Preganglionic neurones are located in the sacral parasympathetic nucleus (in the lateral part of the sacral intermediate grey matter) from where cholinergic nerves (S_2-S_4) travel in the pelvic nerves (nervi erigentes) to the pelvic ganglia (figure 1.2). Postganglionic neurones travel from the pelvic plexus to the detrusor muscle of the bladder to initiate detrusor contraction.
- *Sympathetic pathways* – Lumbar (T_{10}-L_2) sympathetic nerves relax the body of the bladder, either by attenuating activity in the pelvic ganglia or exerting a direct effect on the detrusor muscle. The functionality of this action in the human bladder is debatable. They also excite the bladder base (including trigone) and the urethra, causing their contraction, resulting in efficient storage of urine. Fibres pass through the sympathetic chain ganglia to the inferior mesenteric ganglia and then through the hypogastric nerves to the pelvic ganglia. The male bladder neck is also innervated by noradrenergic, sympathetic nerves which cause contraction of the bladder neck during ejaculation thus preventing the retrograde movement of semen into the bladder. Innervation to the female bladder neck is primarily by means of cholinergic nerves with a little adrenergic input.
- *Somatic pathways* – The lateral border of the ventral horn (Onuf's nucleus) is the origin of the cholinergic motor neurones to the external urethral sphincter. Fibres travel via the pudendal nerves (S_2 and S_3) to contract this sphincter.
- *Afferent pathways* – Afferent axons in the pelvic and pudendal nerves transmit sensory information from the lower urinary tract to the sacral spinal

cord via the dorsal root ganglia (drg). They synapse on interneurones that either mediate connection with local motor pathways or continue their ascent in the lateral spinothalamic tracts to sensory centres in the periaqueductal grey of the pons and also to the prefrontal cortex (figure 1.1). The reflex functions are initiated in the pons and the higher brain centres are paramount in delaying voiding until socially acceptable. This is achieved by an inhibitory influence of the pontine micturition centre arising from the prefrontal cortex, anterior cingulate gyrus and hypothalamus. It is these higher central pathways that are not yet fully functional in infants (Morrison *et al*, 2005).

Pelvic nerve afferents consist of two types of fibre. The myelinated Aδ axons sense bladder fullness or wall tension, and most originate in the mucosa. During inflammation/ irritation of the bladder, these fibres increase their discharge rate at lower pressure thresholds. The unmyelinated C axons in the sub-mucosa also sense bladder volume and respond to stretch. Again they increase their rate of discharge at lower thresholds when the bladder is inflamed and normally mediate pain sensations in response to overdistension. However a proportion of C-fibres are silent afferents and may become recruited during inflammation, or after spinal cord injury to form a new functional sacral spinal reflex that can cause urgency incontinence and possibly bladder pain.

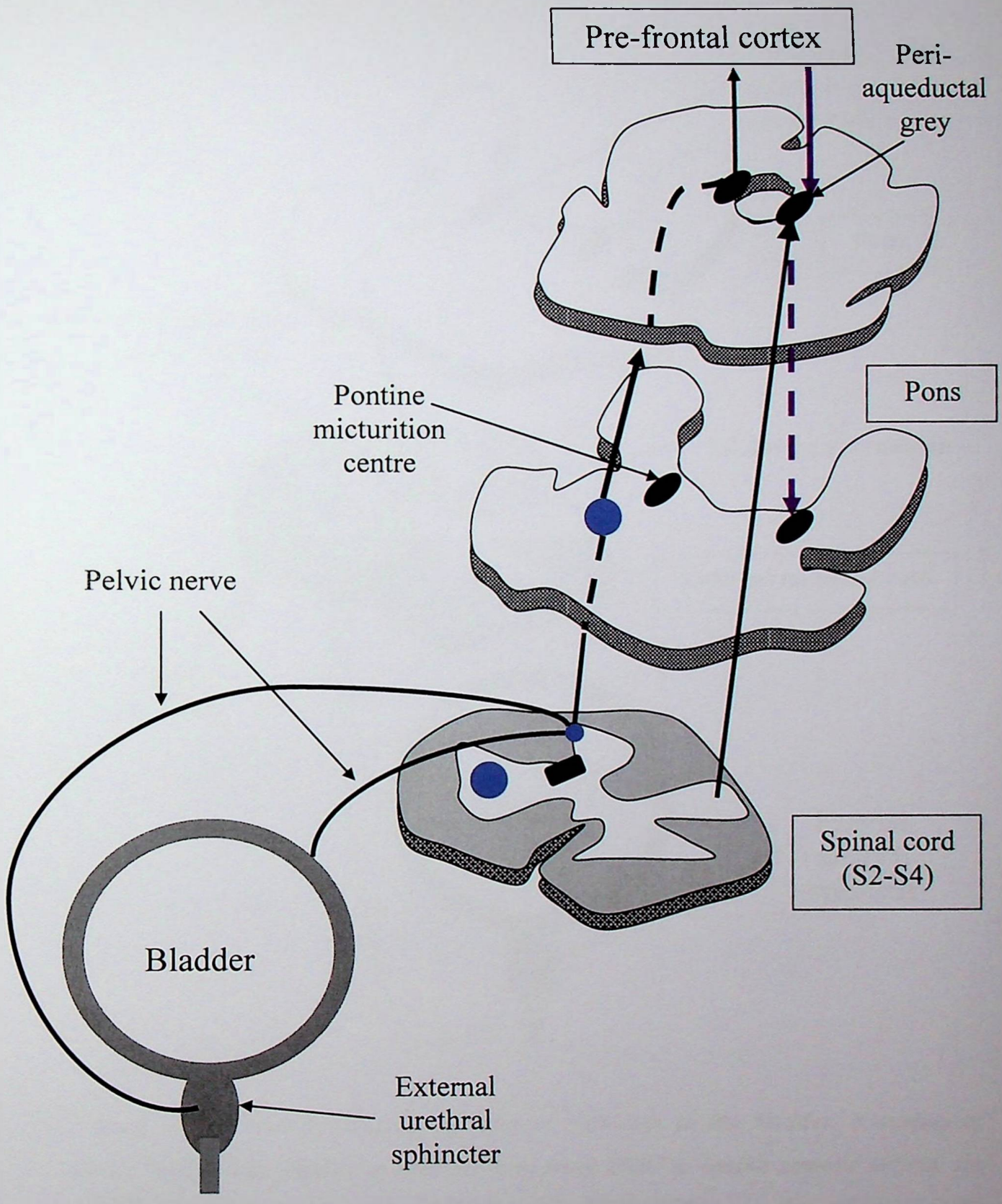


Figure 1.1: Schematic diagram of afferent pathways from the bladder. (Courtesy of Dr. C. McCarthy).

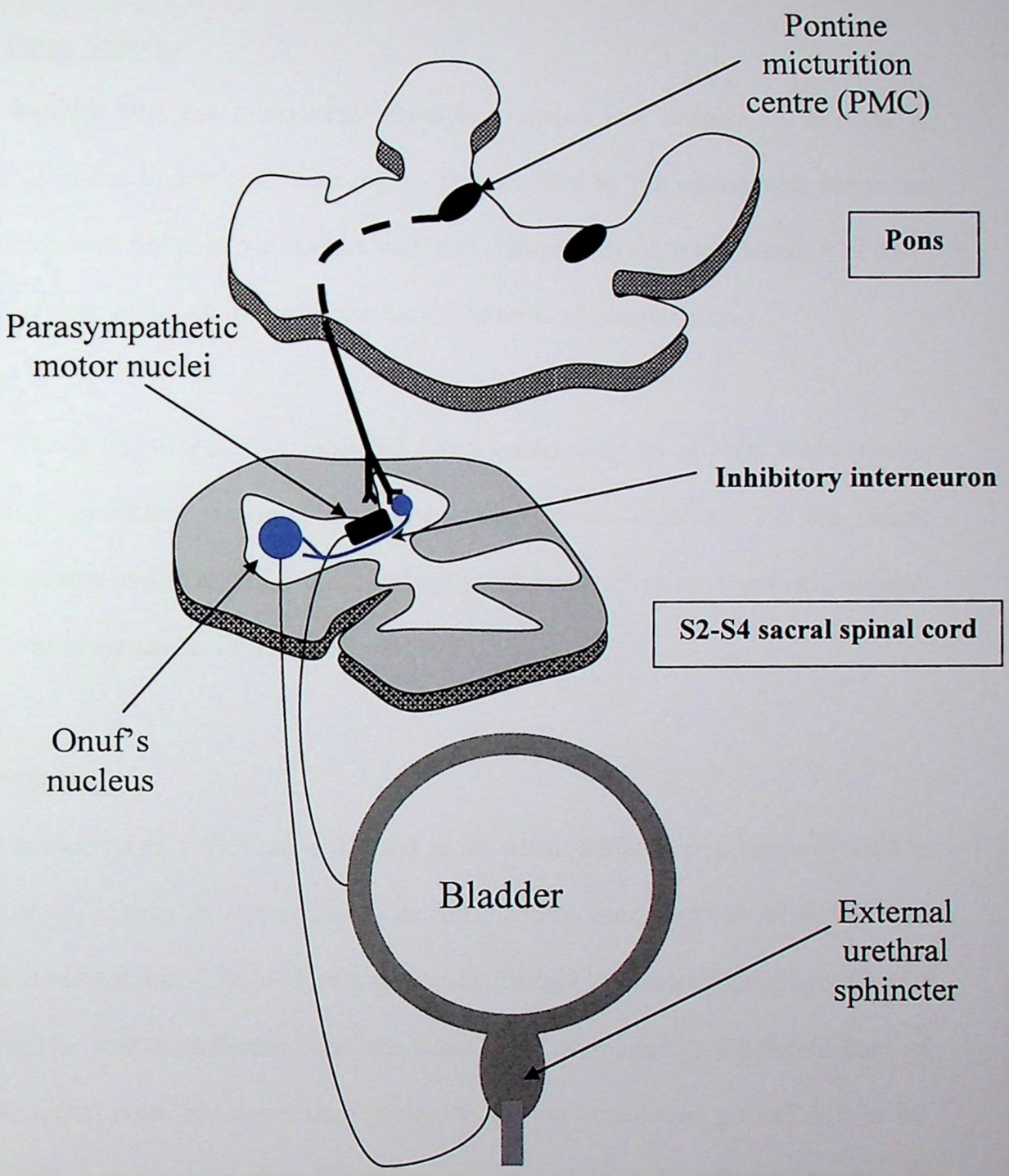


Figure 1.2: Schematic diagram of efferent pathways to the bladder. (Courtesy of Dr.C. McCarthy). Signals to void are sent from PMC to inhibit somatic activity via Onuf's nucleus to relax the urethral musculature. There is also release of the inhibition on the parasympathetic motor nuclei to initiate detrusor contraction.

1.2 The Physiology of Micturition

1.2.1 Urine Storage

As the bladder fills the intravesical pressure remains low despite an increase in volume, i.e. it is a highly compliant organ. This enabled by the viscoelastic properties of the connective tissue in the bladder wall and also possibly due to reduction of basal contractile tone, although the evidence for the latter is not convincing.

During filling the detrusor is inhibited from contracting by several mechanisms: descending inhibitory influences via a bulbo-spinal pathway to the sacral parasympathetic motor nuclei and; a "gating" mechanism on parasympathetic ganglia, possibly via sympathetic fibres (de Groat, 1993).

1.2.2 Micturition

A spino-bulbar-spinal reflex, co-ordinated in the pontine micturition centre (PMC) in the brainstem, results in simultaneous detrusor contraction, urethral relaxation and subsequent micturition. The bladder wall senses filling by monitoring changes to wall tension and/or stretch. Afferent neurones relay this information to the dorsal horn of the sacral spinal cord. Neurones then project to the periaqueductal grey (PAG) in the pons. The PAG and areas such as the limbic system and orbitofrontal cortex influence the PMC, bringing the spino-bulbar-spino reflex under volitional control and decide whether it is appropriate to initiate micturition (figure 1.2).

When socially appropriate, micturition is initiated by relaxation of the external urethral sphincter and the pelvic floor. Urine enters the posterior urethra and the bladder neck funnels. As the pelvic floor relaxes, afferent neurones are activated and

lead to stimulation of the PMC. The PMC then initiates a detrusor contraction by net activation of the cell bodies of pre-ganglionic parasympathetic neurones located in the sacral intermediolateral cell column of S_2-S_4.

Relaxation of the external sphincter slightly precedes detrusor activation as the PMC inhibits somatic motor neurones in Onuf's nucleus via GABA-ergic and glycinergic inhibitory interneurones. The presence of urine in the urethra produces reflex facilitation of the detrusor which helps to sustain its contraction until empty, to constitute a positive feedback mechanism. At the end of micturition urinary flow ceases, intravesical pressure falls and the urethral sphincter contracts. Once these events are complete the higher centres again initiate the "inhibition of the micturition reflex" so that the bladder may then safely refill.

Excitatory transmission to detrusor in the stable human bladder is exclusively via cholinergic nerve fibres. The role of alternative transmitters is considered below (section 1.5). Relaxation of the external sphincter and bladder neck is facilitated by the release of nitric oxide probably from post-ganglionic parasympathetic neurones (Fowler *et al*, 2008).

1.3 The Clinical Problem: Overactive Bladder Syndrome

1.3.1 Definition

As defined by the International Continence Society (Abrams *et al*, 2002), overactive Bladder Syndrome (OAB) is a symptom complex comprising urgency, with or without urgency incontinence, frequency and nocturia, The symptoms must be exhibited in the absence of infection or any other pathology, such as carcinoma (Wein & Rackley, 2006). Urgency is described by the ICS as the complaint of a sudden, compelling desire to void which is difficult to defer; Frequency usually accompanies urgency, with or without urgency incontinence, and is a complaint by the patient who considers that he/she voids too often by day. Nocturia usually accompanies urgency, with or without urgency incontinence, whereby the individual wakes at night one or more times to void.

Urinary incontinence is the involuntary leakage of urine and can be differentiated into certain types. Stress incontinence (SUI) is leakage on effort, exertion, coughing or sneezing. A diagnosis of SUI is made on filling cystometry when there is an involuntary leakage of urine during a rise in intraabdominal pressure (induced by coughing) in the absence of a detrusor contraction. Urge incontinence is leakage immediately preceded or accompanied by a strong urge to void. There can also be a combination of storage symptoms with urgency incontinence and stress incontinence to give mixed urinary incontinence. This contrasts to OAB dry and stress incontinence, producing mixed symptoms.

The SIFO study (a population-based survey) estimated the overall prevalence of OAB in individuals aged 40 years or over to be approximately 17% (Milsolm *et al*, 2001).

The National Overactive Bladder Evaluation Program (NOBLE) found a similar prevalence in the United States (Stewart *et al*, 2003). Furthermore as the incidence of OAB increases proportionally with age (Swithinbank *et al,* 2000), it is reasonable to consider its eeconomic implications on society. The physical constraints imposed by their symptoms have a significant negative impact on patient psychological well being, in effect reducing their quality of life (Stewart *et al.* 2003).

1.3.2 Diagnosis of detrusor overactivity

The symptoms of OAB may be caused by various forms of storage dysfunction. These include: Detrusor overactivity (DO), both idiopathic (IDO) and neurogenic (NDO) in origin, bladder outflow obstruction (BOO) and detrusor sphincter dyssynergia (DSD). Urodynamics is used to study the storage and voiding phase of micturition and thus to diagnose the pathologies causing the symptom complex of OAB. It comprises cystometry (which is the method by which the pressure-volume relationship of the bladder is measured); a pressure-flow study (which involves recording intravesical bladder pressure during voiding); frequency volume chart, symptom assessment, urine flow rate and measurement of post-void residual.

The pressure within the bladder (p_{ves}) and the pressure in the abdominal cavity (p_{abd}) are measured using pressure transducers inserted into the bladder, via the urethra, and rectum respectively. The detrusor pressure (p_{det}) is the component of intravesical pressure that is created by active and passive forces within the bladder wall. It is estimated by electronic subtraction:

$$p_{det} = p_{ves} - p_{abd}$$

As the normal or stable bladder fills, there is very little increase of intravesical pressure, and DO does not occur under any circumstances including provocation tests. Bladder compliance describes the relationship between bladder volume ('V') and p_{det} and is expressed as:

$$\textbf{Compliance} = \frac{\Delta V}{\Delta p_{det}} \ (ml/cmH_2O)$$

If the normal, stable bladder is filled during cystometry at physiological rates, there should be little change to bladder pressure during filling (Klevmark, 1974); normal bladder compliance is ~40 ml/cmH_2O. A micturition reflex is provoked when p_{det} exceeds 10-15 cmH_2O.

It must be stressed that compliance is a steady-state measurement and if the bladder is filled rapidly, p_{det} values are much greater, reaching a steady-state value eventually through a process of stress- relaxation. However, in some patients true compliance is lower so that the volume required to initiate the micturition reflex is also lower. This may be due to the large quantities of fibrous tissue within the bladder as in painful bladder syndrome or post-radiotherapy (Vale *et al*, 1994).

The overactive detrusor is one that contracts spontaneously or on provocation, during the filling phase. The ICS definition states that DO is characterized by phasic contractions in which the pressure rises and then falls, and does not specify a minimum change in p_{det}.

1.3.3 Aetiology of detrusor overactivity

Detrusor overactivity (DO) is defined as idiopathic when there is no discernible cause. There are several mechanisms which are thought to play a role in the pathophysiology of overactive bladder. These include: increased activity in the sensory afferent pathways in the bladder wall and the urothelium, which leads to symptoms of urgency. This increase in afferent activity is thought to be triggered by release of Ach and ATP from non-neuronal as well as post-ganglionic sources (Yoshida *et al*, 2006) and may also facilitate the involuntary contraction of the detrusor muscle (Mostwin *et al*, 2005). Local tachykinin release from sensory nerves in the bladder wall cause detrusor contraction and facilitate neural transmission (Maggi, 1995). Brading (1997) proposed that partial dennervation of the detrusor may alter the properties of the smooth muscle, resulting in increased excitability and coupling between the cells. This fits with "Cannon's Law of Denervation Supersensitivity", which describes the range of effects of the complete loss of nerve input to various organs under experimental conditions. As a result the muscles that are supplied by these nerves shorten and tighten due to the supersensitivity of both the muscle's specialized stretch receptors and motor nerve-muscle junctions, and in the bladder results in DO.

There is evidence that periodic ischaemia and neuronal death may predispose to DO (Greenland *et al,*2001). In vitro experiments show that the bladder wall becomes ischaemic when intravesical pressure rises above capillary pressure. Although there is an initial loss of contractility, it essentially persists. Contractility only declines as glycogen stores are depleted, with permanent damage to intrinsic nerves.

Coolasaet *et al* (1993) have described the micromotion characteristics of bladder wall strips and pressure wave phenomena in total bladders in vitro and in vivo. Local detrusor contractions can occur without an increase in tension or pressure, because

other areas are in antiphase. However, local contractions stretch surrounding tissues, which can stimulate fast stretch receptors. Synchronisation of these micromotion phenomena appears to be possible, and theoretically can cause urge, even in the absence of a pressure increase. This hypothesis could explain why the relationship between urge and pressure is a weak one.

NDO is overactivity due to a proven neurological disturbance such as multiple sclerosis, spina bifida and following spinal cord injury. Patients with supra-pontine lesions such as cerebrovascular disease and Parkinson's disease, lose voluntary inhibition of micturition leading to uninhibited overactive bladder.

1.3.4 Management of OAB

1.3.4.1 Conservative

If the symptoms of OAB are due to a pathological cause such as a UTI, bladder calculi or benign prostatic hyperplasia, then treatment of the underlying aetiology will often resolve the symptoms. A multidisciplinary approach to patient management is best, involving urologists, gynaecologists, general practitioners, continence nurse specialists, physiotherapists and the urodynamics team. The patient is also advised to modify their behaviour, usually after analysis of a frequency-volume diary that they have kept. Generally they are required to reduce the volume of their fluid intake and avoid stimulants such as alcohol and caffeine. They may be sent for bladder re-training where they are taught to delay micturition for increasing periods of time by consciously inhibiting the desire to void (Payne, 2000).

1.3.4.2 Medication

Approximately half the patients suffering with OAB will benefit from drug therapy. These include:

- *Anticholinergic drugs* e.g. oxybutynin, tolterodine, trospium, propiverine. They are thought to act by blocking the muscarinic receptor to which Ach binds and initiates detrusor contraction. Oxybutynin therapy leads to a significant increase in volume to 1st desire to void, maximum detrusor pressure & maximum cystometric capacity (Moore *et al.* 1990). Tolterodine is as effective as oxybutynin, and has the advantage of fewer side effects. It does cause dry mouth in 9% patients and is more expensive than oxybutynin (Larsson *et al,* 1999). The side-effects associated with these drugs are in main due to the low selectivity for muscarinic receptors in the bladder. The new generation antimuscarinic agents such as darafenacin and solifenacin succinate are proposed to be selective M_3 receptor antagonists and may reduce the incidence of dry mouth. In a 12-week double blind trial, compared with placebo, the mean number of urgency episodes per 24 hours was statistically significantly lower with solifenacin but not with tolterodine (Chapple, 2004).
- *Tricyclic antidepressants* e.g. Imipramine. This group acts by increasing urethral contractility and suppressing involuntary bladder contractions via its anticholinergic properties. However, due to its central and sympathomimetic and alpha-adrenergic actions, its side-effects include orthostatic hypertension, dry mouth, nausea and hepatic dysfunction. It is used with caution in the elderly as it can cause cardiac events.
- *Desmopressin* (DDAVP) is a synthetic vasopressin analogue which acts as an antidiuretic and is given to reduce nocturia.

- *Baclofen* is a γ-aminobutyric (GABA) receptor agonist. It targets receptors in the CNS to inhibit micturition. It is of use in patients with DO and limb spasticity and is administerd as an intrathecal pump. Tigabine, a GABA reuptake inhibitor, blocks the micturition reflex in humans and could be used to treat refractory DO (Pehrson & Andersson, 2002).

1.3.4.3 Neuromodulation

This technique involves the electrical activation of afferent nerve fibres in order to modulate their function. The spinal segments involved in lower urinary tract dysfunction are S_{2-4}. There are various methods and sites of stimulation of these segments. The electrical stimulus is applied directly onto or as close as possible to the nerves.

- Sacral nerve root stimulation: The "Interstim" device, which inhibits the sacral afferent nerves thereby inhibiting detrusor activity at the level of the sacral spinal cord (van Kerrebroeck *et al*, 2007).
- Stimulation of dorsal penile/clitoral nerves (afferents of pudendal nerve). This results in a strong inhibition of the micturition reflex and detrusor hyperreflexia in healthy and hyperreflexic patients (Dalmose *et al*, 2003).
- Posterior tibial nerve ($L_{4-5};S_{1-3}$) stimulation as it shares common nerve roots with those innervating the bladder. This may be done trans- or percutaneously at the level of the medial malleolus (Andrews *et al*, 2003).

1.3.4.4 Intravesical botulinum toxin

Botulinum toxin A has been postulated to act primarily by inhibiting the calcium mediated release of Ach at the neuromuscular junction by preventing exocytosis of

the synaptic vesicles (Haferkamp *et al*, 2004). It also inhibits release of ATP and substance P and reduces the axonal expression of capsaicin and purinergic receptors. This may be followed by central desensitization through a decrease in central uptake of substance P and neurotrophic factors. The end result is a profound and lasting inhibition of the afferent and efferent nerves involved in the development of detrusor overactivity (Apostolidis *et al,* 2006).

Injection of botulinum toxin A into multiple sites within the detrusor (excluding the trigone) is done via the cystoscope, under local or general anaesthetic. Side-effects such as mild flu-like symptoms and generalized myalgia can occur, although allergic reactions are rare. It is indicated for the treatment of OAB caused by NDO, but is increasingly being used to treat those with idiopathic DO who have failed medical therapy (Kuo, 2004). Injections need to be repeated every 6-12 months and patients must be aware of the possible need for clean intermittent self catheterization post-operatively. Studies indicate that there are significant increases in maximum cystometric capacity and decreases in the mean maximum detrusor pressure after treatment (Schurch, 2005 and Sahai *et al*, 2007).

1.3.4.5 Surgery

The ultimate aim of any operative intervention is to increase functional bladder capacity and decrease maximum detrusor pressure in order to protect the upper urinary tract from damage.

- **Augmentation cystoplasty.** The bladder is bivalved at the dome down to the trigone and a detubularised segment of ileum is patched onto the

defect, increasing bladder capacity. Its prime use is for the management of severe intractable storage symptoms with demonstrable detrusor overactivity and although extremely effective in treating OAB it is not without its short and long-term complications (Greenwell, 2001).

- **Urinary diversion** is an option for those suffering with intractable urinary incontinence. Usually this takes the form of an ileal conduit which involves anastamosing the ureters into a short ileal pouch which is bought to the skin as an incontinent stoma. Alternatively a continent catheterisable stoma (Mitrofanoff) maybe formed, and although this has a higher long-term complication rate, some patients favour it over an ileal conduit due to its superior cosmetic appearance.

1.3.5 Future therapeutic directions

The criterion of a good drug is that it should be cheap, accessible, efficacious, easy to administer and have minimal side-effects ensuring good patient compliance. All the medications listed above are deficient in one or more aspects. A number of alternative approaches have been proposed.

Capsaicin is a potent neurotoxin that desensitizes the bladder's afferent C-fibres when instilled intravesically (Chancellor, 1999). However, once instilled it can cause temporary acute pain due to neuronal excitation of the afferent neurons. This may be the reason for its poor assimilation into clinical practice. Resiniferatoxin is a naturally occurring capsaicin-like agent derived from the plant *Euphorbia resinifera.* As well as being much more potent than capsaicin it does not cause as much initial excitation. It significantly increases bladder capacity in patients with NDO and is potentially a

much more attractive agent than capsaicin as any bladder discomfort that it does cause is well tolerated (Kim, 2003).

There are three subtypes of β adrenergic receptor. In the detrusor approximately 97% of the β receptor mRNA is of the β_3 subtype. These receptors cause detrusor relaxation, and β_3 agonists are currently under evaluation as a therapeutic option for OAB (Yamaguchi & Chapple, 2007).

Experimental studies in animals suggest that tachykinins such as Substance P, neurokinin A and B (NKA, NKB) may play a role in the micturition reflex (Sellers *et al,* 2006), by activation of specific NK-1 and NK-2 receptors. The NK-1 receptor antagonist, aprepitant was tested versus placebo in a double blind randomised controlled trial (Green *et al,* 2006). In 8 weeks, aprepitant significantly reduced the episodes of urgency and daily micturition events, however powered follow-up studies are necessary to confirm efficacy.

Previous studies have shown that there is an increase in atropine-resistant contractions in patients with DO, and that these contractions may be purinergic in origin (Bayliss *et al*, 1999). This finding could provide a potential drug target and warrants exploration. Further evaluation of this mechanism constitutes the basis of this thesis.

1.4 Detrusor smooth muscle contraction

Smooth muscle is so named because of the absence of striations. It demonstrates spontaneous electrical and mechanical activity which may be phasic (as in the rhythmic activity of the gastrointestinal tract) or tonic (sustained contraction of

vasculature) (Arner *et al*, 2003). This activity may be modified by circulating hormones or autonomic nerves. Detrusor smooth muscle is phasic as it can develop twitch-type contractions in response to stimulation by excitatory nerves and release of excitatory neurotransmitters.

1.4.1 Contractile Proteins

There are three types of filament present in smooth muscle cells, thick, intermediate and thin filaments. The thick filaments consist of myosin type-II molecules. Each is composed of two α-helical heavy chains which make up a myosin head domain, and two regulatory light chain subunits. The former can attach to sites on the actin filament (forming the cross-bridge) and has ATPase activity (Zimmerman *et al*, 1996; Arner *et al*, 2003), providing energy for the two filaments to slide past each other (Craig & Megerman, 1977). The intermediate filaments are composed of desmin and vimentin which form the cytoskeleton of the cell and are not directly involved in the contractile process (Andersson & Arner, 2004). The thin filaments consist of actin monomers (of which there are four different isoforms), and tropomyosin which may aid binding of myosin heads to actin.

1.4.2 Contractile Mechanism

Smooth muscle contraction relies on the interaction between actin and myosin. The actin filaments attach to anchoring points on the cell membrane (dense plates) and within the cell (dense bodies) and are interconnected by myosin filaments. Cross-bridge formation pulls the actin filaments together, by sliding over myosin II filaments, and the cell shortens. This process is ATP driven.

1.4.3 Regulation of contraction by Ca^{2+}

Contraction is initiated by a rise in intracellular [Ca^{2+}], either due to Ca^{2+} entry across the plasma membrane through voltage-dependent Ca^{2+} channels following cell depolarization, from release from intracellular stores, or both. Unlike striated muscle, membrane depolarization does not directly stimulate the release of Ca^{2+} from the sarcoplasmic reticulum (SR). Stored Ca^{2+} maybe released: in response to generation of second messengers such as inositol triphosphate (IP_3) after G-protein mediated receptor activation (see section 1.7); or by Ca^{2+}-induced Ca^{2+} release due to influxing Ca^{2+} acting on ryanodine receptors.

1.4.4 Contractile activation

Ca^{2+} binds to a regulatory protein, calmodulin. The resulting [$Ca^{2+}{}_4$-calmodulin] complex undergoes a conformational change, to expose hydrophobic sites that activate the enzyme myosin light chain kinase (MLCK; Allen & Walsh, 1994). The activated MLCK phosphorylates serine-19 on the light chain of the myosin head (Maita *et al,* 1981) which increases actin-activated myosin ATPase activity and the cross-bridge cycling commences producing detrusor contraction.

1.4.5 Smooth muscle relaxation

Contraction is terminated by a reduction of the [Ca^{2+}], through reuptake into the sarcoplasmic reticulum, or removal from the cell. The [$Ca^{2+}{}_4$-calmodulin] complex dissociates and MLCK is deactivated.

Contraction can also be determined by Ca^{2+}-independent pathways mediated by myosin dephosphorylation via myosin light-chain phosphatase (MCLP), thus

preventing cross-bridge formation. MLCP activity itself may be regulated by its own phosphorylation, whereby phosphorylation reduces activity (Hartshorne *et al*, 2004). In this context MLCP activity is regulated by intracellular rho-associated kinase (Somlyo & Somlyo, 2000) or activation of a smooth muscle specific protein inhibitor CPI-17 (Murthy *et al*, 2003). Thus, rho-kinase inhibition will reduce contraction and the development of such agents has been invoked as an alternative pathway to reduce overactive contraction.

Other signalling pathways exist that decrease Ca^{2+} sensitisation and contractile activity. Protein kinase A (PKA), a cAMP-dependent kinase, can phosphorylate MLCK to decrease its affinity for the Ca^{2+}/calmodulin complex. This leads to fewer phosphorylated myosin light chain heads, and a decrease in cross-bridge formation, hence reducing contraction (Andersson & Arner, 2004). Another pathway which leads to smooth muscle relaxation is activation of protein kinase G by increased levels of cGMP.

1.4.6 Ca^{2+} removal from the sarcoplasm

To maintain a relaxed state, smooth muscle intracellular Ca^{2+} must be minimised. This is achieved by uptake of Ca^{2+} into intracellular organelles, binding of Ca^{2+} to intracellular sites and by removal of Ca^{2+} across the cell membrane, as indicated above. The latter occurs against membrane potential and concentration gradients and is energy dependent. Ca^{2+} is extruded from the cell by an ATP-dependent Ca^{2+}/Mg^{2+} pump and a Na^{+}-Ca^{2+} exchange mechanism (Wu & Fry, 2001). Uptake of Ca^{2+} into the sarcoplasmic reticulum is via Ca^{2+}/Mg^{2+}-ATPase driven pumps which transport

two Ca^{2+} for every ATP molecule hydrolysed (Haynes, 1983) and differ from those on the plasma membrane.

1.5 The role of cholinergic and purinergic neurotransmission in detrusor smooth muscle

Excitation-contraction coupling is the process by which an extracellular event, such as neurotransmitter release, mediates muscle contraction. In the bladder, the prime excitatory input arrives from the sacral parasympathetic outflow (de Groat, 1997). There are cholinergic preganglionic neurones located in the intermediolateral region of the sacral spinal cord, which send axons via the pelvic nerves to ganglionic cells in the pelvic plexus and bladder wall. These cells excite the detrusor smooth muscle by releasing cholinergic (acetylcholine) (Steers, 1997) and non-adrenergic, non-cholinergic (NANC) neurotransmitters from embedded motor nerves. Sympathetic nerves in the bladder release noradrenaline (Andersson, 1993) stimulating adrenergic receptors.

There is no NANC component of detrusor contraction in healthy human or old-world monkey detrusor but evidence for its presence in pathological bladders e.g. interstitial cystitis (Palea *et al,* 1993) arises from the application of atropine, an antimuscarinic agent (Ambache & Zar, 1970). Burnstock (1972) proposed that ATP was the neurotransmitter mediating these atropine-resistant contractions via purinergic receptors. The role of each of these components will be considered in turn.

1.6 Frequency- dependent release of co-transmitters

ATP is released in conjunction with other neurotransmitters, such as noradrenaline (NA), acetylcholine (Ach), neuropeptide Y, nitric oxide and vasoactive intestinal polypeptide (Burnstock, 1999). It is stored both separately and also packaged together with Ach and NA in synaptic vesicles (Bodin & Burnstock, 2001). ATP can be released simultaneously with Ach (Silinsky & Redman, 1996), or separately at different stimulation rates from the motor nerve depending on stimulation frequency. ATP may also be released from smooth muscle in response to autonomic activation (Vizi *et al*, 1992).

Frequency dependent co-transmitter release has been reported in guinea-pig vas deferens (Todorov *et al*, 1996), with preferential release of ATP over NA at low stimulation frequencies (up to 8 Hz). At higher frequencies, such as 16 Hz, the amount of NA released increases, suggesting that the sympathetic nerves of the guinea-pig vas deferens are able to alter the quantities of the neurotransmitter they release in response to different frequencies of stimulation (Todorov *et al*, 1999). A similar phenomenon has been described in the rat superior cervical ganglion, where ATP was co-transmitted with Ach, again with more ATP released at lower stimulation frequencies (Vizi *et al,* 1997).

Furthermore, it has been suggested that the parasympathetic neurones which supply the smooth muscle of the guinea-pig urinary bladder co-release Ach and ATP, as the excitatory responses of the guinea-pig bladder, elicited by field stimulation in the presence of atropine and guanethidine, are abolished by the addition of botulinum toxin (Mackenzie *et al,* 1982). Brading and Williams (1990) later found that in rat detrusor muscle, ATP was more readily released at lower stimulation frequencies.

These phenomena have been further clarified in the guinea-pig detrusor, where ATP-mediated contractions occur at low frequencies (1 Hz) and Ach-mediated contractions occur at 20 Hz (Hashitani *et al,* 2000). This raises the question that if smooth muscle cells are indeed able to recognize, distinguish between and respond to excitatory signals of varying quantities of neurotransmitter, could it be an imbalance in such a system which underlies a pathology such as detrusor overactivity in the human bladder?

1.7 Receptors mediating detrusor contraction

1.7.1 Muscarinic receptors

With the normal human detrusor, contractile activation is exclusively cholinergic as the application of atropine completely abolishes nerve-mediated contractions (Palfrey *et al,* 1984). This is not so in non-primates, such as guinea-pigs, in which nerve-mediated contractions which are resistant to atropine and sensitive to ABMA, can be demonstrated. The initial phase of the contraction is rapid and is thought to be mediated by ATP release, while the second phase is slower and mediated by Ach release (Brown *et al,* 1979).

Ach is released from cholinergic nerve terminals and binds to muscarinic receptors of which five subtypes have been cloned (m_1-m_5) but only four of them (M_1-M_4) have been pharmacologically defined (Eglen *et al,* 1996). M_1, M_3 and m_5 are coupled to $G_{q/11}$ protein. This activates phospholipase C, leading to IP_3 and diacylglycerol (DAG) production (Ferris & Snyder, 1992). IP_3 binds to specific receptors on the sarcoplasmic reticulum, resulting in the mobilisation of Ca^{2+} from intracellular stores, and the activation of the contractile machinery within the smooth muscle cell, as

described previously. A small Ca^{2+} influx may occur at the same time via L-type Ca^{2+} channels or non-selective cation channels (Inoue & Brading, 1990). M_2 and M_4 are coupled to $G_{i/o}$ protein which inhibits adenylate cyclase and hence cAMP accumulation and so limits muscle relaxation (section 1.4.5) (Hedge *et al,* 1997).

In the human detrusor, m_2, m_3 and m_4 receptors have been identified and there is some evidence that there may be a different pattern of receptor distribution in men and women (Mirabella *et al,* 2001). Pontari *et al,* (2004) examined detrusor from patients with NDO and stable bladders and concluded that normal detrusor contractions are mediated by the M_3 receptor and in NDO contractions can be mediated additionally by the M_2 receptor.

In the rat bladder, there is a higher density of m_2 as compared with m_3 receptors in the detrusor membrane (Wang *et al,* 1995). This is also true of the human detrusor, where the ratio of m_2:m_3 is 3:1. Although m_2 dominates quantitatively, it is not thought to be the case functionally, where M_3 play the dominant role in eliciting cholinergic contraction (Longhurst *et al*, 1995; Chess-Williams *et al*, 2001).

Ach is able to control its own release through a positive feedback mechanism, involving prejunctional facilitatory M_1 receptors in rodents (Somogyi *et al*, 1994). Prejunctional inhibitory M_2 and M_4 receptors have also been identified in the rat (D'Agostino *et al,* 1997) and the human bladder (D'Agostino *et al,* 2000).

1.7.2 Adrenergic receptors

The adrenergic receptors (or adrenoceptors) are a class of G protein-coupled receptors that have seven transmembrane domains. They are the targets of the catecholamines, adrenaline and noradrenaline, and are found mainly in the bladder neck and trigone. There are two main groups of adrenergic receptor: α-adrenergic and β-adrenergic. α-receptors bind noradrenaline and adrenaline, though noradrenaline has higher affinity.

They are further subdivided into α1 and α2. The α1 are again subdivided: $\alpha1_A$, $\alpha1_B$, $\alpha1_D$ and $\alpha1_L$ which all increase the activity of phospholipase C. $\alpha1_A$ and $\alpha1_D$ receptors are expressed in human detrusor, with a dominance of $\alpha1_D$ (Malloy *et al.*, 1998). Non-specific α1 stimulation promotes bladder neck closure (de Groat & Yoshimura, 2001). The α2 subclasses are $\alpha2_A$ and $\alpha2_B$, which decrease adenylate cyclase activity.

β -adrenergic receptors are linked to G_s proteins, which stimulate adenylate cyclase (Docherty, 1998). Agonist binding thus causes a rise in the intracellular concentration of the second messenger cAMP. Downstream effectors of cAMP include cAMP-dependent protein kinase (PKA), which mediates net smooth muscle relaxation. There are three different subtypes of β-adrenergic receptors; β1, β2, and β3. All are expressed in human and animal detrusor, with β3 dominating (Fujimura *et al*, 1999). This receptor can mediate trigonal (Yamanishi *et al*, 2003) and detrusor (Yamaguchi, 2002) smooth muscle relaxation.

1.7.3 Purinergic receptors

Purinergic receptors are divided into two major groups according to four criteria: relative potencies of agonists, competitive antagonists, changes in levels of cAMP and

induction of prostaglandin synthesis (Burnstock, 1980). According to such criteria, P1 receptors are most sensitive to adenosine, are competitively blocked by methylxanthines and their occupation leads to changes in cAMP accumulation. P2 receptors are most sensitive to ATP, are blocked (although not competitively) by quinidine, 2-substituted imidazolines, 2,2'-pyridylisatogen and apamin, and their occupation leads to production of prostaglandin. P2 receptors mediate responses of smooth muscle to ATP released from purinergic nerves, while P1 receptors mediate the presynaptic actions of adenosine on adrenergic, cholinergic and purinergic nerve terminals. P2 receptors are further subdivided into two groups. Those which are ligand-gated cation channels are termed P2X receptors and those which are G-protein coupled are P2Y receptors (Abbracchio & Burnstock, 1994).

P3 and P4 receptors have been described but their functions have not been defined. P3 receptors have an affinity for adenine nucleosides and nucleotides (Yoshioka *et al,* 2001). P4 receptors have an affinity for diadenosine polyphosphates only (Pintor *et al,* 2000).

1.7.3.1 P2X receptors

To date, seven separate genes coding for P2X subunits have been identified, and referred to as $P2X_{1-7}$. Each functional P2X receptor is a trimer, with the three protein subunits arranged around the ion-permeable pore. The subunits all share a common topology, possessing two transmembrane domains, intracellular N and C termini and an extracellular loop that contains disulphide bridges between cysteine residues

(North, 2002). It is this loop which is deemed to contain the ATP binding site. With the exception of $P2X_6$, each subunit can readily form a functional homomeric receptor. A P2X receptor made up of only $P2X_1$ subunits is termed a $P2X_1$ receptor. The general consensus is that $P2X_6$ cannot form a functional homomeric receptor when expressed alone, but nevertheless can co-assemble with other subunits to form functional heteromeric receptors. Current data suggest that, with the exception of $P2X_7$, all of the P2X subunits are capable of forming heteromeric P2X receptors with at least one other subunit type e.g. a P2X receptor made up of $P2X_2$ and $P2X_3$ subunits is known as the $P2X_{2/3}$ receptor (Torres *et al,* 1999).

In the bladder there is evidence that it is the $P2X_1$ subtype that is involved in contractile activation. Studies on $P2X_1$ knockout mice indicated a loss of the purinergic component of nerve-mediated contractions which are present in small mammals (Vial & Evans, 2000). Human and monkey bladders do not exhibit this component of contraction, unless pathological. This is thought to be because as humans evolved and became more socially aware, micturition was only performed in socially acceptable situations and environments. Consequently, the rapid detrusor contraction which is mediated by the P2X receptor causing speedy rises in intracellular Ca^{2+} was no longer required. The purinergic component however, is retained by small mammals as they need to void quickly in order to decrease their risk of attack from other animals.

Detrusor examined from patients with bladder pathologies, such as interstitial cystitis and carcinoma, indicated that a considerable amount of the nerve-mediated

contraction was P2X receptor driven (Hoyle *et al*, 1999; Luheshi & Zar, 1990), implying that P2X receptors may be involved in bladder disease.

Northern blot analysis has revealed the presence of multiple P2X receptor isoforms in the rat bladder (Bo *et al,* 1995), but functional evidence supports only the presence of the $P2X_1$ subtype on the smooth muscle membrane. Furthermore, the absence of a residual P2X receptor-mediated response in $P2X_1$ knockout mice suggests that the $P2X_2$ or $P2X_4$ receptor subunits are not expressed functionally at the smooth muscle membrane. This corroborates the hypothesis that, as in vas deferens smooth muscle (Valera *et al,* 1994), the bladder smooth muscle P2X receptor phenotype is composed of the homomeric $P2X_1$ receptor.

Studies on $P2X_3$ knockout mice indicate that ATP released from the urothelium on bladder distension acts on $P2X_3$ receptors on subepithelial sensory nerves to initiate bladder voiding reflexes (Cockayne *et al,* 2000).

1.7.3.2 P2Y receptors

P2Y receptors are a family of G protein-coupled receptors stimulated by endogenous nucleotides such as ATP, ADP, UTP, UDP and UDP-glucose. They have seven transmembrane domains and to date 12 P2Y receptors have been cloned in humans: $P2RY_1$, $P2RY_2$, $P2RY_4$, $P2RY_5$, $P2RY_6$, $P2RY_8$, $P2RY_9$, $P2RY_{10}$, $P2RY_{11}$, $P2RY_{12}$, $P2RY_{13}$ and $P2RY_{14}$ (Abbracchio *et al,* 2006). P2Y receptors are widely distributed throughout the body (Ralevic & Burnstock, 1998) and the biological effects of P2Y receptor activation depends on how they couple to downstream signalling pathways,

either via G_i, G_q or G_s-proteins. Human P2Y receptors and their G-protein couplings are listed in table 1.1.

Table 1.1: P2Y receptors, their G-protein coupling and specific ligands.

Type	Coupling	Nucleotide
$P2RY_1$	$G_{q/11}$	ADP
$P2RY_2$	$G_{q/11}$	ATP, UDP
$P2RY_4$	G_i and $G_{q/11}$	UTP
$P2RY_5$	-	Orphan receptor
$P2RY_6$	$G_{q/11}$	UDP
$P2RY_8$	-	Orphan receptor ? ADP
$P2RY_9$ or GPR23	-	Lysophosphatidic acid (LPA)
$P2RY_{10}$	-	Orphan receptor
$P2RY_{11}$	G_s and $G_{q/11}$	ATP
$P2RY_{12}$	G_i	ADP
$P2RY_{13}$	G_i	ADP

Final details of the P2y receptors remain to be evaluated: human tissues have also yielded a clone with a preliminary identification of $p2y_5$. It is labelled an orphan receptor as its endogenous ligand is yet to be identified, although there has been debate if it is ATP (King & Townsend-Nicholson, 2000). A $p2y_8$ receptor cloned from *Xenopus laevis* shows the rank order of potency: ADPβS>ATP=UTP=GTP=CTP=TTP=ITP>ATPγS (Bogdanov *et al*, 1997). The

$p2y_9$ clone has been described as an LPA receptor (Noguchi *et al.*, 2003). P2Y-like receptors have also been identified on mitochondria (Belous *et al*, 2004).

In mouse detrusor smooth muscle, G-protein coupled P2Y receptors activate a relaxatory pathway (Boland *et al*, 1993), although the subtype is not specified. This has been further investigated in the marmoset bladder, as the relaxatory pathway is thought to be mediated by a cAMP-dependent PKA pathway (McMurray *et al*, 1998). This pathway may contribute to the modulation of contraction via ATP activation. P2Y receptors are yet to be cloned from human detrusor.

It is likely that some of these P2Y receptors will have clinical applications. $P2Y_2$ is a potential drug target for treating cystic fibrosis. $P2Y_{11}$ is a regulator of immune responses, and a common polymorphism carried by almost 20% of North European Caucasians give increased risk of myocardial infarction, making $P2Y_{11}$ a potential drug target for treatment of this condition (Amisten *et al,* 2007). $P2Y_{12}$ is the target of the anti-platelet drug clopidogrel. The role of P2Y receptors in the bladder is unclear, although in detrusor from patients with interstitial cystitis (IC), the levels of $P2X_2$ and $P2X_3$ were increased as compared to healthy control (Tempest *et al*, 2004). In cat detrusor, there was a significant reduction of $P2X_1$ and $P2Y_2$ receptors in cats with IC compared to normal (Birder *et al*, 2004).

1.8 Breakdown of Neurotransmitters

Upon nerve stimulation ATP and Ach are co-released into the synapse. They are rapidly degraded by extracellular enzymes: Ach by acetylcholinesterases and ATP by ectonucleotidases. The breakdown products are taken back into the neuron for re-synthesis but before this, the breakdown products may exert pre- or post-synaptic actions to modulate detrusor contraction.

1.9 Adenosine

The ubiquitous nucleoside adenosine is a product of ATP breakdown. It activates cell surface receptors, causing a variety of physiological effects in multiple organ systems. Adenosine, as well as ATP, controls Ach release through activation of inhibitory A_1 and P2Y receptors located on cholinergic nerve terminals. Adenosine can be further broken down to inosine or taken back into the nerve terminal by a high affinity transporter for neurotransmitter re- synthesis. The pathway of adenosine metabolism is schematically represented in figure 1.3.

Adenosine is only one example of agents that can modulate detrusor contraction. Vasoactive intestinal protein is another such effector, which reduces spontaneous contractions in human detrusor (Kinder *et al*, 1985) and both nerve-mediated and carbachol-induced contractions in human and pig detrusor muscle (Klarskov *et al*, 1984).

1.10 Ectonucleotidases

ATP is sequentially dephosphorylated to adenosine 5'-diphosphate (ADP), adenosine monophosphate (AMP), adenosine and eventually to inosine. The hydrolyses to

adenosine are catalysed by ectonucleotidase enzymes which are bound to the plasma membrane. There are two main types of ectonucleotidase: the ectonucleoside triphosphate diphosphohydrolases (E-NTPDase) and the ectonucleotide pyrophosphate/ phosphodiesterases (E-NPP). Their activity is dependent on the presence of Ca^{2+} and Mg^{2+} with the optimum pH being 7.5 (Kennedy *et al,* 1996). The ectonucleotidases are found both on plasma membranes and in soluble form in body fluids. The latter may be released from vesicles in the synaptic nerves or arise as a result of proteolytic cleavage of membrane-bound enzymes (Zimmerman *et al,* 2000). The E-NTPDase family consists of eight subtypes, four of which are located on the cell surface. They hydrolyse nucleotide 5'-triphosphates and nucleotide 5'-diphosphates. The E-NPP family have both phosphodiesterase and nucleotide pyrophosphatase activity.

In the stable human bladder, no ATP-mediated contractions are evident. However, in the overactive bladder, ectonucleotidase activity decreases and has been implicated in the appearance of atropine-resistant contractions (Harvey *et al*, 2002). Application of the ecto-ATPase inhibitor, ARL-67156, enhanced the nerve-mediated contraction of guinea-pig detrusor strips at the detrusor neuromuscular junction (Westfall *et al*, 1997). The variable role of ATP in contractile activation of detrusor smooth muscle from animal and human bladders maybe due to the differential breakdown of ATP by ectonucleotidases or differential release of extracellular ATP.

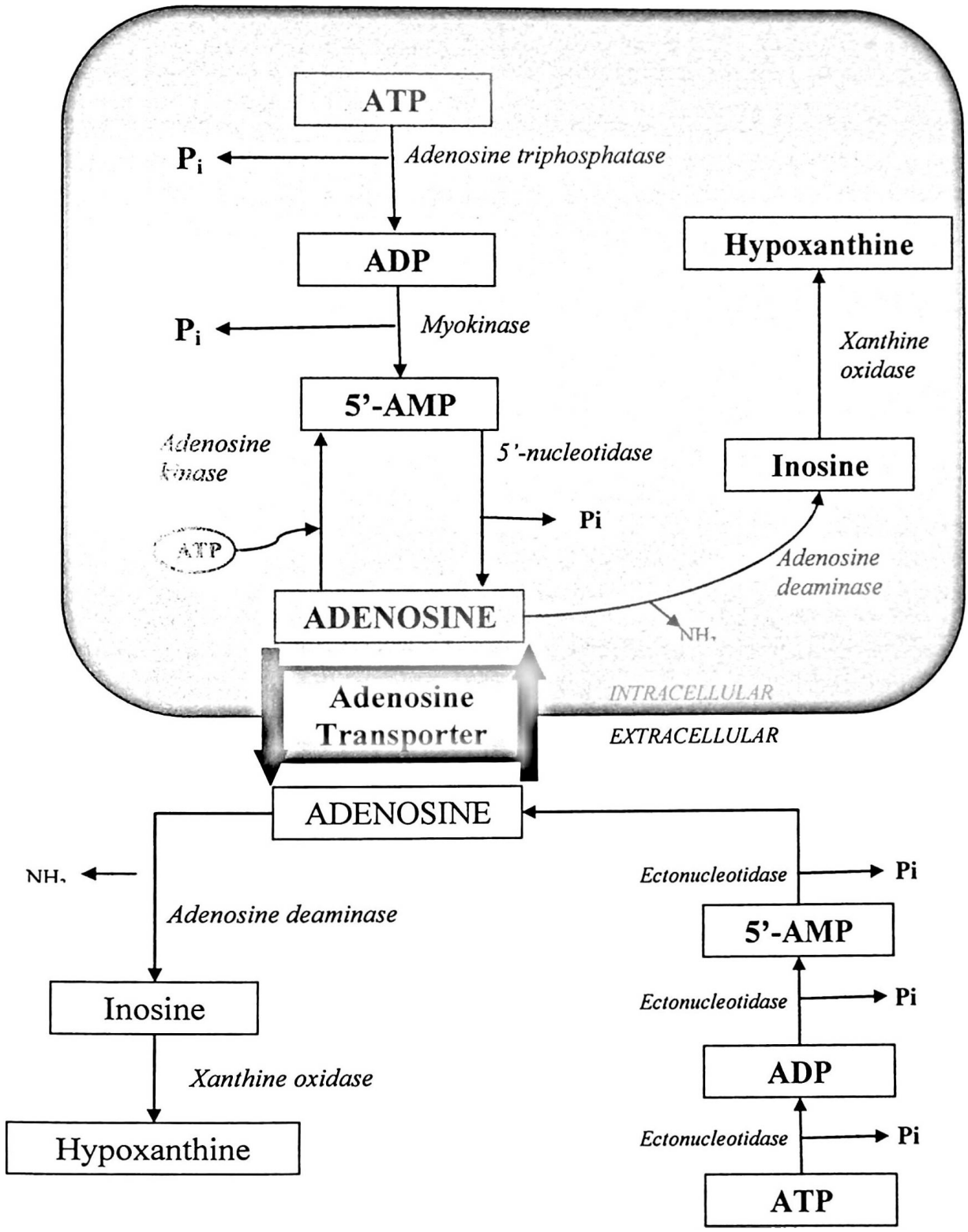


Figure 1.3: Schematic diagram of adenosine metabolism.

1.11 P1 receptors: History, Molecular Biology, Structure and Function

ATP is thought to act directly on P2 receptors and indirectly via its breakdown to adenosine by ectoenzymes on P1 receptors. Four subtypes of P1 receptor have been cloned and classified: A_1, A_{2A}, A_{2B} and A_3 (Olah & Stiles, 1995). A_{2A} and A_{2B} receptors interact with G_s to stimulate adenylate cyclase activity and elevate cAMP levels. A_1 and A_3 receptors however, couple with G_i /G_o, to reduce intracellular cAMP.

Initially the presence of A_1 and the A_2 receptors in particular tissues was identified based on the affinity of the receptor for various adenosine analogues in binding assays. Further definition of this analogue binding affinity led to the identification of two distinct A_2 receptors: A_{2A} (high affinity) and A_{2B} (low affinity), which differ in their distribution (Bruns *et al,* 1986).

All the receptors exhibit seven α-helical transmembrane spanning domains, characteristic of almost all G-protein coupled receptors. Each domain is 20-27 amino acids in length, the carboxy tail being intracellular and the amino terminal faces extracellularly. In total there are three extracellular loops and three intracellular loops (Ji *et al,* 1998). There are consensus sites for N-linked glycosylation which are located on the extracellular regions of the receptor which may modify receptor function.

Human A_1, A_{2B} and A_3 receptors have a similar number of amino acids comprising their primary structures; 326, 328 and 318 respectively. However, the A_{2A} receptor differs in that it has a long carboxy tail, making it 409 amino acids in length. Despite

the differences in length, the human A_{2A} and A_{2B} receptors display 45% sequence homology and the human A_1 and A_3 receptors display 49% (Olah & Stiles, 2000).

1.11.1 A_1 receptors

A_1 receptor expression is most abundant in the human brain, spinal cord, eye, adrenal gland and the atrium of the heart, where its location is predominantly pre-synaptic. There is evidence that A_1 receptor activation inhibits Ach release from cholinergic nerve terminals in the striatum and hippocampus, as well as guinea-pig ileum (Tomaru *et al*, 1995). It can also inhibit the release of the neurotransmitters dopamine, noradrenaline, GABA and 5-HT. It has also been demonstrated post-junctionally on smooth muscle preparations from the rat colon, guinea-pig aorta and trachea.

The A_1 receptor is coupled to a variety of signalling pathways which include activation of phospholipase C (Shim *et al*, 2002), inhibition of phospholipase A_2 and adenylate cyclase, activation of K^+ channels leading to hyperpolarisation and shortening of the action potential in the atria (Liang *et al*, 1996) and inhibition of Ca^{2+} channels.

The gene coding for the human A_1 receptor consists of multiple exons and introns. In particular the 5' untranslated region consists of exons which may undergo alternative splicing events in order to produce two different receptor transcripts (Ren & Stiles, 1994a). High A_1 receptor density in specific human tissues such as the brain may be explained by the expression of the transcript that is more efficiently translated (Olah & Stiles, 2000). This phenomenon of tissue specific splicing regulates A_1 receptor levels.

A_1 receptors can be distinctly identified from the other adenosine receptors on the basis of structure-activity relationships with adenosine analogues. By substitution on the parent adenosine and xanthine molecules, selective high affinity agonists and antagonists, respectively are produced. Three specific positions on the adenosine molecule may be modified to increase affinity to receptor subtypes without destroying agonistic activity: the 5' position on the ribose, and the 2- and N^6 positions of the purine.

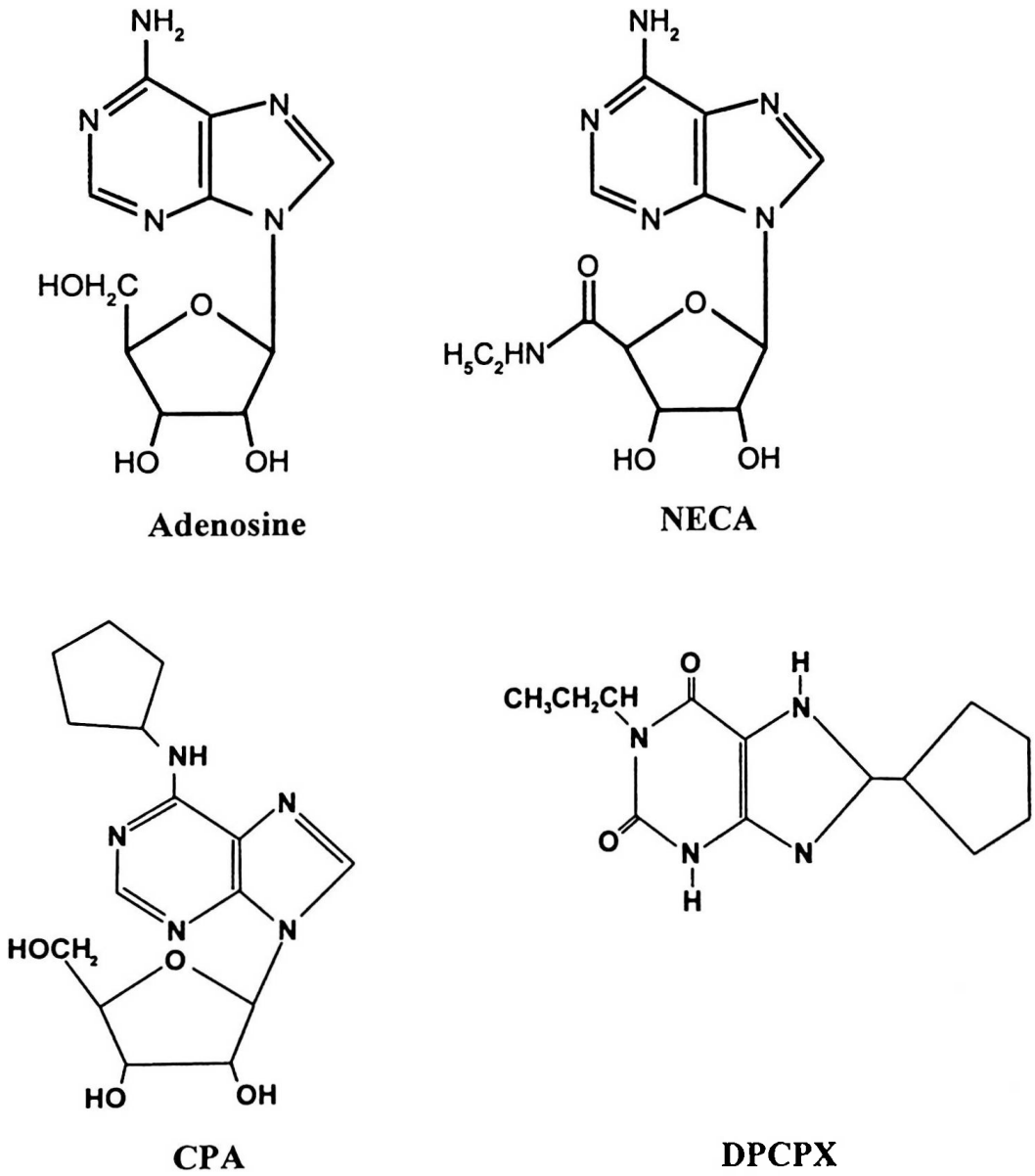


Figure 1.4: Structures of A_1-specific compounds (Klotz, 2000).

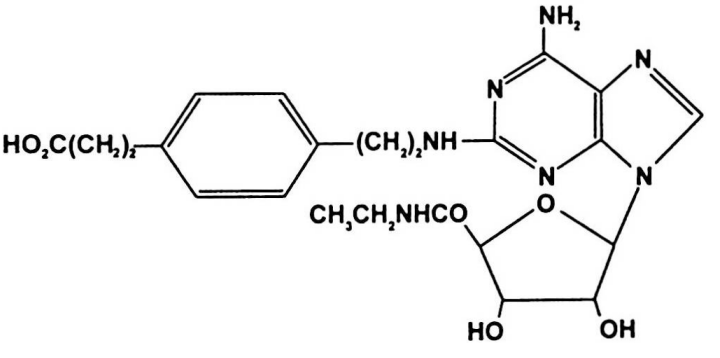
Specific selectivity for the A_1 receptor is achieved by modification of the N^6 position. N^6-cyclopentyladenosine (CPA) is an A_1-specific agonist with an affinity of 0.6 nM. 1,3-dipropyl-8-pentylcycloxanthine (DPCPX) (figure 1.4) is an A_1 selective antagonist with an affinity at A_1, A_{2A} and A_{2B} receptors of 0.9, 0.36 μM and 0.47 μM, respectively (Fredholm *et al*, 1994).

1.11.2 *A_{2A} receptors*

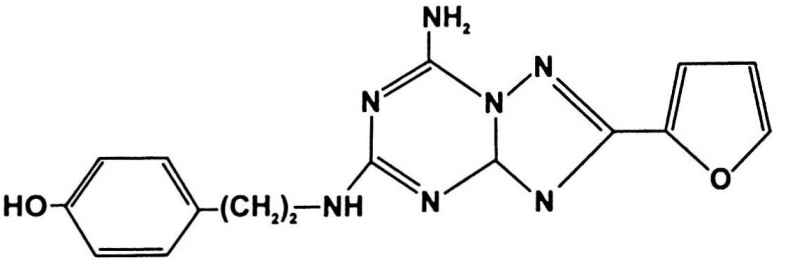
The A_{2A} receptor has been cloned from canine, rat and human (Furlong *et al,* 1992) cDNA libraries. Small alkyl amide substitution at the 5' position, as in 5'-N-ethylcarboxamidoadenosine (NECA) (figure 1.4), provides increased potency at the A_{2A} receptors. Furthermore, NECA is also the most potent agonist at all four subtypes of adenosine receptor and hence non-selective. 3-[4-[2-[[6-amino-9-[(2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl]purin-2-yl]amino]ethyl]phenyl]propanoic acid (CGS-21680) is a truly A_{2A} selective agonist, possessing a 140-fold selectivity for A_{2A} versus A_1. Aryl amines related to CGS-21680, such as PAPA-APEC and a 5-hydroxyl derivative, 2-[2-(4-amino-3[^{125}I]iodophenyl)ethylamino]adenosine (APE) can be radioiodinated to provide A_{2A} receptor-selective radioligands allowing pharmacological characterisation of the receptor, and differentiation between the A_{2A} and A_{2B} receptor subtypes.

Antagonists expressing moderate A_{2A} selectivity include 8-styryl-substitued 1,3,7-alkylxanthines (Jacobson *et al,* 1993) and the triazoloquinoxaline, 4-amino-l-phenyl[1,2,4]triazolo[4,3-a]quinoxaline (CP66,713), a non-xanthine analogue (Sarges *et al*, 1990). However these compounds undergo rapid photoisomerisation when exposed to light, limiting their use. There are three structurally similar non-xanthine

adenosine receptor antagonists which have the same A_{2A} receptor affinity but differ in their adenosine receptor selectivity. 9-chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-c]quinazolin-5-amine (CGS-15943) anatagonises all adenosine receptors, but 4-(2-[7-amino-2-(2-furyl) [1,2,4]-triazolo[2,3-a][1,3,5]triazin- 5-yl amino]ethyl) phenol), (ZM 241385) (figure 1.5) and specifically [7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo [1,5-c]pyrimidine] (SCH 58261), are selective A_{2A} anatagonists (Ongini *et al*, 1999).



CGS-21680



ZM-241385

Figure 1.5: Structures of A_{2A}-specific compounds.

Unlike the other adenosine receptors which are widely distributed in the brain, A_{2A} receptors appear to be concentrated in the striatum. In the rat, in-situ hybridisation studies reveal co-localization of A_{2A} mRNA with dopamine (D2) receptor mRNA on

neurones in the striatum (Fink *et al,* 1992). A_{2A} transcript has also been identified in the human brain, heart, kidney and lung. It has been suggested that post-synaptic A_{2A} receptor stimulation results in a decrease in locomotor and psychomotor activity, resulting in sedation (Fredholm, 1993).

A_{2A} receptors are coupled to G_s proteins and activate adenylate cyclase. The resultant build up of cAMP leads to the inhibition of platelet aggregation and vasodilatation in vascular beds such as the coronary artery (Conti *et al.*, 1997). In the guinea-pig bladder it has been found that relaxation of bladder smooth muscle is mediated by A_{2A} receptor-mediated activation of K_{ATP} channels via adenylate cyclase and elevation of cAMP (Gopalakrishnan *et al*, 2002).

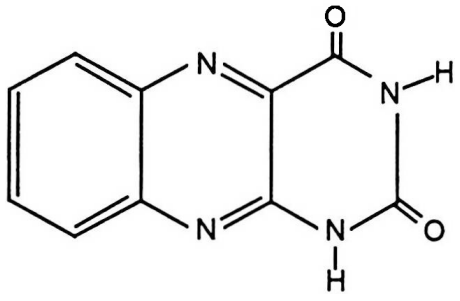
1.11.3 A_{2B} receptors

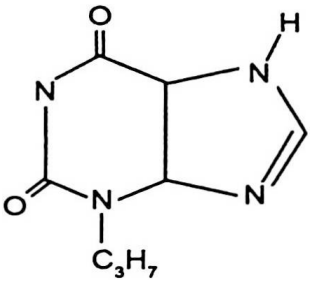
When rat mRNA was subjected to Northern blotting, high levels of the A_{2B} transcript were present in the caecum, large bowel and bladder. Less transcript was identified in the brain, lung, blood vessels, eye and mast cells (Stehle *et al,* 1992). Unlike A_{2A} receptors, A_{2B} are ubiquitously distributed throughout the human brain (Daly *et al,* 1993), found also in glia cells and neurons. The A_{2B} receptor has been cloned from various species, including rat and human, and its sequences are highly similar across species, ranging from 85% identity between human and mouse and 95% identity between rat and mouse.

Activation of A_{2B} receptors leads to a cAMP-dependent potentiation of P-type Ca^{2+} current in pyramidal cells in guinea-pig hippocampus, which maybe inhibited by blocking cAMP-dependent protein kinase A. In contrast, activation of A1 receptors inhibited mainly N-type Ca^{2+} current (Mogul *et al*, 1993).

The A_{2B} receptor has multiple actions when endogenous adenosine is the agonist. These include: mast cell degranulation, vasodilatation, cardiac fibroblast proliferation, inhibition of tumor necrosis factor-alpha (TNFα), increased synthesis of interleukin-6, stimulation of Cl^- secretion in intestinal epithelia and hepatic glucose production. Thus A_{2B} receptor agonists could become therapeutic agents in the treatment of hypertension, myocardial infarction and septic shock. Presently, theophylline is an effective drug for asthma but is far from ideal. The development of specific A_{2B} receptor antagonists maybe useful as an anti-asthmatic agent, due to A_{2B} blockade in mast cells.

The A_{2A} and A_{2B} subtypes arose based on differences in affinity for agonist binding. The A_{2A} is a high affinity receptor, whilst the A_{2B} is low affinity. Hence there are no truly selective agonists, making it difficult to study the functions of the A_{2B} receptor. NECA is one of the most active non-selective agonists. It seems that monosubstitution on the N^6-position of adenosine maybe useful to increase A_{2B} potency (Volpini *et al,* 2003). Interestingly, the A_{2B} receptor demonstrates higher affinity for several of the xanthine antagonists as compared to the A_{2A}. ZM-241385 is a non-xanthine which binds with reasonable affinity (K_D =34 nM) to the human A_{2B} receptor (Ji & Jacobson, 1999). Selective non-xanthine antagonists include alloxazine and enprofylline (figure 1.6). Although they have low potency, alloxazine has a 9-fold selectivity over the A_{2A} receptor. Non-selective A_{2B} antagonists such as 1,3-dipropyl-8-sulfophenyl-xanthine (DPSPX) and DPCPX are more potent. It appears that by substituting at position-8 of the xanthine nucleus, higher A_{2B} antagonist activity is achieved (Feokistov *et al,* 1998).





Alloxazine *Enprofylline*

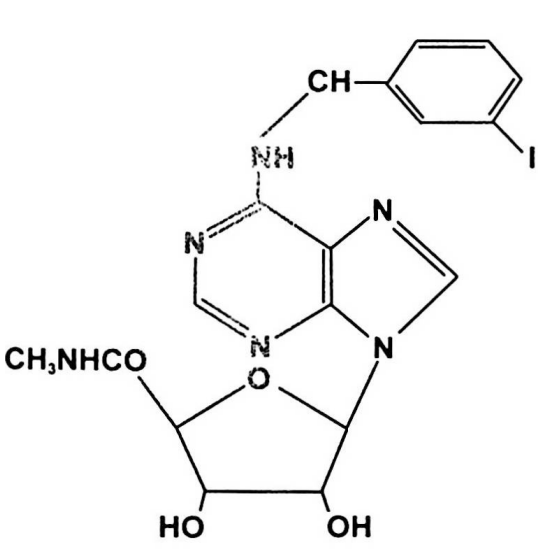
Figure 1.6: Structures of the A_{2B}-selective antagonists

1.11.4 A_3 receptors

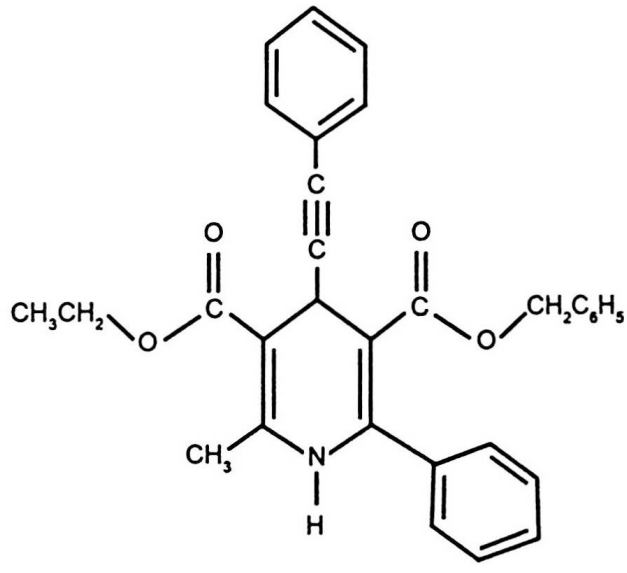
A_3 receptors were initially identified by cloning with degenerate oligonucleotides probes. They display considerable inter-species variation in terms of structure, tissue distribution and ligand-binding characteristics. Sheep and human A_3 receptors display a 72% homology to the rat A_3 receptor and 85% homology to each other. The most abundant quantity of rat mRNA is in the testis, but for the human A_3 mRNA, tissue distribution follows the order lung=liver>brain>aorta>testis>heart (Salvatore *et al,* 1993). Its abundance in the lung offers A_3 receptors a role in the modulation of allergic responses.

The dihydropyridine derivative 3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS-1191) (figure 1.7) and the triazolo-quinazoline; 9-chloro-2-(2-furanyl)-5-((phenylacetyl)amino)-[1,2,4]triazolo[1,5-c] quinazoline (MRS-1220) are both potent human A_3 receptor antagonists, with K_i values of 31 and 0.65 nM, respectively. 3-propyl-6-ethyl-5-[(ethylthio)carbonyl]-2

phenyl-4-propyl-3-pyridine carboxylate (MRS-1523) is a selective A_3 antagonist in the rat. The unifying feature of the A_3 antagonists is that they are all non-xanthines, as the receptor itself has a xanthine-resistant binding site, rendering it insensitive to xanthine antagonists such as DPCPX (Zhou *et al,* 1992).



IB-MECA



MRS-1191

Figure 1.7: Structures of A_3-specific compounds.

Furthermore, some adenosine agonists have a much lower affinity for the A_3 receptor than the A_1 receptor (Zhou *et al,* 1992). However, chloro-N^6-(3-iodobenzyl)-adenosine-5'-N-methyluronamide (IB-MECA) (figure 1.7) is 2500-fold more selective for the A_3 versus the A_1 receptor, and 1400-fold more selective versus the A_{2A} receptor.

It was thought that A_3 inhibits adenylate cyclase, and although this is the case when expressed within cultured Chinese hamster ovary (CHO) cells, it does not seem to

hold true in native cells (Abbracchio *et al,* 1995). Instead A_3 receptor activation leads to activation of phospholipase C and protein kinase C and a rise in inositol phosphate levels (Ramkumar *et al,* 1993).

Adenosine affects cellular proliferation, through interaction with all four adenosine receptor subtypes. The A_3 receptor is of particular interest as in colon cancer cell lines it is the most abundant of the four subtypes and endogenous adenosine, through the interaction with this receptor mediates a tonic proliferative effect. The effect was successfully reproduced by IB-MECA (Gessi *et al,* 2007).

When the A_3 receptor is intensely and repeatedly activated, apoptosis is initiated. Conversely, low levels of A_3 receptor agonists appear to protect against apoptosis (Jacobson, 1998). The cardioprotective effect of A_3 receptor agonists is another area of interest. As A_3 receptors have a lower affinity for adenosine as compared to A_1 and A_{2A} receptors, their activation may only occur in the presence of high concentrations of adenosine, such as during acute ischaemia (Liang & Jacobson, 1998).
Although direct activation or antagonism of rat hippocampal receptors has no effect on synaptic transmission, activation does reduce the sensitivity of presynaptic A_1 receptors which inhibit glutamate release (Dunwiddie *et al*, 1997).

1.12 Future Developments

Although several muscarinic receptor antagonists are available to treat OAB, two of which are M_3 receptor specific; all of them have failed to provide completely successful treatment, with side-effects hindering patient compliance. Hence other avenues need to be explored for those refractory to them. Current and potential

additional approaches to manage OAB have been outlined in sections 1.3.4 and 1.3.5. However, the role of purinergic system in the development of OAB and manipulation of purinergic targets in its management have not yet been considered.

Several lines of evidence summarized in this Introduction suggest that modulation of the purinergic system in particular the P1 receptor (adenosine) system may be an interesting drug target for managing OAB.

- ATP is a functional excitatory neurotransmitter for human detrusor smooth muscle only in certain pathologies associated with overactive bladder.
- ATP breakdown to adenosine is present in detrusor smooth muscle via extracellular ATPases.
- Studies with detrusor from animal bladder show that adenosine indeed modulates contractile function.
- Selective P1 receptor subtype agents are available to permit some indication of the relevant subtypes that may mediate contractile modulation.

1.13 Aims and Objectives of thesis

Hypothesis

Adenosine has a modulatory action upon the contractile process in detrusor smooth muscle, with differential effects of adenosine in stable and overactive human bladders.

The aim of this project was to investigate the distribution, expression and role of P1 receptor subtypes in the modulation of guinea-pig and human detrusor smooth muscle

contraction. Human tissue included detrusor from patients with stable bladders, and those with DO, both idiopathic and neurogenic.

The effects of adenosine and P1-subtype specific agents on contractile function were investigated using isolated detrusor strips, which were contracted by electrical field stimulation or through direct-muscle activation with agonists. P1 receptor subtype expression in human detrusor samples, from both stable and pathological bladders, was determined using mRNA expression using polymerase chain reaction and protein transcription using western blotting.

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 determine whether there is frequency-dependent release of the co-transmitters (ATP and Ach) in guinea-pig and human detrusor smooth muscle, and to quantify this relationship.

iv) To determine if there are any differences in the expression of P1 receptor subtypes in detrusor samples from stable and overactive human bladders.

CHAPTER TWO

Materials & Methods

2.1 Tissue collection

Samples of human detrusor were obtained from patients undergoing cystectomy and ileocystoplasty operations, with local Ethical committee approval (Study Number 00/0039) and fully informed patient consent. Please see Appendix 1 (Patient Information sheet and consent form). A copy of the consent form was kept in the patients' notes and the original was filed in our laboratory.

Dunkin-Hartley guinea-pigs were sacrificed by cervical dislocation and the bladder removed in concordance with Home-Office Guidelines (Schedule 1).

Once obtained all specimens were placed in calcium-free Tyrode's solution, and taken back to the laboratory where they were stored at 4°C for experimentation. Samples were only stored in this way for a maximum of 24 hours. After this time they were either discarded by incineration or frozen in liquid nitrogen for later non-functional work.

2.2 Patient clinical groups

Detrusor biopsies obtained from patients undergoing cystectomy due to bladder carcinoma were presumed to be from stable bladders. The samples were taken distant from the obvious tumour. Patients undergoing clam ileocystoplasty had previously been subjected to urodynamic investigation. Only those with either idiopathic detrusor overactivity or neurogenic bladder dysfunction were selected for this study. In summary, three clinical groups of patients were identified and studied. They are classified as stable, idiopathic overactivity (IDO) and those with neurogenic detrusor overactivity (NDO), and this classification is used throughout.

2.3 Preparation of detrusor specimens

Detrusor specimens were removed from the transportation tube and placed in a dissecting dish, also bathed in calcium-free Tyrode's solution. The specimen was stretched out and secured to Sylgard in the base of the dish using several 14 gauge sterile needles. The urothelium and serosa were carefully removed using fine dissecting scissors, leaving only detrusor muscle as the functional unit under investigation. Use of a binocular microscope (Nikon AL5; Nikon Corporation, Tokyo, Japan) enabled specific identification of muscle bundles which appeared fairly translucent. These bundles were dissected longitudinally in order to form strips approximately 3 mm length x 1 mm width. A 6/0 vicryl (Ethicon, Johnson & Johnson Medical Ltd, Livingstone, UK) suture was tied so as to ensare both ends of the strip.

2.4 General protocol for contraction experiments

Experiments compared contractions generated by application of adenosine receptor agonists (ARAs) versus those elicited by stimulation of excitatory nerves (neurotoxin-sensitive contractions) to gain information about the role played by pre-and post-synaptic mechanisms. The action of adenosine was investigated, as well as agonists specific for one or more subtype of adenosine (A) receptor, such as NECA (A_1/A_2), CPA (A_1 specific), CGS 21680 (A_{2A}) and IB-MECA (A_3 agonist). The effects of alloxazine (A_{2B} antagonist) were also investigated. In each experiment at least six repeats were carried out for each study group.

2.5 Measurement of isometric tension

One end of the detrusor strip was tied to a static hook in the superfusion trough (figure 2.1) and the other to a dynamic isometric tension transducer (Model FT.03, Grass

Instrument Company, USA). The transducer was mounted on a micro-manipulator (Prior Instruments Ltd., Herts., UK), which allowed the length of the strip to be adjusted, such that maximum tension was evoked on electrical field stimulation (EFS). Stimulation current was generated by a stimulator (Model 200, Palmer Bioscience, Sheerness, Kent) and gated by a programmer and finally delivered to the strip via platinum electrodes embedded in the walls of the Perspex superfusion trough. The output of the transducer was connected to a bridge amplifier with variable gain (ORMED Amplifier 5000 Series, ORMED Ltd., Welwyn Garden City, Herts., UK), and in turn was connected to a moving-paper pen recorder.

2.5.1 Delivery of superfusates

All solutions (Tyrode's and other superfusates) were continuously gassed with 95% O_2/ 5% CO_2 and were warmed in a water bath at 37°C. Polythene tubing (2 mm inside diameter; Thermoflow, Coniar Churchill Scientific supplies Ltd, Middlesex, UK) was placed in the superfusate solution, with the other end feeding the superfusion trough under gravity. The tubing was water-jacketed to keep the superfusate at 37°C. The flow rate was approximately 4ml/min with the water bath sitting one metre above the superfusion trough and solution change effected in the water-bath taking approximately 1 minute to reach the strip. The volume of the superfusion trough was 0.0064 cm^3 meaning that exchange time of the superfusate in the trough would be approximately 0.6 seconds.

The strip was entirely submerged in superfusate and was not in contact with the sides of the superfusion trough throughout experimentation. Once the experiment had been completed, the muscle width and length were accurately measured in order that muscle force could be normalised for the cross sectional area of the muscle (mm^2).

The superfusion trough was mounted on a heavy table with neoprene anti-vibration pads.

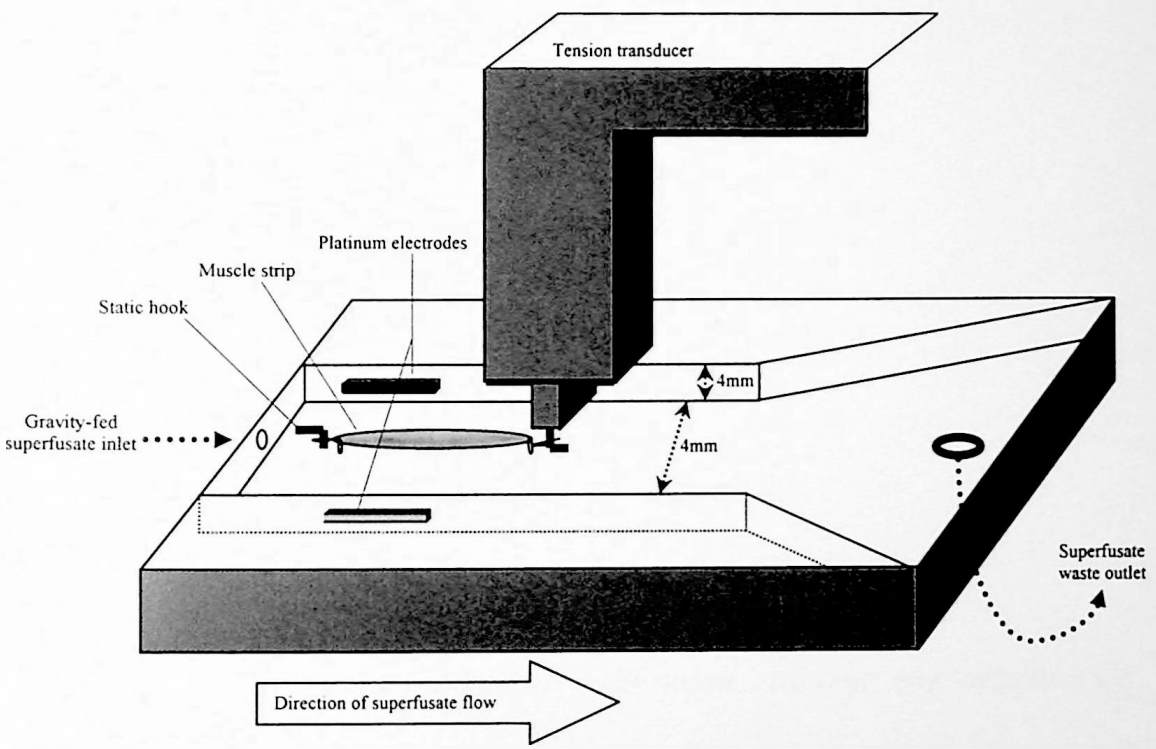


Figure 2.1: Schematic diagram of the micro-superfusion organ bath used to measure isometric tension in detrusor strips.

2.6 Calibration of the tension transducer

Weights of known mass were hung from the tension transducer and the magnitude of the deflection measured by the pen recorder, at each gain setting (0.25, 0.5, 1.0, 2.5, 5.0 volts full scale deflection; fsd).

Force (F) in Newtons (N) was calculated from the equation:

$$F=ma$$

Where m = mass of weight in kg and $a = 9.81$ m/s^2 = acceleration according to earth's gravity. Force (mN) was plotted against deflection (mm), producing a graph for each gain setting. The graph for a gain of 1 volt is shown in figure 2.2.

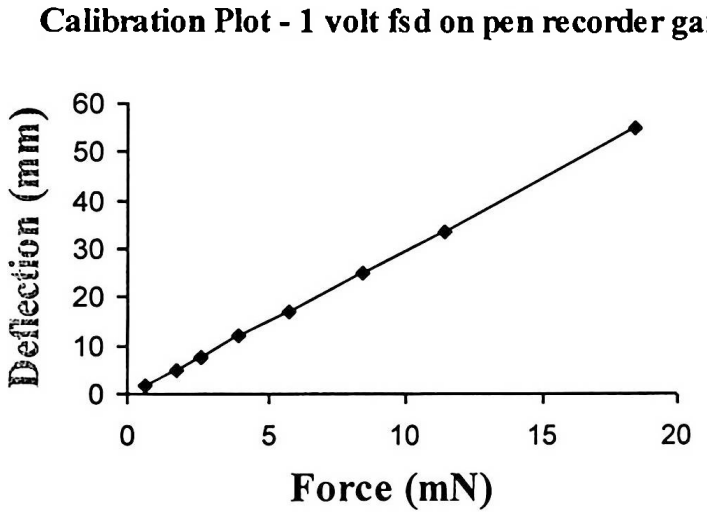


Figure 2.2: Sample tension transducer calibration showing the relationship between deflection on the pen recorder and force. The straight-line was generated by a least-squares fit to the data.

2.7 Experimental solutions

Physiological saline solution (modified Tyrode's) was used to superfuse strips (Table 1). This was gassed with 95% O_2 to ensure that the tissue did not become hypoxic, and 5% CO_2 which along with bicarbonate, acted as a buffer to maintain pH at 7.33 ± 0.22. De-ionized water (Milli-RO 10 plus, Millipore, Croxley Green, Watford, UK) was used as the solvent. All liquid additions were measured using variable volumetric pipettes.

All the chemicals that were used to make up the Tyrode's solution were Analar grade and were obtained from BDH Chemicals Ltd (Poole, Dorset). They were weighed using a Sartorius balance (Sartorius Ltd, Epsom Surrey).

Table 2.1: Composition of Tyrode's Solution.

Chemical	Concentration (mM)
NaCl	118.0
$NaHCO_3$	24.0
$CaCl_2.6H_2O$	1.8
KCl	4.0
$MgCl_2.6H_2O$	1.0
$NaH_2PO_4.2H_2O$	0.4
Glucose	6.1
Sodium pyruvate	5.0

Calcium free (HEPES)-buffered Tyrode's solution was used to transport detrusor samples, but not for the experimental superfusate. Its composition is described in table 2.2. The pH was corrected to 7.6, by titration with 1 M NaOH. This solution was also used for overnight storage of the samples.

Table 2.2: Composition of (HEPES-buffered) calcium-free Tyrode's Solution

Chemical	Concentration (mM)
NaCl	132
KCl	4.0
$NaH_2PO_4.2H_2O$	0.4
Glucose	6.1
Sodium pyruvate	5.0
HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])	10.0

2.7.1 Added reagents

Once a steady level of contractile force had been elicited from the muscle strip in Tyrode's solution, the superfusate was changed and the magnitude of the response was recorded. Values recorded during the intervention were compared to the mean of control values before and after the intervention.

All drugs were from Sigma-Aldrich in dry solid form. Stock solutions of N-ethylenecycloadenosine (NECA), N^6-cyclopentyladenosine (CPA), 2-*p*-((carboxyethyl)phenethylamino)-5'-carboxyamidoadenosine (CGS 21680), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) and 1-deoxy-1-6[6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-N-methyl-β-D-ribofuranuronamide (IB-MECA) were made by dissolving in DMSO and were stored in 20 µl eppendorfs at -5°C. This meant that one eppendorf could be thawed per experiment. All other drugs used were dissolved in Analar grade water; atropine, carbachol, tetrodotoxin (TTX),

α-β-methylene-adenosine triphosphate (ABMA). 1 mM solutions of adenosine were made from solid powder on the day of experimentation, and diluted as necessary.

2.8 Electrical field stimulation (EFS) protocol

The muscle strip was secured in the microperfusion trough and stretched to approximately 1.2-1.5 times its relaxed length using the micromanipulator. The following protocol was used for both human and guinea-pig detrusor strips. Phasic contractions were elicited by field stimulation of the strip from the constant-current stimulator via platinum electrodes in the walls of the trough. The stimulation parameters were 3-second pulse trains (0.1 ms pulse width) every 90 seconds. The stimulation voltage was chosen to be approximately 1.5 times the maximum required to recruit all muscle fibres by adjusting the output voltage (25 to 30 V) of the stimulator.

The strips were stimulated at 8 Hz for guinea-pig and 20 Hz for human until a stable response was obtained, as these frequencies have been shown previously to generate approximately half-maximal tension ($f_{1/2}$) for each tissue (Bayliss *et al,* 1999).
A force-frequency relationship was determined for each muscle strip by using incremental stimulation frequencies from 1 to 20 Hz for guinea-pig and 1 to 40 Hz for human specimens. Once this relationship had been obtained, stimulation frequency was returned to the baseline values until a steady response was regained.

Following this, the superfusate was changed to include different ARAs or antagonists and again force-frequency relationships were established. The percentage change of force for each frequency was compared to the mean of the force in control solution

pre- and post-intervention. Control contractions were determined from the average of 3-5 contractions before and after exposure to the agent. The effects of the ARAs were completely reversed after a washout period of 15 minutes in Tyrode's solution.

2.8.1 Agonist-induced contraction protocol

The acetylcholine analogue, carbachol was used to determine the effects of adenosine receptor modulation on post-synaptic contractile mechanisms. Each carbachol intervention was for 60 seconds and contractions were consistent with repeated applications of the same concentration of carbachol.

To determine the effects of ARAs and antagonists on post-synaptic contractions, the preparation was exposed to maximal concentrations of the agent for five minutes before the application of carbachol. The concentrations of the reagents used were estimated from dose-response results or published data. The carbachol solution also contained the same concentration of ARA. The strip was then exposed to just the ARA solution alone for a further two minutes. The magnitude of the carbachol contracture in the presence of receptor modulators was expressed as a proportion of the carbachol contracture before and after their inclusion.

2.8.2 Protocol to estimate atropine resistance

To obtain the non-cholinergic portion of the nerve-mediated response, the tissue was superfused with 1 μM atropine (muscarinic receptor antagonist) at the end of each experiment, once the responses had settled to a steady state. A force-frequency curve was repeated.

To establish the non-cholinergic non-purinergic portion of the response, the tissue was superfused with 1 μM atropine and 10 μM α, β-methylene-ATP (ABMA). ABMA evokes a large, transient contraction indicating activation of P2X receptors which are then desensitised so that subsequent release of ATP fails to evoke a response.

To determine the extent of residual contraction due to direct muscle stimulation by EFS, the neurotoxin tetrodotoxin (TTX; 1 μM) was applied to the strip. TTX is a sodium channel blocker and prevents the release of neurotransmitters from the nerve terminal by blocking action potential generation in the presynaptic nerve.

The atropine-resistant contraction was the proportion of contraction remaining when the contraction in atropine and TTX was subtracted from that in atropine alone. This was then expressed as a percentage of the control contraction (figure 2.3).

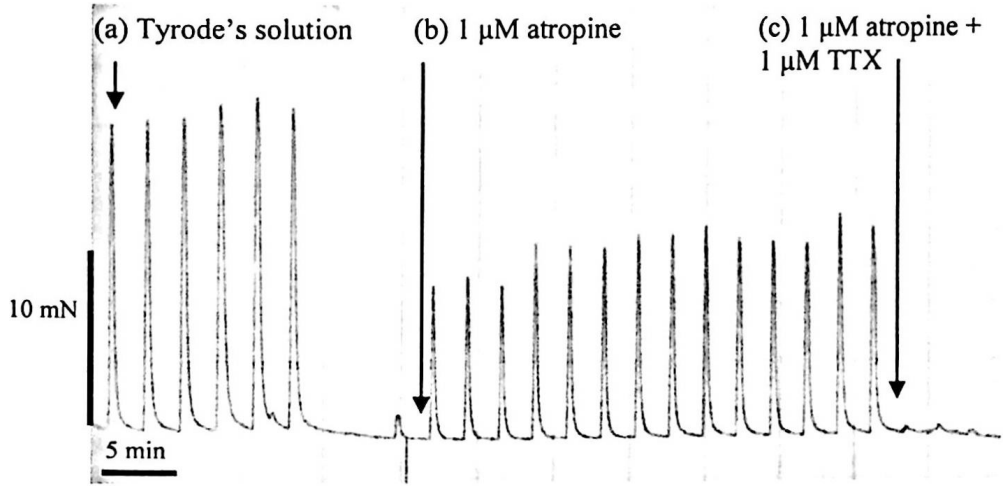


Figure 2.3 Example of atropine-resistant contractions in a human detrusor strip from a patient with IDO. Nerve-mediated contractions in (a) Tyrode's solution, (b) 1 μM atropine, (c) 1 μM atropine and 1 μM TTX, which abolishes contractions.

2.8.3 Data analysis for tension experiments

Empirical fits to force-frequency data; $T = \frac{Tmax \,.\, f^n}{f_{1/2}{}^n + f^n}$

and dose response curves; $T = \frac{Tmax \,.\, [A]^n}{EC_{50}{}^n + [A]^n}$ 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. For dose-response curves n is also known as the Hill coefficient. The curve fits were performed in Kaleidagraph (Synergy software).

2.8.4 Statistical analysis

Data were analysed by paired Student's *t*-test between pre/post-control and intervention contractions. Comparison of adenosine receptor efficacy between human and guinea-pig detrusor was made using an unpaired Student's *t*-test. Atropine resistance data do not follow a normal distribution as there are several zero values, therefore these data were tested for significance using the Wilcoxon signed-rank test. *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) 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 either as mean ± standard deviation (SD) or median values with 25 and 75% quartile values.

2.9 Adenosine receptor expression experiments

Following the contractile experiments all the detrusor samples were labelled and stored in liquid nitrogen. When required for the second phase of experimentation ie. to identify adenosine receptor expression in the samples, the samples were prepared for total RNA extraction.

2.9.1 Total cellular RNA (tcRNA)

RNA that is extracted from cells comprises 3 different types:

- Ribosomal RNA (rRNA): of which there are two forms (28S and 18S). These are components of the ribosome, the cell's protein synthesis organelle.
- Transfer RNA (tRNA): of which there are multiple forms. These molecules decode the mRNA and are essential for protein synthesis.
- Messenger RNA (mRNA): these are the RNA molecules that encode protein.

Of the tcRNA extracted from a cell, mRNA comprises only 5%, with rRNA and tRNA together comprising 95%. In order to specifically select out the mRNA from the others, experimental protocols exploit the fact that it possesses a poly-adenylate (poly-A) tail. This poly-A tail will hybridise to poly-dT oligonucleotides (oligo-dT). The oligo-dT is attached to agarose which acts as a solid support, and is used to bind the mRNA. As the rRNA and the tRNA do not bind to the oligo-dT, they can be easily washed away.

2.9.2 Sample Preparation and Single-Step Liquid-Phase separation

Frozen detrusor samples were weighed and then crushed using a pestle and mortar, keeping them cold by pouring liquid nitrogen into the mortar. tcRNA, DNA and protein were isolated from each detrusor specimen using an adaptation of the single-

step RNA isolation method (Chomczynski *et al,* 1987). TRI REAGENT™ (Sigma; guanidine thiocyanate and phenol) was added to the samples (1 ml per 50-100 mg of tissue) causing cell disruption and denaturation of the endogenous nucleases, thus preserving the integrity of the RNA and DNA in the sample. The sample was centrifuged at 12,000 g for 10 mins at 4 °C resulting in three distinct layers within the tube as in figure 2.4.

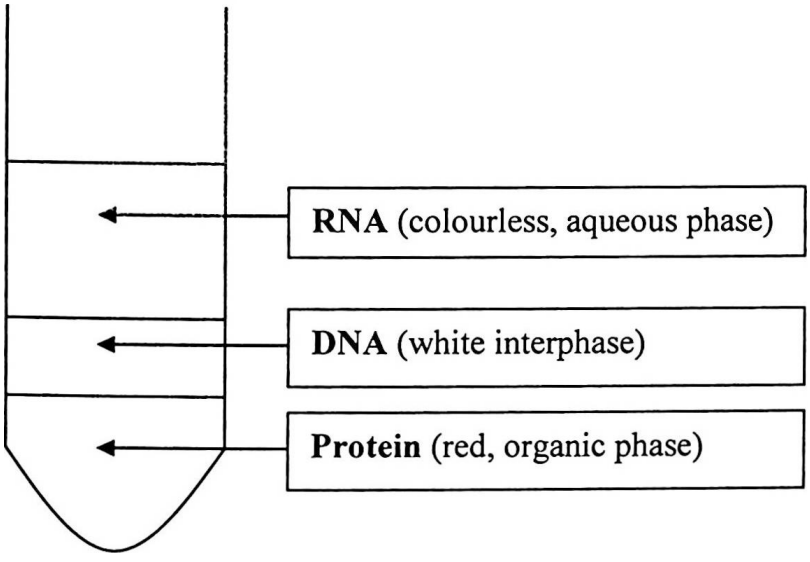


Figure 2.4: Three distinct phases can be seen following single-step phase separation.

2.9.3 RNA isolation

The aqueous phase was transferred to a 1.5 ml eppendorf tube (Starsted, UK) using a pipette and taking care not to contaminate with the interphase. The organic and interphases were frozen at 4°C for future use (section 2.16- Western blotting). Added to the aqueous phase was 0.5 ml of isopropanol per ml of TRI REAGENT™ used in sample preparation. The sample was allowed to stand for 10 minutes at room temperature then centrifuged at 12000 g for 5 minutes. The RNA precipitate formed a

white pellet at the bottom and side of the tube. The supernatant was discarded and the RNA pellet was washed by adding 1 ml 75% ethanol (BDH Lab Supplies) per 1 ml of TRI REAGENT™ used in sample preparation. The sample was vortexed and then centrifuged at 7,500 g for 5 minutes. The tcRNA pellet was then air-dried for 10 minutes to remove excess ethanol, but not completely as this reduces its solubility. 100 µl of 0.1% diethylpyrocarbonate [DEPC] treated RNase free H_2O (USB Corporation, Cleveland, Ohio, U.S.A.) was added to each RNA pellet and left to sit for 20 minutes (section 2.9.4). This was followed by gentle vortexing and passing the solution through a pipette several times until the tcRNA was dissolved. The samples were then frozen at -80°C until required.

2.9.4 Hazards when handling RNA

RNA is a single-stranded molecule which is prone to hydrolytic cleavage by the enzyme ribonuclease (RNase) and is far less stable than DNA. RNases are ubiquitous enzymes secreted from the skin which are inherently stable and retain activity even after autoclaving and after exposure to chaotropic agents such as guanidium thiocyanate. The only method of preventing the effects of RNase is by chemical inactivation by use of the methylating agent, 0.1% diethylpyrocarbonate (DEPC). This irreversibly modifies the active site histidine residue of the enzyme. Consequently all water used during RNA work must be DEPC treated. Furthermore to avoid contamination by RNase, other precautions should be taken, as listed below:

- Gloves must be worn at all times.
- Gloves must be changed frequently.
- Solutions should be dedicated for RNA work only.

- All pipette tips and eppendorf tubes should not be handled by hand prior to autoclaving.
- If possible pre-packed RNase free materials should be purchased.
- Glassware should be baked pre-use.

2.9.5 Quantification of RNA

The concentration and purity of the tcRNA was determined by measuring the optical density or light absorbance of nucleotides in solution in the ultraviolet region of the spectrum, at wavelengths of 260 nm and 280 nm, using the UV1101/T Biotech photometer (WPA, U.K). RNA absorbs light at 260 nm. The ratio A_{260}:A_{280} is an indication of the purity of the RNA. The ratio for pure RNA should be 1.8-2.1. However, even if the ratio is less than 1.8, the RNA quality maybe acceptable and can be confirmed by subsequent gel electrophoresis.

2.10 Priniciples of Photometry

The Beer/Lambert law states that the absorption of monochromatic energy eg. light of one colour or wavelength, is directly proportional to the molar concentration of the absorbing compound. A photometer measures Absorbance (A) by a solution and by applying Beer's Law allows Concentration (C) to be estimated:

$$C = A \text{ x constant.}$$

If A =1 at 260 nm for the photometer used in these experiments, this means that the sample contains 50 mg/ml double stranded DNA, 40 mg/ml single stranded DNA or RNA or 20 mg/ml single stranded oligonucleotides (Sambrook *et al,* 1989). The concentration of nucleic acid in the sample can be calculated from the empirical formula:

$$C\ (\mu g/\mu l) = A_{260} \times [0.5/\text{ volume RNA assayed } (\mu l)] \times Y.$$

Where Y= 50, 40 or 20 depending on the types of nucleic acid listed above, to be assayed. In this case, Y = 40, as RNA was used.

Prior to making test measurements the instrument was zeroed. This was done by filling a clean quartz cuvette with 245 µl of water and selecting the filter wavelength of 260 nm. Following this 5 µl of the detrusor tcRNA was added to the cuvette, and an absorbance reading was obtained. The cuvette was washed with purified water and the procedure was performed for each of the 20 detrusor samples used in this study (table 3.16).

2.11 Reverse Transcription – Polymerase Chain Reaction (RT-PCR)

By determining the steady-state levels of mRNA within cells or tissues one is able to measure the level of gene expression. Because RNA is an inherently unstable molecule, we chose RT-PCR as the technique for quantifying mRNA levels. Currently this is the most sensitive technique for mRNA detection and quantitation available. Compared to the two other commonly used techniques, Northern Blotting and the RNase protection assay, RT-PCR can be used to quantify mRNA levels from much smaller samples as a result of exponential amplification. RT-PCR is essentially a two-step procedure heavily reliant on the action of two particular enzymes. The first is reverse transcriptase which makes a cDNA copy of mRNA. The second is Taq polymerase which amplifies the cDNA copy by PCR.

2.11.1 Reverse Transcription

The first stage of RT-PCR is known as the "first-strand reaction" and involves three basic steps:

1. Denaturation of secondary mRNA structures into single-stranded mRNA.
2. The addition of gene-specific sense and anti-sense primers, (Sigma-Aldrich, Dorset, U.K.), which bind to the mRNA strands at specific sites. They are short strands of DNA which are complementary to specific sequences on the mRNA and initiate polymerization by MMLV (Moloney Murine Leukaemia Virus; Sigma-Aldrich, Dorset, U.K.), the viral reverse transcriptase enzyme (Gerard *et al*, 1986). Furthermore, the primers (oligo-dNTP's) have a polyT tail that recognises the polyA tail of mRNA, ensuring that only mRNA and not t/rRNA's are reverse transcribed.
3. Completion of reverse transcription using deoxyribonucleoside-triphosphates (dNTPs), producing cDNA.

2.11.2 Protocol for Reverse Transcription Reaction

Water was added to an eppendorf tube along with the test mRNA and gene specific primers were added (see table 2.3 for quantities). The samples were then incubated at 70°C for ten minutes in a Progene Thermal Cycler (Techne, Cambridge, U.K.), in order to denature any secondary structures into single strands.

Samples were chilled on ice for 10 minutes to allow the primer to bind to the mRNA. A master-mix of 5x MMLV reverse transcriptase buffer; 0.1 M dithiothreitol (a reducing agent and 10 mM dNTP Mix (containing dATP, dCTP, dGTP and dTTP; Sigma-Aldrich, Dorset, U.K.) was added to each sample and kept at 42°C in the thermocycler for two minutes. Reverse transcription was triggered by adding the

MMLV reverse transcriptase enzyme and maintaining the temperature at 42°C for 50 minutes. The enzyme action was halted by increasing the temperature to 75°C for 15 minutes. The final product was eppendorf tubes of stable cDNA which were stored at -20°C for future PCR. The reverse transcription reaction was optimised for pH, salt concentrations and incubation temperature to give ideal conditions for the MMLV reverse transcriptase to act. The final protocol is outlined in table 2.3.

2.11.3 Polymerase Chain Reaction

After the reverse transcriptase reaction was complete, and complementary DNA had been generated from the original single-stranded mRNA, the polymerase chain reaction, termed the "second strand reaction," was initiated. It enabled the amplification of DNA molecules from a DNA template.

PCR involves three stages (figure 2.5).

1. Denaturation – the two strands of DNA are separated by heating.
2. Annealing – the temperature is rapidly dropped to allow the forward and the reverse primers to adhere to the DNA.
3. Extension – the temperature is raised to optimum for the DNA polymerase enzyme; DNA synthesis will commence in the presence of dNTPs.

Table 2.3: Protocol for first strand reaction

	Concentration	Volume (µl)	Temp (°C)	Time (mins.)
Denaturation: make up to a total volume of 22µl				
RNA	4µg/µl	x	10	70
Distilled water	Nuclease free	22-x-1-1		
Primer	Sense	1		
	Anti-sense	1		
Chill on ice for ten minutes and then add:				
5 'x' first strand buffer	250 mM Tris, 375 mM KCl, 15mM $MgCl_2$	8	42	2
DTT	0.1 M	4		
dNTP	10mM	2		
Reverse Transcription				
MMLV-RT	1 U/µl	2	42	50
Enzyme inactivation			72	15
Storage of cDNA			-20	Indefinitely

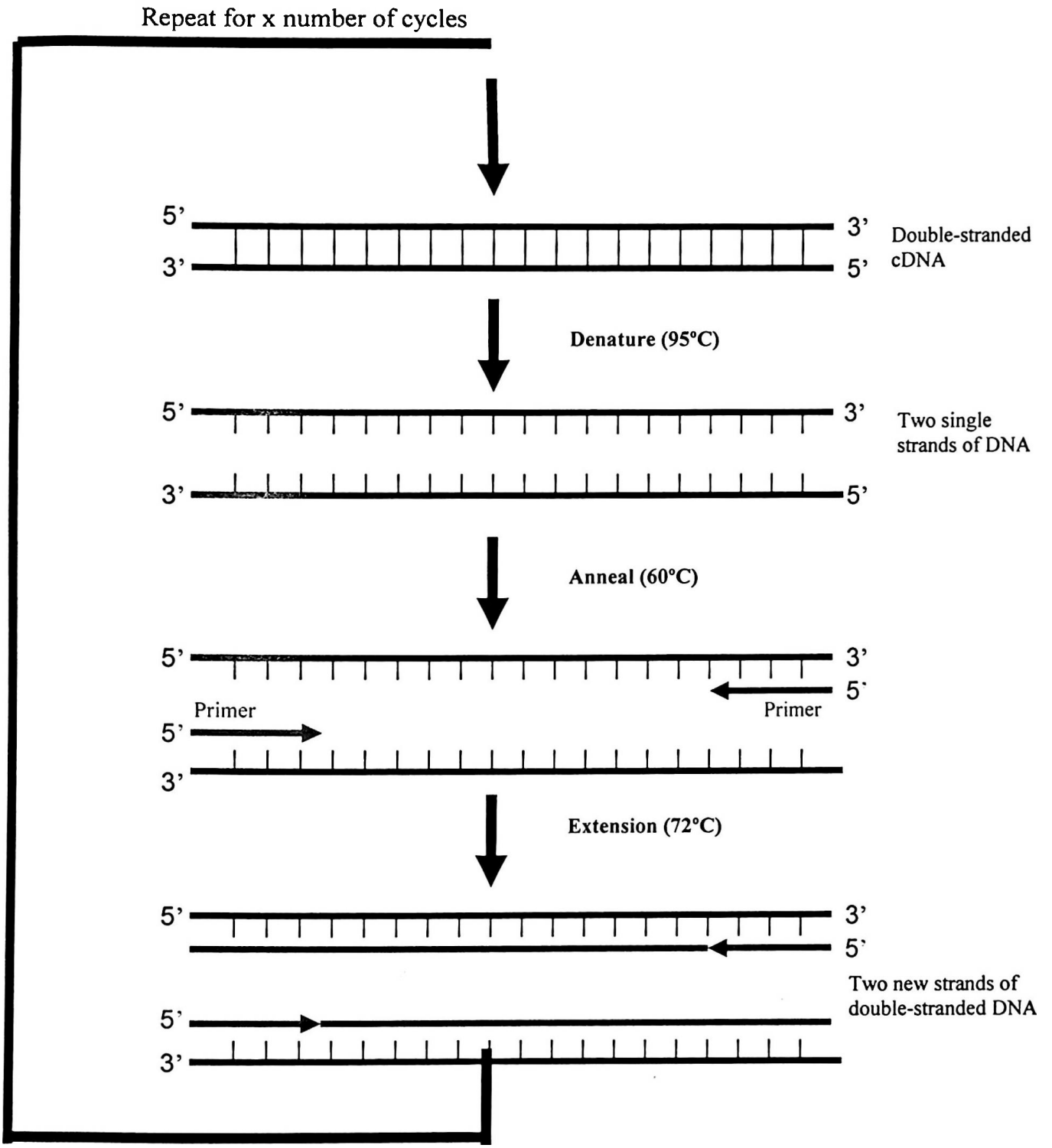


Figure 2.5: Diagram depicting the three steps of the Polymerase Chain Reaction

2.11.4 Optimisation of RT-PCR Protocol

This protocol (table 2.4) was titrated after several trials to ensure that optimal conditions were being used for the RT-PCR reaction, in terms of primer, template, $MgCl_2$ and other salt concentrations. The time and temperature at each of the three steps of the PCR reaction were also critical.

2.12 Preparation of reagents and materials

In order to avoid contamination by the enzyme ribonuclease (RNase), the working surfaces were wiped clean with 70% ethanol whilst wearing gloves, prior to experimentation. All eppendorfs and pipette tips were autoclaved. All reagents were handled on ice. The water used for RNA work was DEPC treated.

2.12.1 *Taq* Polymerase

PCR is reliant on thermostable DNA polymerases in thermophilic bacteria. The enzyme most commonly used is *Taq* polymerase which is isolated from *Thermus aquaticus* species, originally found in Yellowstone National Park, U.S.A. The property of these enzymes which makes them ideal for PCR is that their optimum temperature for DNA synthesis is >72°C and they are not denatured by repeated heating and cooling. The concentration of the enzyme was titrated until the optimum was achieved. *Taq* polymerase has endogenous 5' to 3' exonuclease activity and when used in excess will result in the production of degradation artefacts which cause smearing of the PCR product when it is displayed on the agarose gel. The rate of primer extension by *Taq* polymerase is about 50-100 nucleotides/sec. Thus, the time required for primer extension depends on the length of the sequence to be amplified.

*Table 2.4: Titrated PCR Protocol explaining reagents and time at each step. **The heated thermocycler lid contacts closely with samples, allowing rapid PCR. *dNTP stock contains 120µl water and 20µl each of dGTP, dATP, dCTP, dTTP.*

PCR Step	Reagent	Concentration	Volume	Temp (°C)	Time (mins)
Heat thermocycler lid**				105	5
Denaturation					
	Forward Primer	100 pM/µl	0.5	94	4
	Reverse Primer	100 pM/µl	0.5		
	dNTP stock 100mM*	200 µM	1.0		
	10x PCR Buffer		4.5		
	$MgCl_2$	25 mM	4		
	Water		32.5		
	cDNA	100 ng/µl	2		
Hot Start				65	5
Add *Taq* Mastermix					
	10x RED *Taq* PCR buffer		0.5		
	Taq polymerase	1 unit/ µl	0.5		
	Water		40		
Extension					
Repeat for 30 cycles				95	1
				60	1
				72	1

2.12.2 DNA template

Using high quality, purified DNA templates greatly enhances the success of PCR reactions. It is also critical that contamination from previous PCR reactions be avoided.

Approximately 10^4 copies of the target DNA are required to detect a product in 25-30 cycles of PCR. This means that 1-10 μg/ml of genomic template is required. In general, higher DNA concentrations decrease the specificity of the reaction (i.e., extra bands are seen), particularly when a large number of cycles are employed. High DNA concentrations can be advantageous when fewer cycles are desired, for example to increase the fidelity of the reaction.

2.12.3 The PCR thermocycler

The same realtime PCR thermocycler (section 2.11.2) for each reaction was used and always used eppendorfs of the same size in order to minimize variations in temperature distribution which could affect the PCR reaction.

2.12.4 Control tubes

For each experiment a control tube was used containing the master mix, with water replacing the cDNA. This ensured that there was no RNA/cDNA contamination within the mastermix.

2.12.5 Hot start programme

The tubes were loaded into the thermocycler and rapidly heated to 94°C for 4 minutes to promote denaturation. Whilst the Taq polymerase was being prepared, the

temperature was kept at 65 °C in order to keep the two strands of DNA apart. This is called a 'hot start', and reduces both template and primer degradation.

2.12.6 Annealing temperature

The annealing temperature is critical to the PCR reaction. It depends on the length of the DNA, the number of G/C pairs and the number of mismatches in the sequence. If the temperature is too high, the primers will not anneal to the DNA. If the temperature is too low, false priming may occur.

2.12.7 Melting temperature (*Tm*)

This is the temperature at which the two DNA strands will anneal, and there are various formulae to calculate *Tm*. For these experiements, the first formula was used *Tm*.

1. The Wallace Rule – used for short primers, <18 bases:

$$Tm = 2\,x\,(A + T) + 4\,x\,(G + C)$$

2. $Tm = 81.5 + 16.6\,(log_{10}\,[Na]) + 0.41\,(\%GC) - (625/N)$

where [Na] = sodium concentration, N = length of the primer, %GC =% GC of total base content.

3. $Tm = H\,[S + R \ln (C/4)] - 273.15°C + 16.6\,log_{10}\,[Na^+]$

where H = enthalpy, S = entropy (for helix formation for the particular DNA strand), R = molar gas constant, C = concentration of the primer.

Several forces determine the stability of the primer between the oligonucleotide and its complementary strand and ultimately the *Tm*. These are as follows:

1. Hydrogen bonds between complementary purine and pyrimidine bases (to form A:T and G:C base pairs). There are two hydrogen bonds between A:T base pairs but three between G:C which means the G:C pair is more stable as relatively more energy is required to separate the bond. Consequently the recommended G:C content of the primers is between 40% and 60%.
2. Hydrophobic interactions exist between staggered bases of the nucleic acid sequence.
3. Electrostatic forces exist which are created by the phosphate molecules of the nucleic acid backbone. The identical ionic charge of the two phosphate base backbones results in their repulsion of each other leading to primer destabilisation. To counteract this, salt cations can be added to the reaction to mask the phosphate charges thereby increasing the ionic strength resulting in the stabilisation of the double stranded sequences. Those primers with a higher ionic strength have a higher *Tm*.

2.12.8 Magnesium concentration

DNA Taq polymerase relies on an optimum magnesium chloride ($MgCl_2$) concentration to function. It was important to thaw the frozen stock solution and to vortex it to ensure uniform distribution in the stock prior to adding it to the PCR reaction. Other reagents within the reaction also can affect the actual $MgCl_2$ concentration and reduce its availability for Taq DNA polymerase. These include template DNA, chelating agents such as EDTA within the sample, dNTPs and primers, which form complexes with Mg^{2+} ions. The optimal $MgCl_2$ concentration must be selected for each reaction. Too low a $[Mg^{2+}]$ results in a low yield of PCR product, and too high, may increase the yield of non-specific products. Hence if

ultimately no PCR product is generated, the stringency of the PCR reaction could be reduced by increasing the magnesium concentration. In contrast if multiple non-specific PCR products were generated the stringency could be increased by reducing the $MgCl_2$ concentration. In these experiments, the [$MgCl_2$] was 25 mM as established after several trial runs. If the DNA samples contain EDTA or other chelators, the $MgCl_2$ concentration in the reaction mixture should be raised proportionally.

2.12.9 Number of PCR cycles

The number of PCR cycles depends on the amount of template DNA in the reaction mix and on the expected yield of the PCR product. For less than 10 copies of template DNA, 40 cycles should be performed. If the initial quantity of template DNA is higher, 25-35 cycles are usually sufficient. It is important to select the appropriate number of cycles so that the amplification product is clearly visible on an agarose gel and can be quantified. This is because there are three distinct phases to a PCR reaction:

- Exponential phase – the product exactly doubles at every cycle.
- Linear phase – reaction reagents are being consumed, hence the reaction begins to slow down.
- Plateau phase – the reaction stops and no more product is formed. The existing products will degrade if left.

Therefore as constant amplification only occurs during the exponential phase, the number of cycles to a PCR reaction should be altered to fall within this phase. In these experiments 30 cycles were carried out. The original RNA template was degraded by RNase H, leaving cDNA and spare primers.

2.13 Primer Design

Primers are short, made to order, stretches of oligonucleotides which prime the DNA template for the attachment of the polymerase. This is the first step towards duplicating the template to which the primer hybridizes. The primer directs the polymerase to move 5' to 3' on the polynucleotide chain i.e. from the phosphate group to the hydroxyl group.

The primer is antisense to the mRNA of interest and the best way to design a specific primer is to employ a primer design program available on the Web Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). To determine specificity, all sequences were compared with the Genbank using the Basic Local Alignment Search Tool (BLAST) programme available at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). When both primer sequences showed homology to the same gene, but different from the one of interest, they were discarded.

Primers are usually 15-30 nucleotides (base pairs) in length. Longer primers provide higher specificity. The GC content should be 40-60% and the primers sequence should start and end with at least one or two purine bases. More than three G or C nucleotides at the 3'-end of the primer should be avoided, as nonspecific priming may occur due to the strong nature of the C-G hydrogen bonds. The GC content and length must be chosen such that the melting temperature (*Tm*) of flanking sense and anti-sense primers should not differ by more than 5°C, using the equations above (section 2.12.7).

It is advisable to select PCR primers in such a way that the 5' and the 3' primers span different exons, so that the amplification product obtained from the cDNA would be of different length from that obtained from any contaminant genomic DNA comprising intronic sequences.

2.13.1 Adenosine receptor primers

Primers specific for all four adenosine receptors were constructed, ensuring that only the target product would be amplified. The actual gene sequence for each receptor was downloaded from the Human Genome Project website (http://www.genome.gov/10001772) and the primers were designed, ensuring exon-exon boundaries were crossed to ensure the primer was mRNA specific. The sequence was then subjected to the BLAST programme. All the primers used were made by Sigma–Genosys Ltd. The sequences are listed in table 2.5.

2.13.2 Glyceraldehyde-3-phosphate dehydrogenase primers

A housekeeping gene is typically a gene that is transcribed at a relatively constant level. The housekeeping gene's products are typically needed for maintenance of the cell. It is generally assumed that their expression is unaffected by experimental conditions. Examples include actin, ubiquitin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH-3). In these experiments GAPDH-3 was chosen as the housekeeping gene and was used to compare against the gene product of interest. The GAPDH-3 gene has nine exons and its primer pair was designed to cross exon-2 and exon-3 and generate a PCR product size of 103 base pairs (bp).

Table 2.5: Primer sequences for adenosine receptors and GAPDH-3

PRIMER		Direction	Primer Sequence	Exon	Number of Base Pairs	Tm (°C)
Adenosine Receptor	A1	*Sense*	5'-gccacagacctacttccaca-3'	5	304	62.8
	A1	*Antisense*	5'-ccttctcgaactcacacttg-3'	6	304	65.0
	A_{2A}	*Sense*	5'-aacctgcagaacgtcaccaa-3'	1	244	65.7
	A_{2A}	*Antisense*	5'-gtcaccaagccattgtaccg-3'	2	244	65.2
	A_{2B}	*Sense*	5'-gatcattgctgtcctctgg-3'	-	298	63.4
	A_{2B}	*Antisense*	5'-tcctcgagtggtccatcag-3'	-	298	64.1
	A3	*Sense*	5'-accactcaaagaagaatatg-3'	2	327	53.9
	A3	*Antisense*	5'-acttagctgtcttgaactcc-3'	2	327	59.9
GAPDH-3		*Sense*	5'-gagtcaacggatttggtcgt-3'	2	103	63.9
		Antisense	5'-ttgaggtcaatgaaggggtc-3'	3	103	63.8

2.14 Semi-quantitative analysis of gene transcription

This technique relies on the presence of an internal control which was GAPDH-3, the house-keeping gene. This is expressed in all cells. Its transcription was assumed to be at a constant level, and was used as comparison with the gene transcript of interest (the A_1, A_{2A}, A_{2B} and A_3 receptor transcripts).

Controls which were positive and negative for the mRNA of interest were included in the PCR allowing validation of the reaction. The positive control ileal mRNA from an

established cell line). The negative control contained all the reagents required for PCR except the mRNA of interest.

2.15 Agarose Gel Electrophoresis

Agarose is a seaweed extract consisting of a chain of sugar molecules. Electrophoresis through agarose gels is a method used to separate, identify and purify DNA molecules. When the gel is cooled, agarose polymersises and the sugar molecules cross-link to form a semi-solid matrix through which negatively charged DNA fragments can move when an electric current is applied to the gel. Fragments of linear DNA migrate through agarose gels with a mobility that is inversely proportional to the $\log_{10}$ of their molecular weight. Thus, a plot of the distance from the starting point (the well) to the end-point where the DNA fragments migrate, as a function of $\log_{10}$ (molecular weight) or $\log_{10}$ (base-pair number) will yield a straight line plot. Several other factors have important effects on the mobility of DNA fragments in agarose gels:

- *Agarose concentration* - Higher concentrations (2-3%) of agarose facilitate separation of small DNA fragments, whilst low agarose concentrations (0.5%) allow resolution of larger fragments (200 bp-50 kb) in length.
- *Voltage* - As the voltage applied to a gel is increased, larger fragments migrate proportionally faster than small fragments.
- *Electrophoresis buffers* - The most commonly used for dsDNA (double-stranded DNA) are TAE (Tris-acetate-EDTA) and TBE (Tris-borate-EDTA). DNA fragments migrate at different rates in these two buffers due to differences in ionic strength. Buffers maintain pH and provide ions to support conductivity. Water alone will not allow DNA migration.

- *Ethidium Bromide* - The location of the DNA within the gel can be determined by staining with low concentrations of ethidium bromide. This is a fluorescent dye that intercalates between bases of nucleic acids. It is a potent mutagen and a suspected carcinogen so must be handled with care. When it binds to DNA it alters its mass and therefore its mobility. Bands containing 1-10 ng DNA can be detected by direct examination of the gel in UV light.

2.15.1 Protocol for agarose gel electrophoresis

Agarose (3mg) was added to 150 ml TBE, and dissolved by boiling in an 800 W microwave for two minutes. TBE contains Tris which maintains a constant pH, borate which provides the ionic concentration of the buffer and EDTA, which chelates divalent cations, (stock solution, Sigma, U.K.). Ethidium bromide (10 µl; 10 mg/ml stock, Sigma, U.K.) was added to the cooling solution.

The solution was then mixed by swirling and the melted agarose was poured, with comb in place into the sealed gel former, ensuring the tray was on a flat surface. The teeth of the comb form the wells of the solidified gel once removed. The gel was left to solidify for 30 minutes.

The PCR products were then mixed with a DNA-loading buffer, prior to loading in the wells. The loading buffer contains 0.25% bromophenol blue making the products easy to identify. It also contains 30% glycerol which serves to increase the density of the PCR product.

The gel was transferred into the electrophoresis chamber, filled with 1x TBE buffer.

All samples were loaded into the wells and a record was kept of which samples were in which well.

A 100 base-pair DNA ladder was also electrophoresed simultaneously in order that the size of DNA fragments in the gels could be appropriately measured.

A 60 mA current at 120 V was applied for 30 minutes from the positive to the negative electrodes so that the DNA, which is negatively charged is encouraged to move towards the positive electrode.

The entire gel was then transferred to the UV transilluminator and visualized at 365 nm wearing protective eye wear. A Polaroid photograph was taken (section 3.7).

2.15.2 Scanning densitometry

Polaroid photographs of all the gels following electrophoresis were scanned onto a computer for analysis using an Epson GT-9500 scanning densitometer (Epsom, Japan). The image was then exported to an image analysis programme (Labworks™ Image acquisition and analysis software, Cambridge UK). The Integrated Optical Density (IOD) of each band was estimated and the level of gene transcription was measured as a ratio of the IOD of the gene compared to GAPDH-3. The levels of gene transcription were analysed for the relative levels of adenosine receptor subtypes in the bladder specimens of differing pathology.

2.16 Western Blotting

This is a semi-quantitative immunological technique by which specific proteins from a tissue sample are detected. Proteins are first separated by size on an acrylamide gel, transferred to a solid support and then probed with antibodies that are specific for antigenic epitopes displayed by the target protein.

The bladder protein samples were solubilised with denaturants and reducing agents, separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was subsequently exposed to an unlabelled antibody specific for the target adenosine receptor protein.

This primary antibody was then detected by a secondary antibody, conjugated to horseradish peroxidise (HRP). The membrane was soaked briefly in a detection reagent which elicited an HRP-catalysed oxidation of luminol. The extent of the oxidation of H_2O_2 by HRP is taken to be proportional to the amount of HRP and hence the protein of interest.

$$\text{Target protein} + \text{Luminol} + H_2O_2 \rightarrow \text{3-APA}[\Diamond] \rightarrow \text{3-APA} + \text{photon}$$

Where 3-APA is 3-aminophthalate; 3-APA[◊] is the excited state, fluorescing as it decays to a lower energy level.

The emitted light was detected on photographic film and appeared as a black band. Light peaked after 5-20 minutes and decayed slowly thereafter with a half life of 60 minutes (figure 2.6).

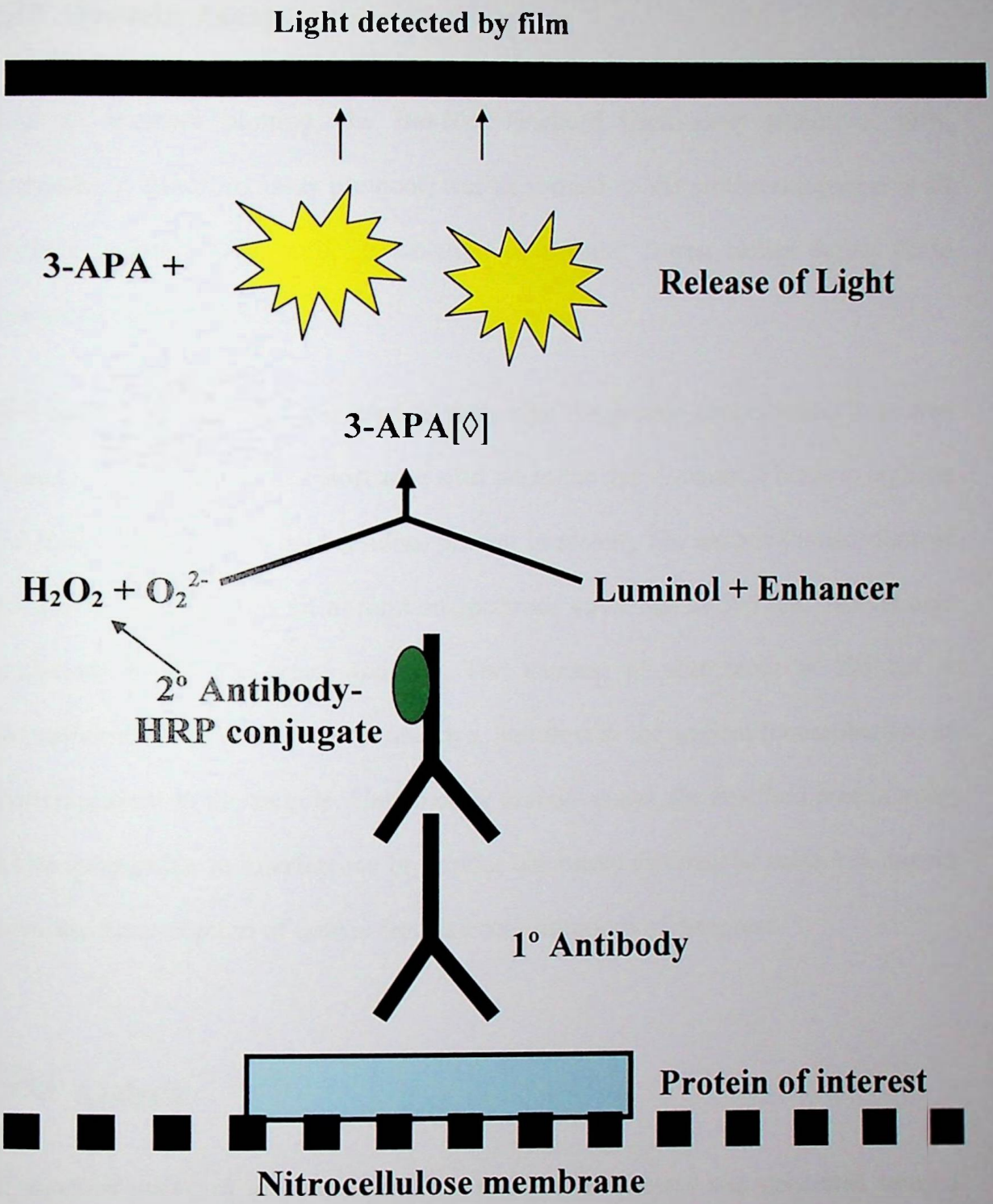


Figure 2.6: The Western Blot Detection System.

2.17 Protein Assay

Prior to Western blotting, the Bio-Rad Bradford micro-assay (Bradford, 1976; Appendix 3: Bradford assay protocol) was performed on the protein component of all detrusor samples (Appendix 2: Isolation of protein) frozen earlier during Phase separation (section 2.9.2).

This colourimetric assay was used to determine the protein concentration in protein extracts. It is based on an absorbance shift when the dye Coomassie binds to arginine and hydrophobic amino acid residues present in protein. The anionic (bound) form of the dye is blue and has an absorption spectrum maximum at 595 nm. The cationic (unbound) forms are green and red. The increase of absorbance at 595 nm is proportional to the amount of bound dye, and thus to the amount (concentration) of protein present in the sample. Unlike other protein assays, the Bradford protein assay is less susceptible to interference by various chemicals that may be present in protein samples. An exception of note is elevated concentrations of detergent.

2.17.1 Analysis

A standard curve of absorbance versus micrograms protein was generated using a commercially available standard, bovine serum albumin (BSA), figure 2.7.

Volume BSA (µl)	0	2	4	6	8	10	12
Concentration (µg/µl)	0.000	0.014	0.028	0.042	0.056	0.070	0.084
Absorbance 1	0.308	0.389	0.471	0.552	0.624	0.69	0.747
Absorbance 2	0.307	0.414	0.478	0.567	0.64	0.704	0.758
Mean Absorbance	0.308	0.402	0.475	0.560	0.632	0.697	0.753
Final Absorbance = Mean absorbance – Absorbance blank tube.	0.000	0.094	0.167	0.252	0.325	0.390	0.445

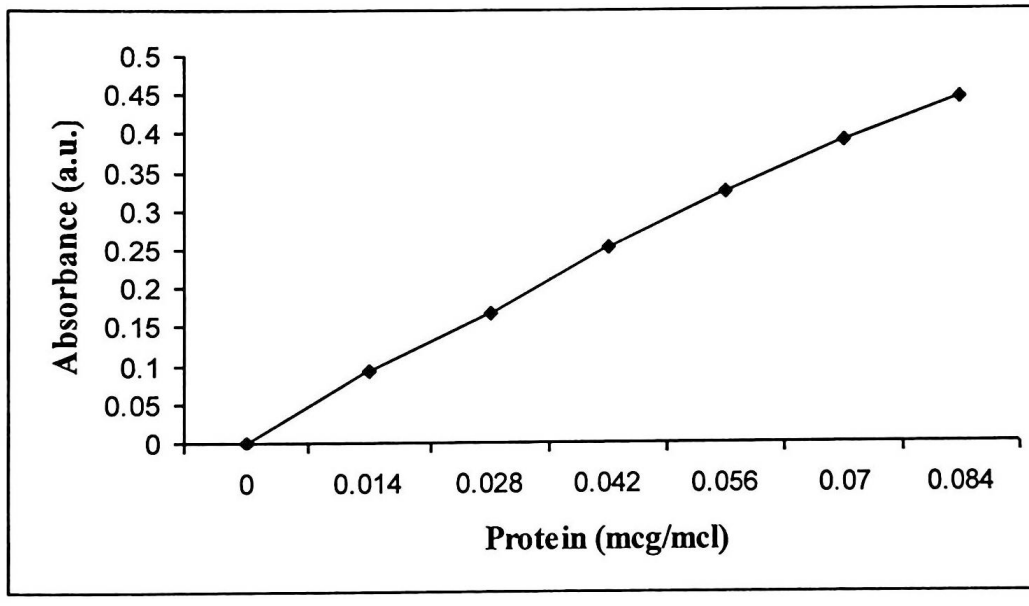


Figure 2.7: Top; Protein standard absorbance data for Bradford assay.

Concentration of protein standard solution = 1.4 µg/ µl. Mean absorbance blank tube at 595 nm = 0.3075 (Mean 0.308, 0.307).

Bottom; Protein standard curve, with absorbance measured in arbitary units (a.u.).

The curve was then used to determine concentrations of protein in the original detrusor samples (see Appendix 4: Protocol to determine protein concentration of detrusor specimen and Appendix 5: Raw data used for calculation of the protein concentration of each human detrusor specimen).

2.18 Preparing for SDS-PAGE

A molecular weight marker and the protein isolated from the detrusor specimens of 20 patients were defrosted. Twenty μg of each protein was placed into an eppendorf tube. The actual volume of each sample used depended on the protein concentration calculated from the Bradford assay. The volume was made up to a total of 16.25 μl with de-ionised water. To this 6.25 μl of 4x NuPAGE LDS Sample buffer (blue) was added. It has a pH of 8.4 which encourages a reducing agent to reduce protein disulphide bonds, thus denaturing it. It contains Coomassie G250 and Phenol Red as tracking dyes giving a sharp dye front by migrating very close to the moving front, ensuring that small peptides do not run off the gel. It also contains glycerol which increases its overall viscosity. Finally 2.5 μl of NuPAGE reducing agent was added to the sample and heated at 70°C for 10 minutes. The reducing agent was 500 mM dithiothreitol (DTT) at a 10x concentration.

The running buffer (NuPAGE SDS MOPS 20x) was then prepared, (Appendix 6: preparation of running buffer) and 600 ml used to fill the lower chamber of the electrophoresis system. The presence of SDS, a detergent, ensures dissociation of proteins into individual subunits and minimises aggregation. The upper chamber was filled with undiluted 1x MOPS running buffer (200 ml) to which 50 μl NuPAGE antioxidant was added. All agents were from Sigma.

The gel itself has a neutral pH and hence the reducing agent, DTT, does not migrate through the gel with the sample, tending to remain at the top of the gel. In the absence of an antioxidant in the upper chamber, the disulphide bonds may reoxidise during the electrophoresis, resulting in diffuse bands.

2.18.1 SDS-Polyacrylamide Gel Electrophoresis

The NuPAGE Electrophoresis system was used (Invitrogen™ life technologies, CA, U.S.A). The gel tank was washed in detergent and dried, as were the spacers. The XCell SureLock™ Mini-Cell is a vertical gel system where the gel is polymerised between two plates and the thickness of the gel is determined by the spacers used.

The gel itself was a NuPAGE Novex bis-Tris [bis(2-hydroxyethyl)imino-tris (hydroxymethyl) methane-HCl] pre-cast gel. The percentage of polyacrylamide within the gel determines the range of protein molecular weights that are separated according to size (table 2.6).

Table 2.6: Molecular weight of proteins separated by SDS-PAGE depends on % acrylamide content of the gel.

% Acrylamide	Range of proteins separated (kDa)
15	12-43
10	16-68
7.5	36-94
5.0	57-212

The comb was carefully removed from the gel cassette, as explained above, and the wells irrigated with running buffer and then the samples were loaded using a Gilson pipette. The orientation of the molecular weight markers and the samples were noted down immediately. The gel was run until the markers reached the bottom of the gel (Appendix 7: SDS-PAGE Protocol).

Two types of markers were loaded together into the well used as the ladder.

1. Prestained Protein Marker (New England Biolabs #P7708S). This contains a mixture of purified proteins covalently bonded to a blue dye, which separates into eight bands when electrophoresed. This marker is visible on the gel after Western blotting but does not show up on the membrane during chemiluminescent Western procedures. For this reason a second marker was run in conjunction.
2. Biotinylated Protein Ladder (Cell Signalling Technology™ #7727). This is a mixture of purified proteins that are covalently bonded to biotin and which resolve to ten bands on electrophoresis. The bands range in size from 10-200 kDa. This ladder may be detected by the anti-biotin antibody that is used as the secondary antibody in the Western procedure.

2.18.2 Controls

The control specimens used for the Western blots were human detrusor lysate (ab4008, Abcam Ltd., Cambridge, U.K.). These were run along-side the detrusor specimens and the ladder.

2.18.3 Transfer protocol

Once the gel had been run for 60 minutes it was carefully removed from the gel tank and placed onto a polyvinylidene difluoride (PVDF) membrane that had been pre-wet with methanol, to improve transfer of low molecular weight proteins. A gel membrane sandwich was formed (figure 2.8) and this was placed in a small square tank containing transfer buffer (Appendix 8: Preparation of transfer buffer). The black plastic grill comprised the negative electrode. Overnight transfer was performed at 16 V in a cold room. As the proteins were eluted out of the gel, they moved down to the membrane and became covalently bonded. After transfer the membrane was carefully removed wearing gloves. The corner was cut off to help orientate it and the membrane was kept protein side up during all the subsequent steps.

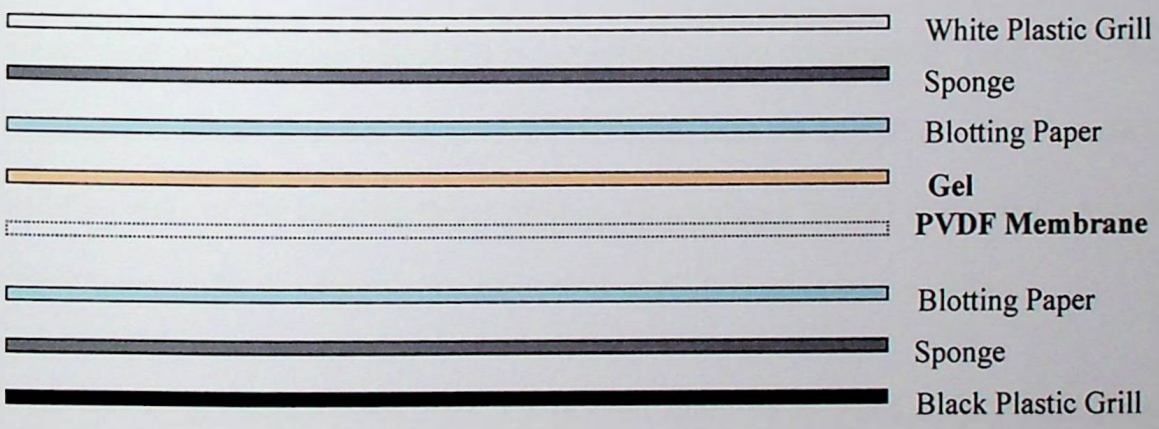


Figure 2.8: Assembly of gel membrane sandwich prior to transfer.

2.18.4 Ponceau staining of the membrane

The membrane was stained with Ponceau S Solution (0.1% Ponceau S in 5% acetic acid, Sigma chemicals). This enabled me to check transfer of proteins and to assess protein loading. Ponceau does not interfere with subsequent antibody probing and its

presence is transient so will wash away easily. The membrane was soaked in Ponceau for three minutes and then washed three times with ultrapure water, which cleared the background leaving the proteins stained pink. A photograph of the membrane was then taken.

2.18.5 "Blocking the membrane"

Since the membrane has been chosen for its ability to bind protein, and both antibodies and the target are proteins, steps must be taken to prevent interactions between the membrane and the antibody used for detection of the target protein. Blocking of non-specific binding is achieved by placing the membrane in a dilute solution of protein – typically BSA or non-fat dry milk, with a minute percentage of detergent such as Tween 20. The protein in the dilute solution attaches to the membrane in all places where the target proteins have not attached. Thus, when the antibody is added, there is no room on the membrane for it to attach other than on the binding sites of the specific target protein. This reduces "noise" in the final product of the Western blot, leading to clearer results, and eliminates false positives.

The blocking reagent used was 5% non-fat dried milk (Marvel) in PBS/Tween 20 (0.1%) buffer (Appendix 9: Preparation of blocking solution).

The membrane was left to incubate in blocking solution in a small freezer box for 1 hour. Gentle agitation was provided by a Rotomax 120 – Setting 3. The blocking solution was then rinsed off the membrane, by gentle agitation with 15 ml PBS/Tween 20 for five minutes, five times.

2.18.6 Primary Antibody Hybridisation

During the detection process the membrane is "probed" for the protein of interest with antibodies. The antibody is often linked to a reporter enzyme, which drives a colorimetric or photometric signal. Traditionally this takes place in a two-step process, hence the term primary antibody.

Antibodies are generated when a host species or immune cell culture is exposed to the protein of interest ie. the adenosine receptor (or a part thereof). They are then harvested and used as sensitive and specific detection tools that bind the protein of interest directly. The choice of primary antibody depends on availability and literature review, and should be species-reactive. The dilution factor of the antibody was obtained empirically after several repeats.

Blots were incubated overnight at 4°C on ice with the primary adenosine receptor antibody (Alpha Diagnostic chemicals, Cambridge), 1:2000 concentration in 15 ml diluent (5% blocking solution) and gentle agitation on Rotomax 120-Setting 5. This allowed the antibody to bind specifically to the immobilised protein on the membrane.

The membranes were then washed five times with gentle agitation (Setting 3), for five minutes at a time with 15 ml TBS/ Tween 20 solution. The aim was to wash away unbound antibody and to remove non-specific binding, minimising background noise.

Table 2.7: Antibodies for adenosine receptors. aa=amino acid, EC=extracellular domain, IC=intracellular domain. (All primary antibodies from Alpha Diagnostic chemicals, Cambridge, U.K.).

1^{o} Ab	Ag peptide location	Antibody Host	Type	Ab cross-reactivity
A1	Rat, 14aa, EC3	Rabbit	Polyclonal	Rat, Human, Rabbit, Chicken, Cow
A_{2A}	Canine, 30aa, IC4	Rabbit	Polyclonal	Rat, Human, Chicken, Cow
A_{2B}	Human ,16aa, EC2	Rabbit	Polyclonal	Rat, Human, Mouse
A3	Human, 15aa, EC3	Rabbit	Polyclonal	Human

2.18.7 Secondary Antibody Hybridisation

The membrane was rinsed to remove unbound primary antibody and exposed to a secondary antibody, directed at a species-specific portion of the primary antibody; due to its targeting properties referred to as "anti-mouse" or "anti-goat" etc. Secondary antibody that will bind to almost any primary antibody is the next step, to enable visualisation of primary antibody binding. The secondary antibody is usually linked to biotin or to a reporter enzyme such as horseradish peroxidase (HRP). This step confers an advantage in that several secondary antibodies will bind to one primary antibody, providing enhanced signal. The HRP-linked secondary is used in conjunction with a chemiluminescent agent, and the reaction product produces luminescence in proportion to the amount of protein. A sensitive sheet of photographic film is placed against the membrane, and exposure to the light from the reaction creates an image of the antibodies bound to the blot. The enzyme can be

provided with a substrate molecule that will be converted by the enzyme to a coloured reaction product that will be visible on the membrane.

Secondary antibody (1:5000 concentration) in 15 ml 5% blocking solution was added to each tray containing a membrane. Antibodies used were:

1. Anti-biotin, HRP-linked Antibody (Cell Signalling Technology™ #7075). This detects the biotinylated protein marker on the Western blots.

2. Goat -Anti-Rabbit IgG Antibody-HRP –linked (Cell Signalling Technology™)

Trays were gently agitated for 1 hour at room temperature (Rotomax 120-Setting 3). The trays and membranes were again washed five times for five minutes with PBS/Tween 20.

2.18.8 Tertiary Hybridisation

The biotinylated secondary antibodies were detected with HRP-labelled streptavidin (Cell Signalling Technology, Danvers, MA, U.S.A.) , which was diluted 1:1000 with blocking buffer and incubated with the membrane for 30-60 minutes at room temperature and gentle agitation. The membranes were then washed as described earlier.

2.18.9 Detection of bound proteins

The membranes were incubated at room temperature for one minute with a 10 ml Lumiglo solution (Amersham Pharmacia; 9 ml purified water, 0.5 ml peroxide, 0.5 ml Lumiglo), with gentle agitation. The membrane was carefully lifted from the tray with minimal handling using forceps, and excess fluid was shaken off onto blotting paper. The membrane was then placed face down onto cling-film (Saran wrap), and a parcel made. The outer wrap was completely dried. Kitchen towel was used to smooth the

wrap over the membranes so as to eliminate any air bubbles between the membrane and the wrap. The parcel was then placed face up onto a photographic plate and Kodak film placed on top of it. The lid of the plate was closed and the films were exposed for 5-30 minutes. After this time they were removed from the photographic plate.

This entire procedure was performed in a dark room with the use of safety lights. After exposure the film was placed into developing solution for two minutes, then into Stop solution (water) for 30 seconds and finally into Fixative solution for five minutes. Excess fluid was shaken off the films which were then hung to dry and examined on a light box. The pre-marked corners of the film were aligned with the marked corners of the Western blot in order to allow orientation and molecular weight determination of any bands present.

2.19 Statistical Analysis

Descriptive statistics were used to summarise the observations from the experiments. Gender differences between the sets were analysed using a Mann-Whitney-U test. The Kruskall-Wallis test was used to compare differences of adenosine subtype receptor expression in each of the different bladder pathologies. Pearson's correlation coefficient was used to measure the strength of the relationship between bladder pathology and subtype expression. Statistical significance was accepted at $p<0.05$. The levels of gene transcription were shown graphically and results were analysed for uniformity and skewness and by the one-sample Kolmogorov-Smirnov test for normality. Comparisons between groups were then made by one-way analysis of variance (ANOVA) with Bonferroni's correction, or unpaired Student's t-test, to identify differences between individual group means.

APPENDIX 1:

Patient Information Sheet – Bladder Physiology Study

You are being invited to take part in a research project and this Information Sheet explains what will happen if you agree. You do not have to take part in this study and you can withdraw at any time without reason or justification – you just need to tell your doctor.

Your decision to take part or otherwise will not affect your treatment in any way. If you do agree to participate your details will remain entirely anonymous. The study is being carried out in conjunction with the Institute of Urology. If you need further information please telephone Professor Christopher Fry at the Institute of Urology (020 7679 9376) who will be pleased to help.

Investigation of bladder physiology

At the Institute of Urology the major direction of our research is to understand why the bladder contracts in an uncontrollable way to cause a frequent desire to go to the toilet and even incontinence. If we can understand this process it will be easier to generate treatments to minimise the problem and to improve your quality of life.

Your operation will not be changed in any way by taking part in this study. During the operation a few extremely small pieces of the bladder are routinely sampled for the hospital pathologist and one will be allocated to our laboratory project. The tissue

sample will be broken up into its individual cells and either used immediately or after a few days. After the experiments all samples are destroyed and none is kept for future studies by us or any other group. All of these tests are directed towards understanding why the bladder contracts abnormally, and prevention of the problem in the future.

You will not benefit directly from this study but by participating you will help to find treatments that prevent the condition occurring in others. Please be sure that you are entirely happy before you agree, and ask the doctor any questions you may wish, before signing the consent form.

You will in no way be identifiable and your participation in this study is confidential, but data from the experiments using your tissue may be used in peer-reviewed publications of medical and scientific journals.

Your help and cooperation is greatly appreciated.

Patient Consent Form

Investigation of BLADDER pathophysiology

The patient should complete this form himself/herself

Have you read the 'Patient Information Sheet'? YES / NO

Have you had the opportunity to ask questions and discuss this study? YES / NO

Have you received satisfactory answers to all your questions? YES / NO

Have you received enough information about this study? YES / NO

With whom have you spoken Dr / Mr / Miss .....

Do you understand that you are free to withdraw from this study at any time without having to give reason, and without affecting your medical care?

YES / NO

Do you agree to take part in this study? YES / NO

Name (please print) .....

Signed ..... Date .....

If you require any further information please feel free to contact us on the telephone number given on the Patient Information Sheet (020 7679 9376).

APPENDIX 2: Isolation of Protein

Adapted from Roche Molecular Biochemicals

1. The organic phase was mixed thoroughly with 1.5 ml isopropanol for each 1 ml Tripure reagent required in initial homogenization (section 2.9.2).
2. Incubate for 10 minutes at room temperature to allow protein precipitate to form.
3. Centrifuge at 12,000 g for 10 minutes at 4°C.
4. Discard the supernatant.
5. Resuspend the protein pellet with 0.3 M guanidine hydrochloride in 95% ethanol. Use 2 ml guanidine hydrochloride/ ethanol for each 1 ml Tripure reagent required in initial homogenization.
6. Mix and incubate at room temperature for 20 minutes.
7. Centrifuge at 7500 g for 5 minutes.
8. Discard the supernatant. (Repeat steps 5-8 twice).
9. Add 2 ml 100% ethanol to each protein pellet.
10. Vortex to wash the pellet.
11. Incubate at room temperature for 20 minutes.
12. Centrifuge at 7500 g for 5 minutes at 4°C.
13. Discard the supernatant.
14. Remove the excess ethanol by air-drying the pellet.
15. Dissolve pellet by adding 1% SDS and repeatedly pass solution through a pipette.
16. Sediment any insoluble material by centrifuging the resuspended protein at 10,000 g for 10 minutes at 4°C.
17. Transfer the supernatant containing the protein to a new tube.

APPENDIX 3: Bradford Assay Protocol

Adapted from BioRad Corporation Information sheet.

1. Warm up the spectrophotometer for 15 minutes before use.
2. Make up the Bradford reagent: Dissolve 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol, add 100 ml 85% phosphoric acid. Dilute to one litre when the dye has completely dissolved, and filter through paper just before use.)
3. To generate a standard protein curve, place 0, 2, 4, 6, 8, 10,12 µl of the protein standard solution / bovine serum albumin (BSA), known concentration of 1.4 µg/µl. into glass cuvettes. Dilute to a total volume of 200 µl with dye.
4. Incubate 5 minutes.
5. Measure the absorbance at 595 nm.
6. Calculate the mean absorbance$_{595nm}$ of two repeats and use this mean value to generate the protein standard curve.

APPENDIX 4: Protocol to determine the protein concentration of detrusor specimens

1. Dilute 2 µl of protein sample to make a total of 16 µl with sterile water.
2. Take 5 µl of this new solution and dilute in 200 µl Bradford reagent.
3. Controls are BSA only and 0.01%SDS only.
4. Incubate 5 minutes.
5. Measure the absorbance at 595 nm.
6. Calculate the mean absorbance$_{595nm}$ of two repeats and use this mean value to calculate the protein concentration of the sample from the standard curve.

APPENDIX 5: Raw data used for calculation of the protein concentration of each human detrusor specimen, (*SDS = Mean Absorbance of SDS = 0.299).

Sample No.	Abs 1	Abs 2	Mean Abs	Mean-SDS	Protein conc.(µg/ µl)
1	0.328	0.321	0.325	0.026	0.004
2	0.429	0.388	0.409	0.110	0.019
3	0.42	0.413	0.417	0.118	0.020
4	0.333	0.324	0.329	0.030	0.005
5	0.335	0.32	0.328	0.029	0.005
6	0.353	0.346	0.350	0.051	0.009
7	0.385	0.38	0.383	0.084	0.014
8	0.499	0.494	0.497	0.198	0.033
9	0.324	0.329	0.327	0.028	0.005
10	0.413	0.391	0.402	0.103	0.017
11	0.332	0.323	0.328	0.029	0.005
12	0.325	0.338	0.332	0.033	0.006
13	0.324	0.328	0.326	0.027	0.005
14	0.409	0.414	0.412	0.113	0.019
15	0.327	0.328	0.328	0.029	0.005
16	0.325	0.324	0.325	0.026	0.004
17	0.325	0.312	0.319	0.020	0.003
18	0.317	0.314	0.316	0.017	0.003
19	0.36	0.357	0.359	0.060	0.001
20	0.321	0.32	0.321	0.022	0.004

APPENDIX 6: Preparation of running buffer

NuPAGE MOPS SDS Running Buffer (20x)	50 ml
De-ionised water	950 ml
Total Volume	**1000 ml**

Use 600 ml to fill lower chamber.

APPENDIX 7: SDS-PAGE Protocol

1. Cut open gel pouch with scissors and remove cassette.
2. Peel off tape from bottom of cassette.
3. Gently pull comb out of cassette in one smooth movement taking care not to damage the wells.
4. Rinse each well with running buffer with a pipette several times. Invert gel to remove buffer.
5. Fit gel(s) into tank and secure in place – use plastic buffer dam if only running one gel.
6. Check seals by filling upper chamber first and looking for leaks. If leaking, remove buffer and re-arrange gel(s) or dam.
7. Load prepared samples.
8. Fill outer chamber with 600 ml running buffer.
9. Run at 200 V (100-115 mA) for 60 minutes.

APPENDIX 8: Preparation of transfer buffer

Transfer (blotting) buffer (10X):	Tris Base	24.29 g
	Glycine	112.5 g
	Water	1 litre
Dilute 1:10 before use and add 20% Methanol:	10X Blotting Buffer	150 ml
Store at 4°C	Water	1050 ml
	Methanol	300 ml

APPENDIX 9: Preparation of blocking solution

Prepare PBS/Tween 20 (0.1%) buffer:	PBS	500 ml
	Tween 20	0.5 ml

Prepare blocking solution:	PBS/Tween 20 (0.1%) buffer	200 ml
	5% non-fat dried powdered milk	10 g

CHAPTER THREE

Results

3.1 Tissue responses to nerve mediated stimulation

3.1.1 Guinea-pig detrusor

Detrusor strips were allowed to equilibrate at 8 Hz for 20-60 minutes, prior to any interventions. This frequency has previously been determined as that which produces about half-maximal contractions. The mean nerve-mediated tension of guinea-pig at 8 Hz was 23.0 ± 13.8 $mN.mm^{-2}$ (n=27) and was 54.6 ± 18.2% of the maximal tension generated from the force-frequency experiments. Group data for $f_{1/2}$ values (frequency of half maximal tension) are expressed at log_{10} values (log_{10} $f_{1/2}$ ± SD) as these have been shown to be the normally distributed data set. The transformed $f_{1/2}$ value from the mean log_{10} $f_{1/2}$ ($f_{1/2}$ = $10^{\wedge logf1/2}$) is given in parenthesis. The averaged force-frequency relationship is shown in figure 3.1. Under control conditions, the mean log $f_{1/2}$ was 0.95 ± 0.32 (8.9 Hz) - figure 3.1. The maximum tension is that estimated from the curve-fit (section 2.83). A sample tracing is shown in figure 3.2.

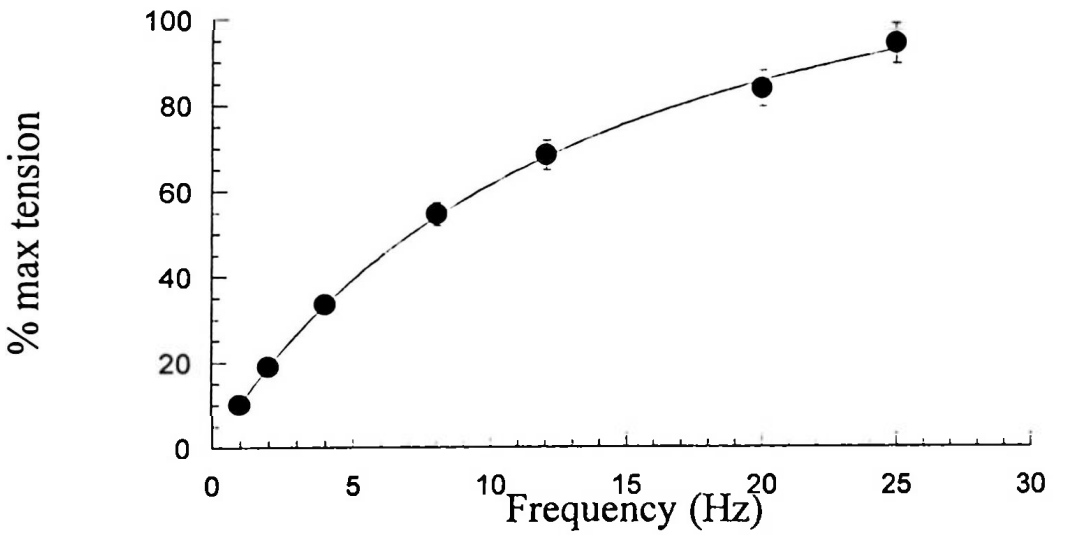


Figure 3.1: Force-frequency plot of guinea-pig detrusor in control conditions. Mean data ± SD.

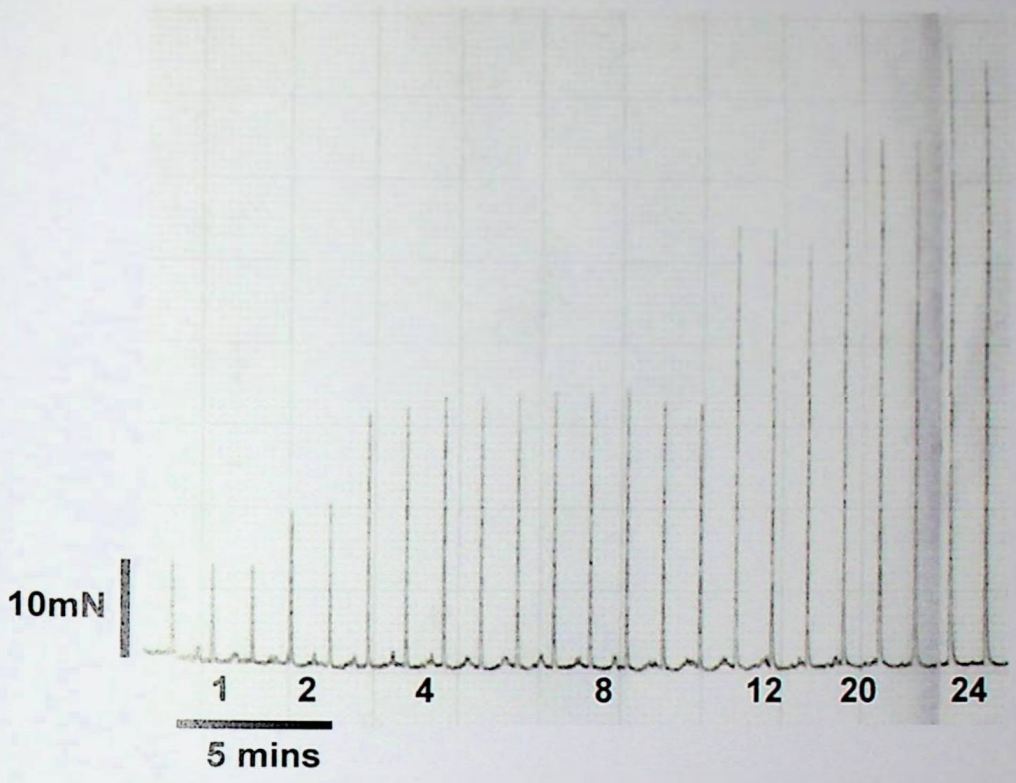


Figure 3.2: Guinea-pig detrusor: trace of tension generated in Tyrode's solution, demonstrating the force-frequency relationship.

3.1.2 Human detrusor

Detrusor strips were allowed to equilibrate at 20 Hz for 20-60 minutes, prior to any interventions. This frequency has previously been determined as that which produces approximately half-maximal contractions (Bayliss *et al*, 1999). The mean nerve-mediated tension of human stable detrusor at 20 Hz was 32 ± 16 $mN.mm^{-2}$ (n=17) and was 71 ± 13% of the maximal tension generated from the force-frequency experiments (figure 3.3). The mean log $f_{1/2}$ was 1.27 ± 0.91 (18.8 Hz). A sample tracing is shown in figure 3.4.

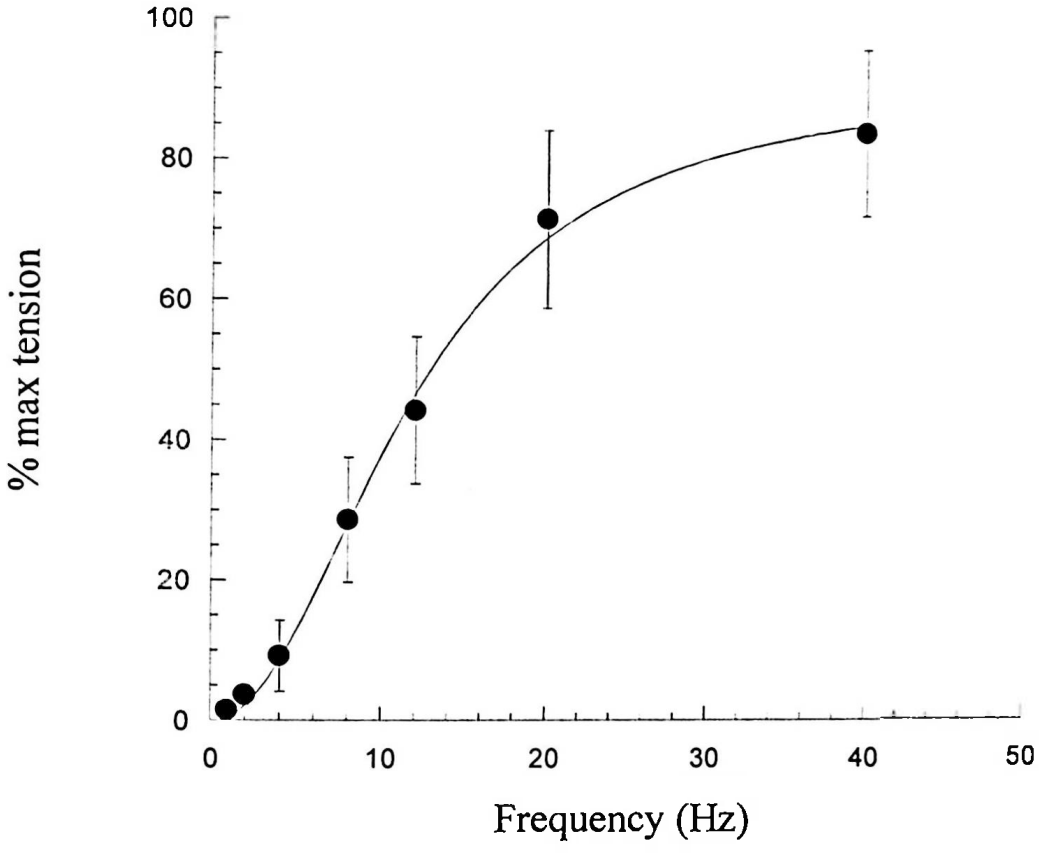


Figure 3.3: Force-frequency plot of human detrusor from stable bladders in control conditions. Mean data ± SD.

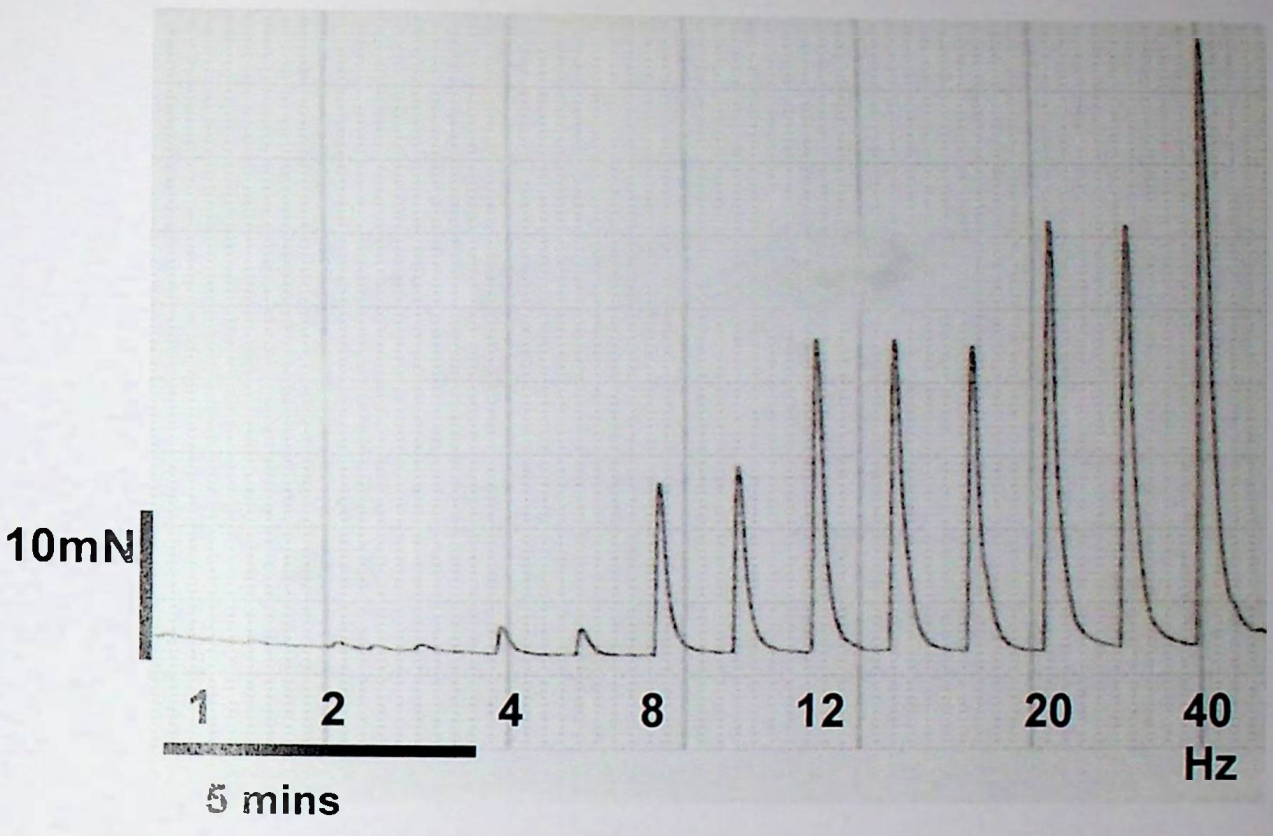


Figure 3.4: Human detrusor from a stable bladder; trace of tension generated in Tyrode's solution, demonstrating the force-frequency relationship.

With tissue from the human idiopathic overactive (IDO) group, the mean nerve-mediated tension of detrusor at 20 Hz was 39 ± 17.1 $mN.mm^{-2}$ (n=12) and was 79.4 ± 9.6 % of the maximal tension generated from the force-frequency experiments (figure 3.5). The mean $logf_{1/2}$ was 1.00 ± 0.52 (10.0 Hz). A sample tracing is shown in figure 3.6.

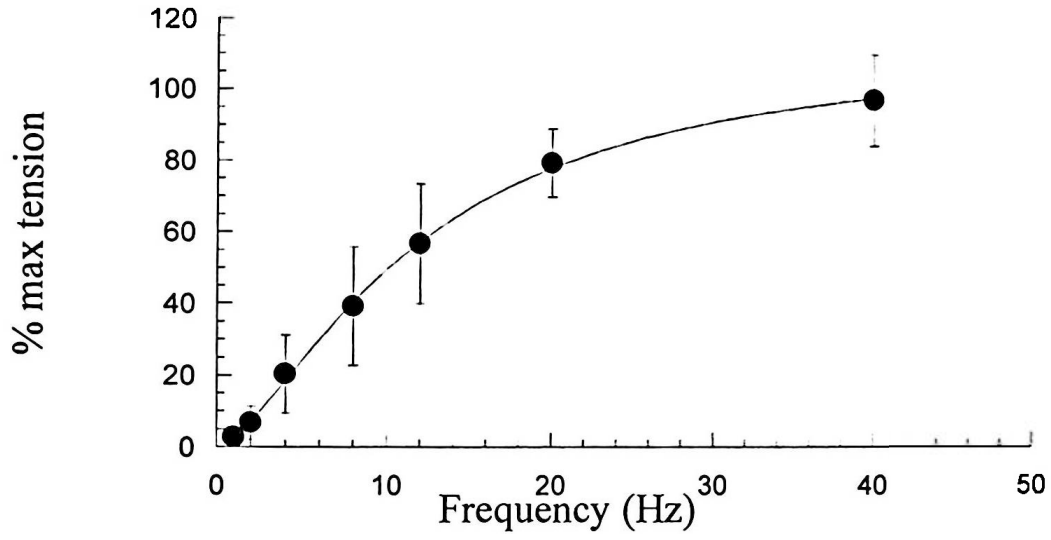


Figure 3.5: Force-frequency plot of human detrusor from idiopathic overactive bladders in control conditions. Mean data ± SD.

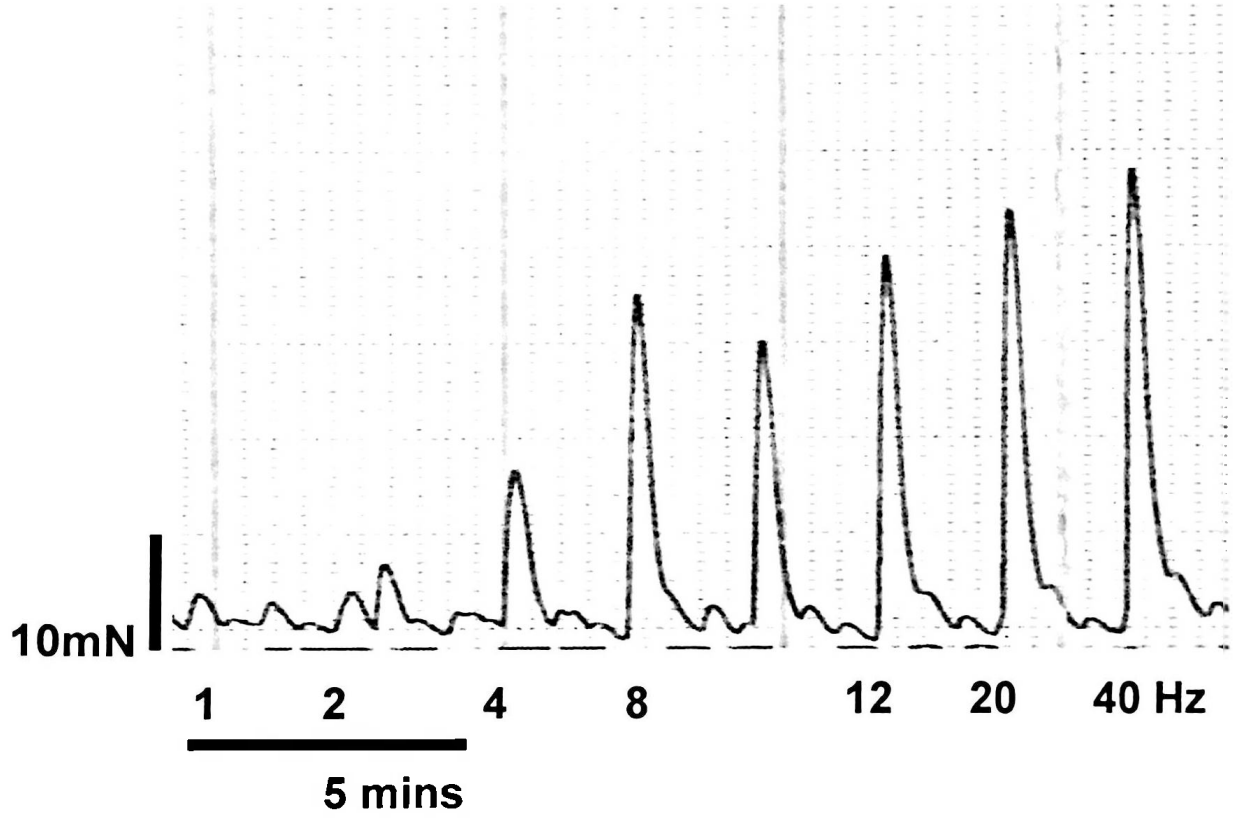


Figure 3.6: Human detrusor from an idiopathic overactive bladder; trace of tension generated in Tyrode's solution, demonstrating the force-frequency relationship.

With tissue from the neurogenic detrusor overactivity (NDO) group, the mean nerve-mediated tension at 20 Hz was 36.6 ± 22.2 $mN.mm^{-2}$ (n=18) and was 77.2 ± 12.6 % of the maximal tension generated from the force-frequency experiments (figure 3.7). The log $f_{1/2}$ was 1.07 ± 0.49 (11.7) Hz. A sample tracing is shown in figure 3.8.

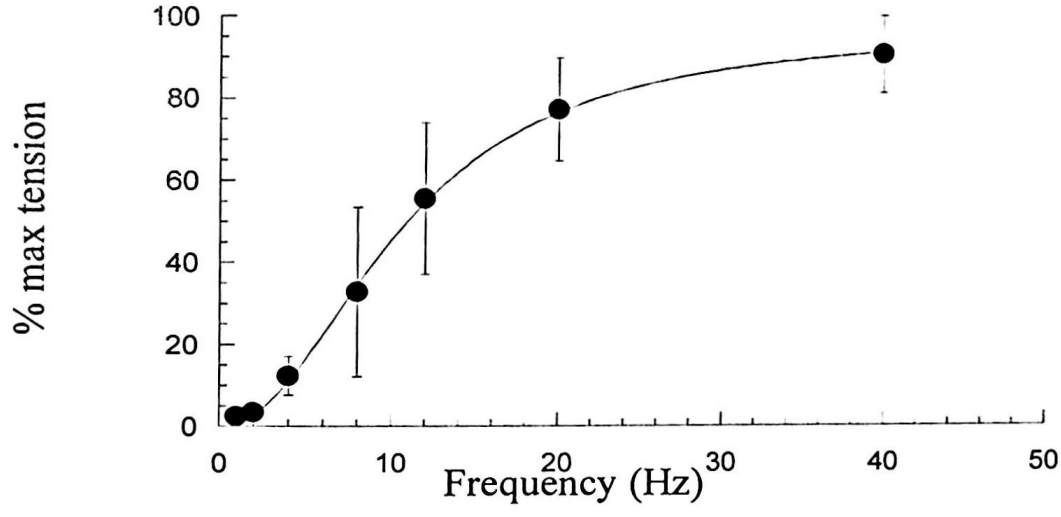


Figure 3.7: Force-frequency plot of human detrusor from neurogenic overactive bladders in control conditions. Mean data ± SD.

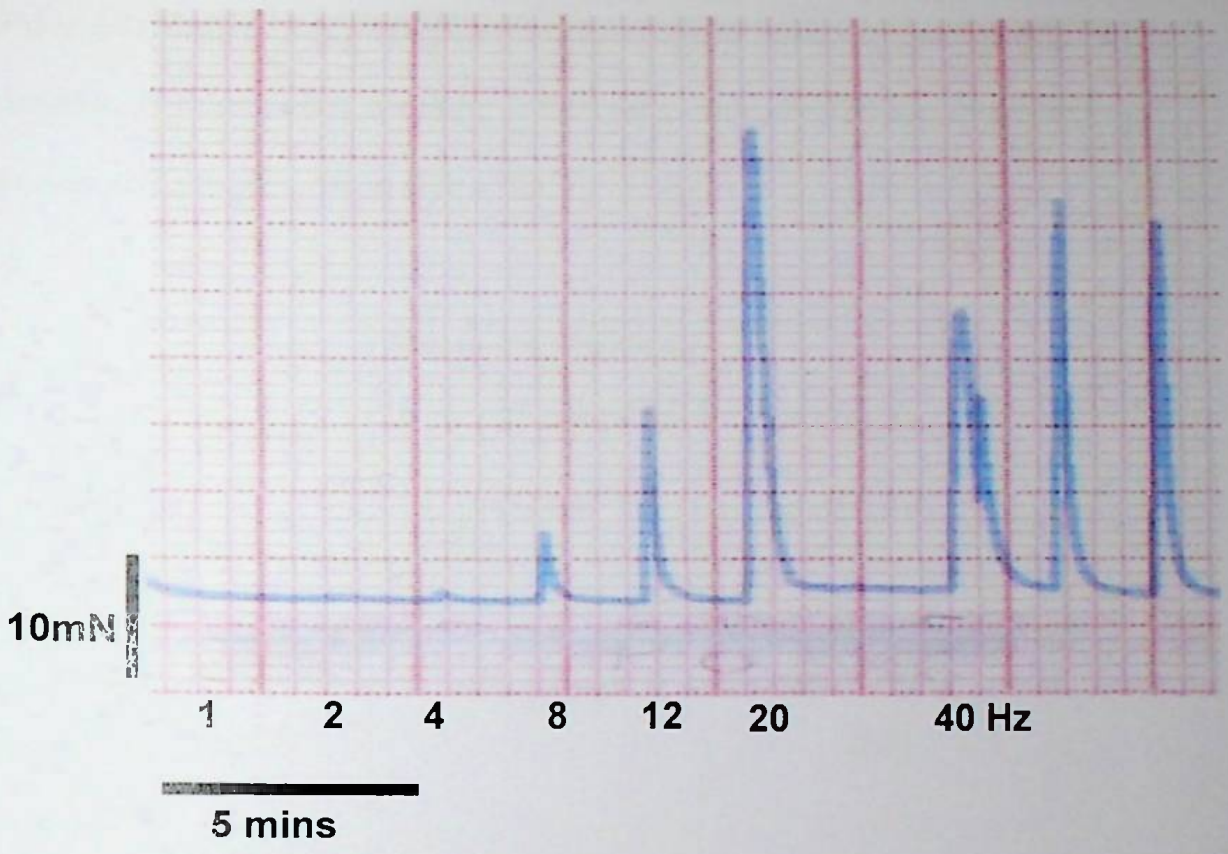


Figure 3.8: Human detrusor from a neurogenic overactive bladder; trace of tension generated in Tyrode's solution, demonstrating the force-frequency relationship.

3.1.3 Summary of force-frequency plots

Table 3.1 shows the tension values generated by guinea-pig and human samples at 8 or 20 Hz respectively and the half-maximal stimulation frequencies ($f_{1/2}$) in Tyrode's solution, as log $f_{1/2}$ values with the transformed mean value in parenthesis.

Table 3.1: Summary of force-frequency results from human and guinea-pig samples. Tension values are those at 8 Hz for guinea-pig (GP) and 20 Hz for human (H) samples. Mean data ± SD.

Tissue	Maximum tension at 8 Hz (GP) or 20 Hz ($mN.mm^{-2}$)	log $f_{1/2}$ ($f_{1/2}$, Hz)
Guinea Pig (n=27)	23.0 ± 13.8*	0.95 ± 0.32 (8.9)
Human; stable (n=17)	32.0 ± 16.2	1.27 ± 0.91 (18.8*)
Human; overactive, idiopathic (n=12)	39.0 ± 17.1	1.00 ± 0.52 (10.0*)
Human; overactive, neurogenic (n=18)	36.6 ± 22.2	1.07 ± 0.49 (11.7*)

These results indicate that the human samples all achieve similar maximum tension values, with the guinea-pig achieving a significantly lower maximum nerve-mediated contraction. Of interest however is how the $f_{1/2}$ values of the human pathological

bladders are similar to that of the guinea-pig $f_{1/2}$ and significantly different to the $f_{1/2}$ of human stable bladder.

3.2 Atropine resistance

3.2.1 Atropine resistance in guinea-pig detrusor

With guinea-pig detrusor there are two functional neurotransmitters, acetylcholine (Ach) and ATP (Bayliss *et al*, (1999). Under the stimulation conditions used here, in control experiments the neurotoxin, tetrodotoxin (TTX, 1 μM) virtually abolished contractions at all frequencies, indicating that they were nerve-mediated. On addition of 1 μM atropine to the superfusate at the end of each force-frequency experiment, tension declined leaving an atropine-resistant component. This residual fraction was in turn abolished by addition of the non-hydrolysable ATP analogue, α,β-methylene ATP (ABMA), indicating that it was due to ATP release. ABMA, after an initial excitation of muscle purinergic receptors, desensitizes them. Thus, any subsequent nerve-mediated ATP release will not excite the detrusor smooth muscle. Any small residual contractions, when present, were resistant to TTX. The atropine-resistant component (due to release of Ach) was calculated as the TTX-sensitive contraction after the addition of atropine, the residual component due to the release of ATP. Force-frequency curves were generated before and after the addition of atropine and $f_{1/2}$ values estimated.

Note: In this section concerned with atropine-resistance the values of nerve-mediated tension after the addition of atropine are quoted as median values (25%, 75% inter-quartiles). This is because in human detrusor samples from stable bladders there was generally no atropine-resistant contraction and so the group values would not be

normally distributed. In all other sections where tension values are quoted, mean ± SD values are given.

At 8 Hz, median tension was reduced significantly from 31.8 (20.2, 39.7) $mN.mm^{-2}$ to 12.3 (10.9, 29.9) $mN.mm^{-2}$ in 1 μM atropine. This was equivalent of a reduction to 53.8 (27.8, 72.3) % of control. Figure 3.9 shows the mean force-frequency plots in the absence and presence of atropine.

The log $f_{½}$ values (with mean transformed $f_{½}$) in atropine (n=20) was 0.72 ± 0.54 (5.2 Hz); and was significantly smaller from that in the same preparations prior to the addition of atropine (0.91 ± 0.76, 8.2 Hz, paired t-test).

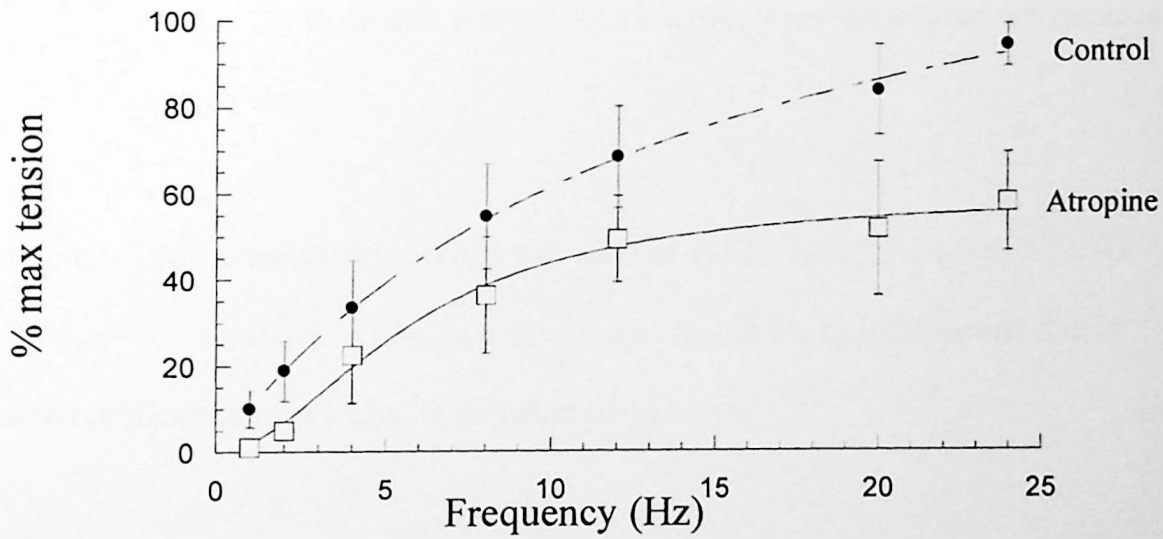


Figure 3.9: Force-frequency plot for guinea-pig detrusor strips in the presence of 1 μM atropine as a percentage of estimated maximal tension in control conditions. Mean values ± SD.

The reduction of the $f_{1/2}$ value in the presence of atropine suggests that contractions elicited at higher stimulation frequencies are more susceptible to the antagonist than at lower frequencies. One explanation may be that Ach is released preferentially at higher frequencies, and by inference ATP is released preferentially at lower frequencies.

This thesis has devised an additional method to evaluate frequency-dependent changes to tension generation by different transmitters. Tension values were recorded at two frequencies: one below $f_{1/2}$ (at 2 Hz, T_2) and one above $f_{1/2}$ (at 20 Hz, T_{20}). The ratio T_{20}/T_2 was also recorded. If an agent were to depress the magnitude of nerve-mediated contractions:

- a reduction of T_{20}/T_2 would indicate that high frequency-dependent contractions were affected more.
- an increase of T_{20}/T_2 would indicate that low frequency-dependent contractions were affected more.

Atropine (1 μM) reduced significantly the value of T_{20}/T_2 from 5.3 ± 1.0 to 2.7 ± 0.8 ($p<0.05$, n=10; table 3.2). These data are consistent with the hypothesis that Ach is released preferentially at higher stimulation frequencies.

3.2.2 Atropine-resistance in human detrusor

Detrusor from urodynamically-proven stable human specimens demonstrated minimal atropine resistance, 2.6 (0.7, 4.5)% of control at 20 Hz. This indicates therefore that Ach is likely to be the sole neurotransmitter responsible for mediating detrusor contraction in stable bladders.

Conversely, atropine-resistance was demonstrated in specimens from pathological bladders. In the idiopathic group, 1 μM atropine reduced median maximum force to 44.6 (38.8, 48.8) % of that in control conditions. The log $f_{½}$ values before and after atropine were 1.00 ± 0.52 (10.0) and 0.95 ± 0.50 (8.9 Hz), and were significantly smaller after atropine addition (paired t-test).

The median tension generated at 20 Hz was reduced from 43.2 (30.3, 47.2) $mN.mm^{-2}$ in control to 20.1 (10.8, 23.1) $mN.mm^{-2}$ in atropine. Figure 3.10 displays the force-frequency relationship of human idiopathic detrusor in the presence of 1 μM atropine as a percentage of estimated maximal tension in control conditions.

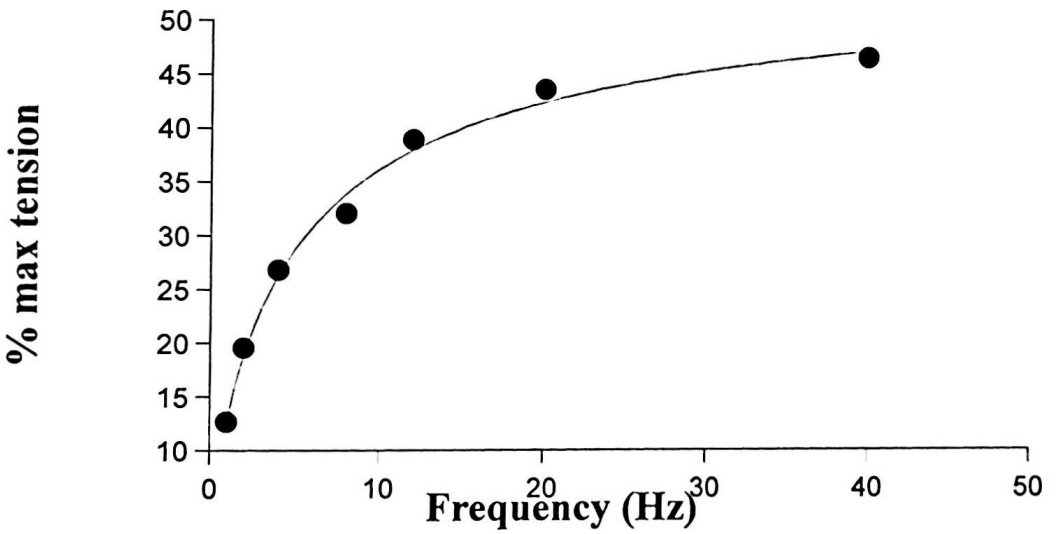


Figure 3.10: Force-frequency plot for human idiopathic detrusor in the presence of 1 μM atropine as a percentage of estimated maximal tension in control conditions. Closed circles = Mean values ± SD.

With the NDO group (n=18), 1μM atropine reduced the median maximum force to 37.4 (35.4, 40.5) % of that in control conditions. The log $f_{1/2}$ values before and after

atropine were 1.11 ± 0.38 (12.8) and 1.01 ± 0.52 (10.2), and were significantly smaller (paired t-test) after atropine addition.

The median tension generated at 20 Hz was also reduced from 33.5 (19.6, 53.9) $mN.mm^{-2}$ in control to 12.5 (5.0, 21.8) $mN.mm^{-2}$ in atropine. The force-frequency relationship of the NDO group in the presence of 1 μM atropine is shown in figure 3.11.

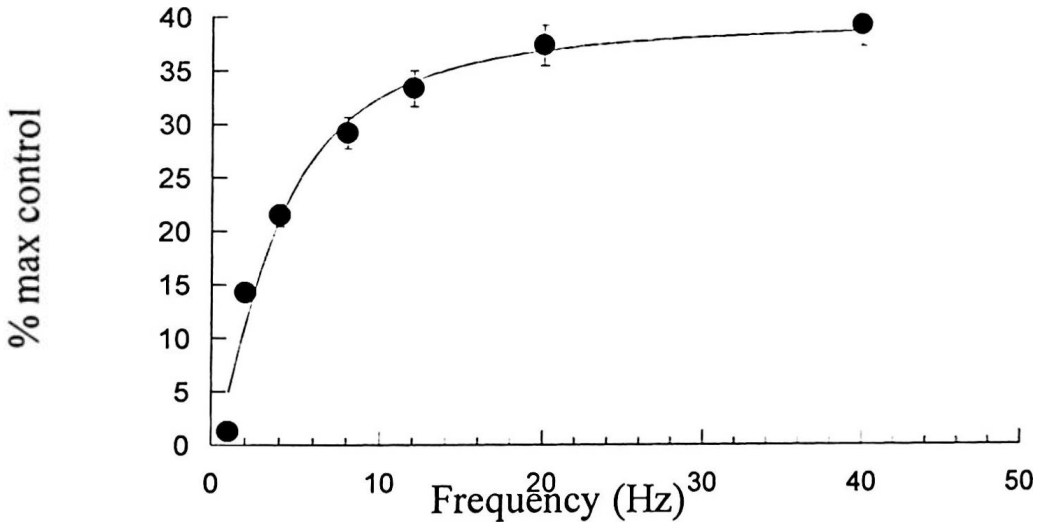


Figure 3.11: Force-frequency plot for human detrusor; NDO group, in the presence of 1 μM atropine as a percentage of estimated maximal tension in control conditions.

Atropine-resistant contractions were present in both the IDO (9/12) and NDO (16/18) groups, with significantly fewer occurrences in stable bladders specimens (2.6; 0.7, 4.5)% (figure 3.12). These results indicate that detrusor overactivity in the human bladder is associated with the presence of atropine-resistant contractions.

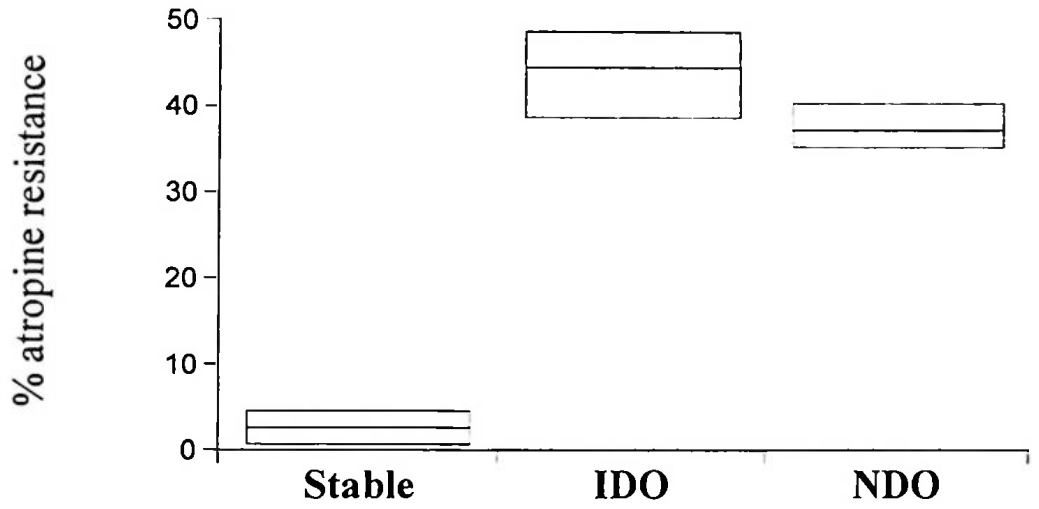


Figure 3.12: Box plot of atropine-resistant contractions as a percentage of control, in human bladder samples from the three pathological groups at 20 Hz stimulation. The median, 25% and 75% percentiles have been plotted.

3.3 Frequency-dependent neurotransmitter release

3.3.1 Guinea-pig detrusor

To test further the hypothesis that there is a frequency-dependent differential release of neurotransmitters, the effects of ABMA, (a non-hydrolysable analogue of ATP) was also examined at 2 Hz (low frequency) and 20 Hz (high frequency) as for atropine, section 3.2.1. ABMA was used to desensitise P2X receptors. Any resulting nerve-mediated contractions would therefore be due to activation of muscarinic receptors by acetylcholine. That Ach and ABMA were the sole transmitters were shown by the combined addition of atropine and ABMA when TTX-sensitive contractions were completely abolished. Data are shown in table 3.2.

*Table 3.2: Ratio of tension measured at 20 Hz and 2 Hz (T_{20}/T_2) in control guinea-pig detrusor and in the presence of 10 μM ABMA or 1 μM atropine. *p<0.05.*

	Control	ABMA (n=8)		Control	Atropine (n=10)
T_{20}/T_2 ratio	4.4 ± 0.8	18.3 ± 10.9*		5.3 ± 1.0	2.7 ± 0.8 *

When 10 μM ABMA alone was added to the superfusate, the T_{20}/T_2 was significantly increased, from 4.4 ± 0.8 to18.3 ± 10.9. Table 3.2 summarises the T_{20}/T_2 measurements.

In summary, the effect of ABMA was to significantly reduce nerve-mediated contraction at low and half-maximal frequencies, but not at high frequencies of stimulation (figure 3.13). This implies that ATP predominates as the neurotransmitter at low stimulation frequencies. The effect of atropine was to cause significant reduction of contraction at 20 Hz but not at 2 Hz, implying that detrusor contraction is mediated via a cholinergic pathway at high stimulation frequencies (figure 3.14). These results indicate that there is preferential release of ATP at low stimulation frequencies and preferential acetylcholine release at higher frequencies.

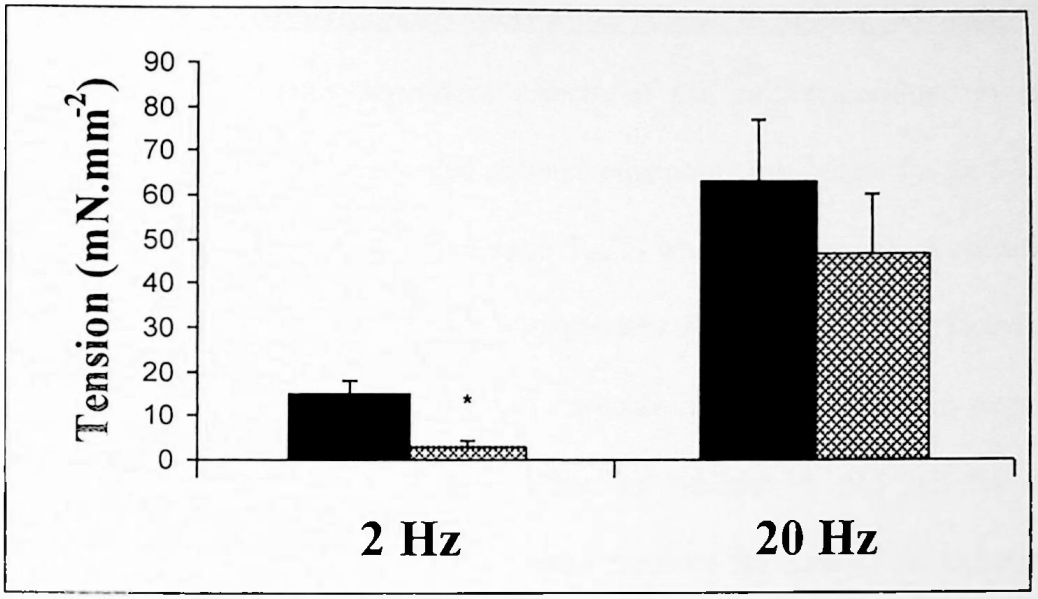


*Figure 3.13: Bar chart of tension generated at different field-stimulation frequencies in the presence of 10 μM ABMA, in guinea-pig detrusor. Solid bars = Control tension in Tyrode's solution; Hatched bars = Tension generated in ABMA, (n=8). *p<0.05.*

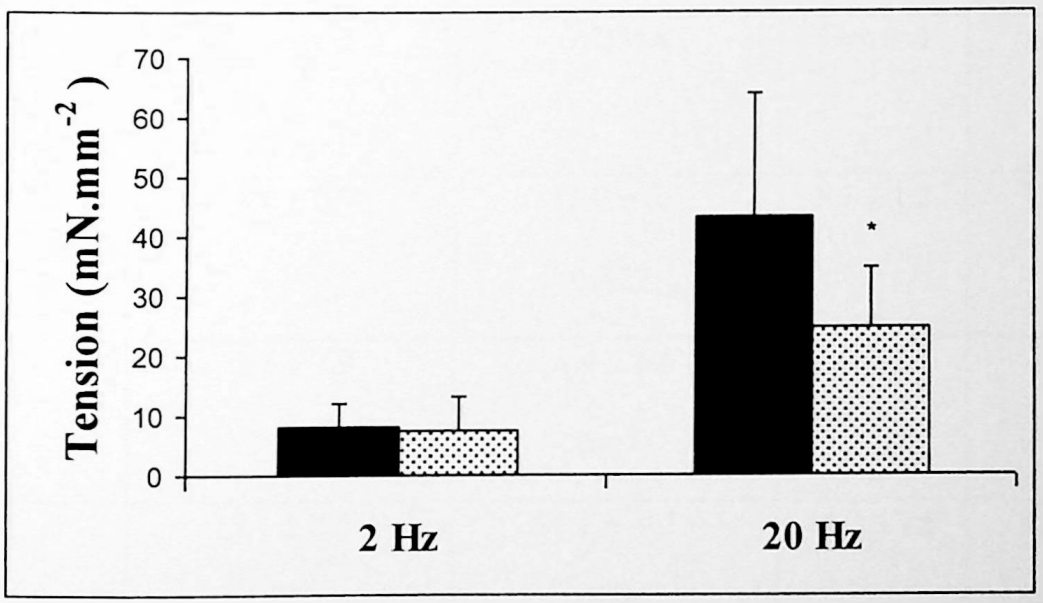


*Figure 3.14: Bar chart of tension generated at different field-stimulation frequencies in the presence of 1μM atropine, in guinea-pig detrusor. Solid bars = Control tension generated in Tyrode's solution; Hatched bars = Tension in atropine, (n=10). *p<0.05.*

3.3.2 Frequency-dependent neurotransmitter release in human detrusor

To quantify the frequency-dependent effects of the neurotransmitter in human detrusor, tension values were recorded at two frequencies: one below $f_{1/2}$ (at 4 Hz, T_4) and one above $f_{1/2}$ (at 40 Hz, T_{40}). The ratio T_{40}/T_4 was also recorded. A reduction of T_{40}/T_4 would indicate that high frequency-dependent contractions were affected more. However, an increase of T_{40}/T_4 would indicate that low frequency-dependent contractions were affected more. The effect of adding 10 μM ABMA to the superfusate was examined and the T_{40}/T_4 ratio recorded for each of the three human sub-groups (table 3.3).

Table 3.3: Summary of T_{40}/T_4 ratios for human detrusor.

T_{40}/T_4 ratio in human detrusor				
Detrusor	**Control**	**ABMA**	**Control**	**Atropine**
Stable	4.4 ± 0.7	13.9 ± 5.7 (n=11)	5.1 ± 1.2	13.5 ± 7.1 (n=7)
IDO	5.7 ± 2.9	9.4 ± 6.6 (n=7)	4.9 ± 2.1	4.7 ± 1.9 (n=9)
NDO	5.1 ± 2.5	21.1 ± 9.7* (n=6)	14.2 ± 7.8	9.6 ± 7.1* (n=16)

In stable human detrusor samples there was no demonstrable significant difference between the T_{40}/T_4 ratios in ABMA or atropine. The values in ABMA and atropine

exhibited a wide variability; in the presence of atropine this was due to the near abolition of contraction. With ABMA, contractions at 4 Hz were nearly abolished; this is consistent with a small ATP-dependent component at this frequency, but the reliable estimate of a T_{40}/T_4 ratio was made very difficult. Overall for human detrusor from stable bladders Ach can be regarded as the predominant neurotransmitter generating force.

In the IDO group there was no significant difference between the T_{40}/T_4 ratios for ABMA or atropine. However, in the NDO group, ABMA significantly increased the T_{40}/T_4 ratio indicating that ABMA preferentially reduced the nerve-mediated contractions at low frequencies, implying that ATP predominates as the neurotransmitter at low stimulation frequencies (figure 3.15). In the same group of patients, but with different experiments atropine significantly reduced the T_{40}/T_4 ratio (figure 3.16), implying that detrusor contraction is mediated by acetylcholine at high frequencies of stimulation. The NDO group demonstrates similar differential frequency-dependent neurotransmitter release as does the guinea-pig.

It is noted in table 3.3 that the control values for T_{40}/T_4 were different in the ABMA and atropine experiments. However because the T_{40}/T_4 values in the presence of ABMA or atropine are paired with their respective controls then the conclusions drawn from the experiments remain significant.

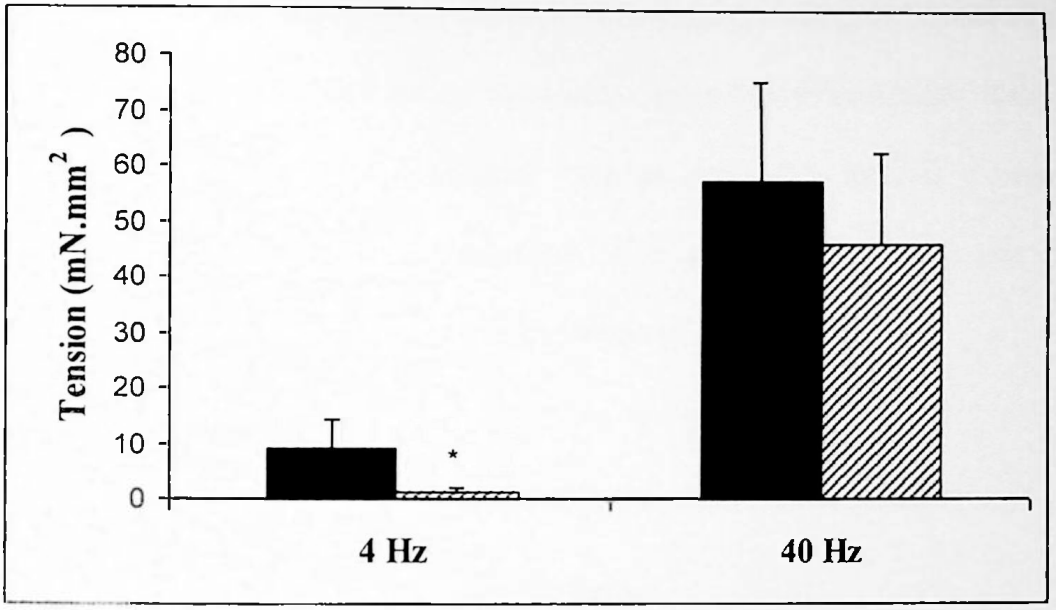


*Figure 3.15: Bar chart of tension generated at different field-stimulation frequencies in the presence of 10 μM ABMA in human NDO bladders. Solid bars = Control tension in Tyrode's solution; Hatched bars = Tension in ABMA, (n=6). *p<0.05.*

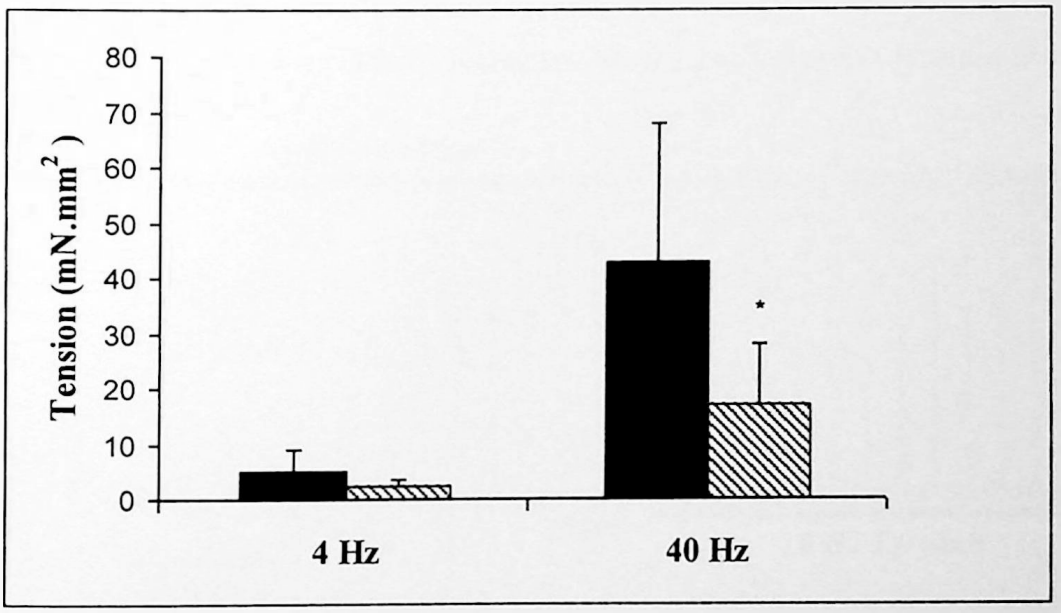


*Figure 3.16: Bar chart of tension generated at different field-stimulation frequencies in the presence of 1 μM atropine in human NDO bladders. Solid bars = Control tension in Tyrode's solution; Hatched bars = Tension in atropine, (n=16). *p<0.05.*

These results indicate that ATP-mediated contractions can occur in human detrusor, but they are more frequently found in samples from overactive rather than stable bladders. These data are also consistent with the hypothesis there is a frequency-dependent differential release of transmitter: ATP at lower frequencies and Ach at higher frequencies at least in detrusor from human NDO bladders.

3.4 Effect of P1-specific compounds on nerve-mediated contractions in guinea-pig detrusor

3.4.1 Effect of adenosine on the force-frequency relationship

After establishing the frequency-dependent release of neurotransmitters in guinea-pig and NDO human detrusor, the effect of P1 compounds was analysed. Adenosine, an ATP breakdown product, has been hypothesized to depress detrusor contraction by negative feedback via P1 receptors. A sample tracing is shown in figure 3.17, when after removal of adenosine, tension increased when stimulation was resumed at 8 Hz..

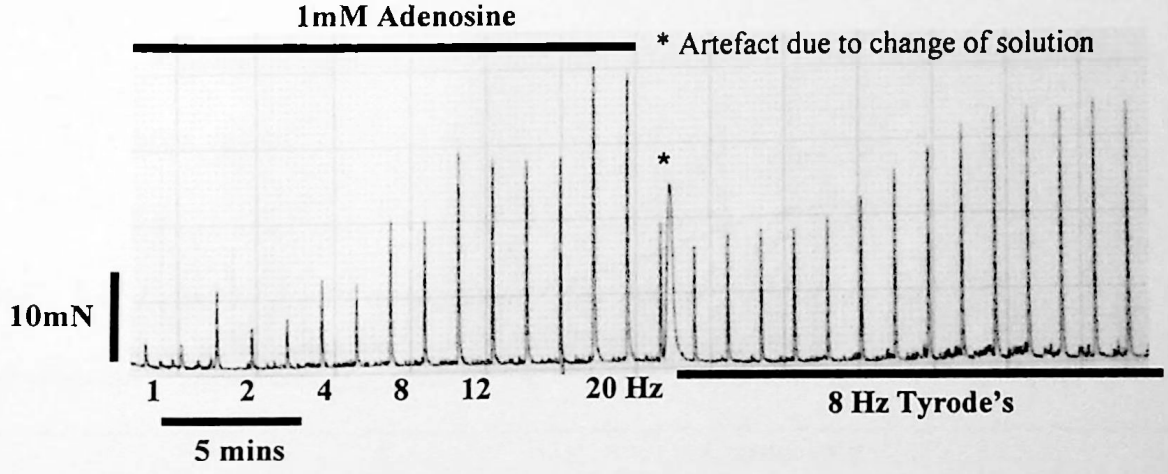


Figure 3.17: Tracing showing tension generated from a force-frequency relationship in guinea-pig detrusor in the presence of 1 mM adenosine; stimulation frequencies (Hz) are listed. After removal of adenosine, stimulation is resumed at 8 Hz.

A concentration-dependent effect of adenosine and the A1-specific agonist, CPA, on EFS contractions has been previously determined (Y. Ikeda; unpublished data) and maximum effective concentrations of 1 mM and 10 μM were found. These concentrations were used in subsequent experiments. Consequently, detrusor strips were exposed to the agonists for 10-minute intervals at 8 Hz and the percentage change in EFS-mediated contractions during interventions, compared to the average of pre and post-control periods, was determined.

The maximum reduction of EFS contractions was to 52.6 ± 12.4% of control at 8 Hz with adenosine (n=16). However, table 3.4 shows that 1 mM adenosine reduced force relatively more at low stimulation frequencies, i.e. 2 Hz (below $f_{1/2}$ for half-maximal activation) compared to high frequencies (20 Hz). 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 (0.98 ± 0.54; 9.6 Hz adenosine vs. 0.80 ± 0.49; 6.3 Hz control; figure 3.18, table 3.5). Note: to calculate $f_{1/2}$ in the presence and absence of adenosine each force-frequency curve was normalised to its own maximum value.

Table 3.4: Effect of 1 mM adenosine on contractile force at low and high stimulation frequencies ($f_{1/2}$ guinea-pig ≈ 8 Hz). (*p<0.05, high vs. low frequency).

	Guinea-pig (n=17)	
Frequency (Hz)	**2**	**20**
% control	10.8 ± 0.7*	33.8 ± 2.6

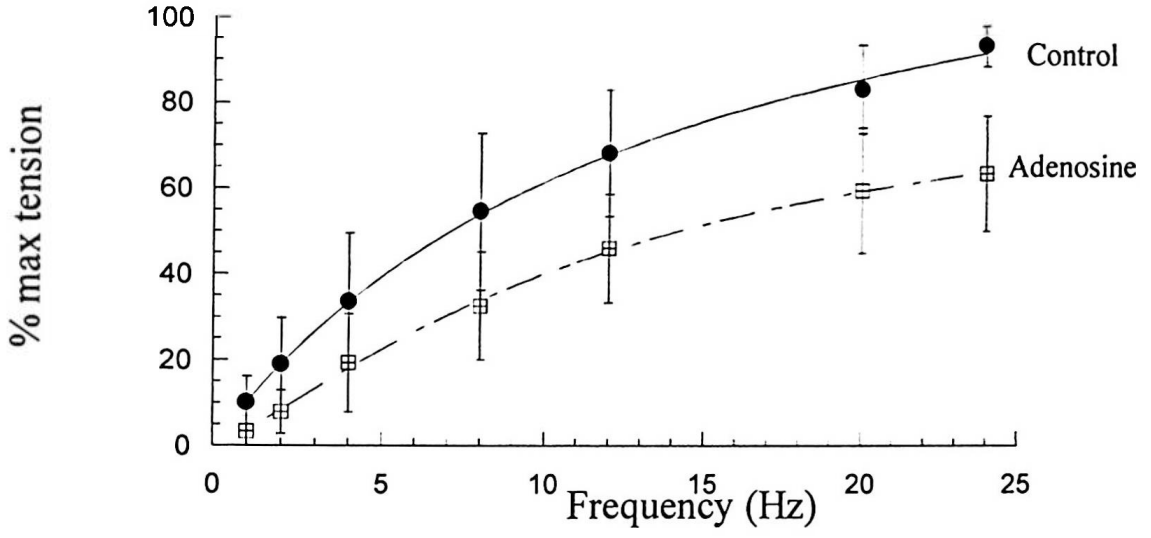


Figure 3.18: Force-frequency of guinea-pig detrusor in the presence of 1 mM adenosine. Control = circles, Adenosine = squares. Data as mean ± SD. Tension values are normalized to maximum values in the absence of adenosine.

3.4.2 Effect of P1 agonists and antagonists

After establishing that adenosine has a depressant effect on nerve-mediated contractions especially at low stimulation frequencies, further experiments were conducted to elucidate if there was a specific P1 receptor that was involved. Detrusor strips were exposed to subtype-selective agonists such as CPA (A_1 agonist), CGS-21680 (A_{2A} agonist) and IB-MECA (A_3 agonist). and force-frequency relationships were generated (table 3.5). In addition the A_{2B} antagonist alloxazine was used because of the relative non-selectivity of available A_{2B} agonists. The concentrations used were maximum values derived from preliminary experiments or from the literature.

*Table 3.5: Summary of force-frequency results for guinea-pig detrusor,*p< vs. control.*

Compounds	Mean %-tension of control at 8 Hz	log control $f_{1/2}$ ($f_{1/2}$ Hz)	log $f_{1/2}$ in presence of compound ($f_{1/2}$ Hz)
1 mM adenosine (n=16)	52.6 ± 12.4*	0.80 ± 0.49 (6.3)	0.98 ± 0.54 (9.6)*
10 μM CPA (n=9)	70.3 ± 20.7*	0.86 ± 0.51 (7.2)	0.84 ± 0.52 (7.0)
10 μM CGS-21680 (n=9)	77.7 ± 16.9*	0.82 ± 0.51 (6.6)	0.85 ± 0.55 (7.1)
1 μM alloxazine (n=14)	84.6 ± 16.9*	0.82 ± 0.51 (6.6)	0.96 ± 0.64 (9.1)*
10 μM IB-MECA (n=10)	99.5 ± 8.0	0.85 ± 0.54 (7.1)	0.75 ± 0.54 (3.5)

10 μM CPA reduced contractions (70.3 ± 20.7% of control), but to a significantly smaller extent than adenosine – table 3.5. However, the $\log f_{1/2}$ value was not altered significantly by CPA. The smaller effect of CPA than adenosine implies that it is not only the A1 receptor subtype that is contributing to adenosine-modulated reduction of nerve-mediated contractions. This phenomenon was investigated further.

10 μM CGS-21680 has high A_{2A} affinity, combined with good A_{2A} vs. A1 selectivity. The agonist significantly reduced contractions to 77.7 ± 16.9 % of control at 8 Hz (n=9). The $\log f_{1/2}$ value however was not significantly changed significantly (table 3.5)

1 μM alloxazine (n=14), significantly reduced nerve-mediated contractions to 84.6 ± 16.9% of control, at 8 Hz stimulation. The $logf_{1/2}$ value was significantly increased by alloxazine (0.96 ± 0.64 (9.1 Hz) vs. 0.82 ± 0.51 (6.6 Hz)

The A3-specific agonist, IB-MECA (10 μM) had no significant effect on the force generated at 8 Hz; 99.5 ± 8.0% of control, (n=10) or on the $logf_{1/2}$ value.

The T_{20}/T_2 atropine and ABMA experiments suggested a differential frequency-dependent release of neurotransmitters. To investigate the hypothesis that P1-receptors modulate a frequency-dependent reduction of nerve-mediated contraction, the T_{20}/T_2 ratio was calculated and compared, in the presence and absence of P1-compounds including adenosine (Table 3.5). If the P1-compound increased the T_{20}/T_2 ratio this would indicate a preferential reduction of force at low frequencies, and by inference an effect on the ATP pathway.

Adenosine, CPA, CGS-21680 and alloxazine significantly reduced the force of contraction more at lower (2 Hz) stimulation frequencies than higher ones (20 Hz) as seen by increases in the T_{20}/T_2 ratio. IB-MECA displayed no evidence of altering the T_{20}/T_2 ratio (table 3.6).

Table 3.6: Ratio of tension measured at 20Hz and 2Hz (T_{20}/T_2) in control guinea-pig detrusor and in the presence of various P1-compounds.

Compound	Control T_{20}/T_2 ratio	T_{20}/T_2 Ratio
Control (n=38)	4.50 ± 1.41	-
1 mM adenosine (n=17)	4.14 ± 1.00	6.38 ± 2.22*
10 μM CPA (n=9)	3.55 ± 1.04	5.55 ± 1.69*
10 μM CGS-21680 (n=14)	4.09 ± 1.14	5.10 ± 1.75*
1 μM alloxazine (n=14)	3.95 ± 1.18	4.66 ± 1.31*
10 μM IB-MECA (n=10)	4.07 ± 1.27	4.04 ± 1.31

These results show that adenosine significantly reduced nerve-mediated contractions in detrusor through a combination of A_1, A_2 receptor activation. The differential effects at low frequencies (i.e. an increase of T_{20}/T_2 and T_{40}/T_4 values) is consistent with the hypothesis that there is, at least in part, a preferential effect on ATP release. Moreover, measurement of the T_{20}/T_2 and T_{40}/T_4 ratios may be a more sensitive way to demonstrate frequency-dependent effects on contraction, compared to estimation of $f_{1/2}$ values. This is because differential effects on T_{20}/T_2 and T_{40}/T_4 values were seen

with adenosine and specific A1 and A2 compounds. These ratios will be used henceforth to evaluate frequency-dependent effects of agonists.

3.5 Effect of P1-specific compounds on nerve-mediated contractions in human detrusor

3.5.1 Effect of adenosine on the force-frequency relationship

Force-frequency curves were analysed in all 3 human groups (figure 3.21), to determine whether adenosine had the same effect as in guinea-pig detrusor. Consequently, detrusor strips were exposed to 1 mM adenosine for 10-minute intervals at 20 Hz stimulation and the percentage change in EFS-mediated contractions during interventions, compared to the average of pre and post-control periods was determined.

Adenosine significantly reduced force to 58.0 ± 14.8% of control (n=18) at 20 Hz stimulation with samples from stable bladders. With IDO samples force was also significantly but modestly reduced (86.9 ± 16.2% of control, n=12). The NDO samples again showed a significant reduction of force at 20 Hz (40.2 ± 11.6% of control, n=18) – see also table 3.7.

Values of T_{40}/T_4 were also calculated for the effect of adenosine in the three groups – the data are also shown in table 3.7. The ratio was increased in the stable and NDO group, but not in the IDO group.

*Table 3.7: The effect of 1 mM adenosine on nerve-mediated contraction at 20 Hz stimulation and on T_{40}/T_4 ratios. Data expressed as mean ± SD, *p<0.05 compared to control.*

Human detrusor Group	Tension % of control	T_{40}/T_4 control	T_{40}/T_4 1mM adenosine
Stable (n=18)	58.0 ± 14.8*	19.2 ± 12.2	40.2 ± 19.2*
IDO (n=12)	86.9 ± 16.2	5.0 ± 2.1	5.3 ± 0.8
NDO (n=18)	40.2 ± 11.6*	5.1 ± 2.1	15.5 ± 6.3*

3.5.2 Effect of P1 agonists and antagonists

Having established that adenosine significantly reduced nerve-mediated contraction in the stable and NDO groups, further experiments were conducted to elucidate if there was a specific P1 receptor that was involved in this modulation of nerve-mediated detrusor contraction.

Detrusor strips were exposed to CPA, CGS-21680, alloxazine and IB-MECA (as per guinea-pig experiments), and force-frequency relationships were established. Data from stable bladders is shown in table 3.8, which lists the percentage tension compared to control at 20 Hz stimulation as well as values of the T_{40}/T_4 ratios.

*Table 3.8: Stable human bladder samples; The effect of adenosine and P1 receptor subtype compounds on nerve-mediated contraction (20 Hz) and the T_{40}/T_4 ratio. Mean data ± SD. *p<0.05 compared to control.*

Compound	% tension of control at 20Hz	T_{40}/T_4 control	T_{40}/T_4 Compound
1 mM adenosine (n=16)	58.0 ± 14.8*	19.2 ± 12.2	40.2 ± 19.2*
10 μM CPA (n=6)	93.9 ± 5.7	21.7 ± 7.0	20.5 ± 10.1
10 μM CGS-21680 (n=6)	86.9 ± 20.6	21.7 ± 7.0	29.5 ± 8.9*
1 μM alloxazine (n=10)	92.2 ± 17.9	17.9 ± 13.0	18.6 ± 13.1
10 μM IB-MECA (n=6)	93.6 ± 3.7	23.6 ± 7.8	26.8 ± 8.4

The data in table 3.8 indicates that apart from adenosine, none of the other P1-compounds tested exhibited any significant reduction in force of nerve-mediated contractions. Adenosine and CGS significantly increased T_{40}/T_4 values. However, none of the other compounds exhibited any significant effect on nerve-mediated contractions, or T_{40}/T_4 ratios. This implies that adenosine has a significant effect on the force-frequency relationships and tension. It has a negative inotropic effect on detrusor contraction that is frequency-dependent. One explanation is that with human

stable bladders the effects of adenosine are partially modulated via A_{2A} receptors, but that adenosine may have an additional non-specific effect .

For IDO bladder samples data are shown in table 3.9. These results are consistent with only modest effects of adenosine-receptor compounds on samples from IDO bladders.

Table 3.9: IDO human bladder samples; The effect of adenosine and P1 receptor subtype compounds on nerve-mediated contraction (20 Hz) and the T_{40}/T_4 ratio. Mean data ± SD. *p<0.05 compared to control.

Compound	% tension of control at 20 Hz	T_{40}/T_4 control	T_{40}/T_4 Compound
1 mM adenosine (n=12)	86.9 ± 16.2*	5.0 ± 2.1	5.3 ± 0.8
10 μM CPA (n=6)	97.7 ± 5.6	4.4 ± 1.0	4.4 ± 1.6
10 μM CGS-21680 (n=6)	93.9 ± 8.1	4.4 ± 0.9	4.6 ± 0.6
1 μM alloxazine (n=7)	97.8 ± 4.1	5.5 ± 2.7	5.1 ± 2.1
10 μM IB-MECA (n=5)	99.7 ± 6.1	6.1 ± 2.3	5.7 ± 1.5

There was a small, but modest, reduction of force by adenosine in the IDO group, but was not repeated with any of the selective compounds.

For samples from NDO bladders data are similarly shown in table 3.10.

Table 3.10: NDO human bladder samples; The effect of adenosine and P1 receptor subtype compounds on nerve-mediated contraction (20 Hz) and the T_{40}/T_4 ratio. Mean data ± SD. *p<0.05 compared to control.

Compound	% tension of control at 20 Hz	T_{40}/T_4 control	T_{40}/T_4 Compound
1 mM adenosine (n=10)	42.1 ± 9.8*	5.1 ± 2.1	15.5 ± 10.8*
10 μM CPA (n=7)	49.4 ± 16.2*	6.6 ± 0.6	9.8 ± 7.6
10 μM CGS-21680 (n=7)	93.7 ± 23.1	6.6 ± 0.6	8.5 ± 3.2
1 μM alloxazine (n=8)	68.1 ± 23.6*	6.5 ± 3.4	10.7 ± 5.6
10 μM IB-MECA (n=5)	82.7 ± 16.9	6.9 ± 0.8	7.6 ± 0.8

Adenosine, CPA and alloxazine all reduced nerve-mediated force, but only adenosine increased the value of the T_{40}/T_4 ratio.

3.6 Post-synaptic P1-receptors

P1-receptors may modulate detrusor contraction either pre or post-synaptically i.e. by affecting transmitter release or by acting on the muscle itself. By activating muscarinic (M3) receptors which cause direct detrusor contraction, it is possible to avoid the release of neurotransmitters which accompanies EFS. This enabled the effects of P1-compounds on muscle receptors to be studied.

Contractures were generated by 0.3 μM carbachol in the absence or presence of P1-receptor compounds. The effect of these compounds was determined as a percentage change in the force of pre- and post-intervention control carbachol contractions.

3.6.1 The effect of P1-compounds on carbachol-induced contractures of guinea-pig detrusor

The addition of 1 mM adenosine to carbachol (n=10), caused a significant reduction of the contraction as compared to control, 63.6 ± 19.9 % (p=0.03).

10 μM CPA (n=8), caused a small but significant reduction in the force of the carbachol contracture, 89.6 ± 15.0% (p=0.001).

10 μM CGS-21680 (n=10), 1 μM alloxazine (n=8) and 10 μM IB-MECA (n=8) did not significantly affect the carbachol contracture as compared to their paired controls (96.9 ± 6.8, 96.7 ± 1.1 and 96.8 ± 1.2% respectively). These results are summarised in table 3.11.

*Table 3.11: Effect of P1-compounds on the carbachol contracture in guinea-pig detrusor; *p<0.05 as compared to control (paired Student's t-test).*

Compound	% of control carbachol contracture
1 mM adenosine (n=10)	63.6 ± 19.9 *
10 μM CPA (n=10)	89.6 ± 15.0*
10 μM CGS-21680 (n=10)	96.9 ± 6.8
1 μM alloxazine (n=8)	96.7 ± 1.1
10 μM IB-MECA (n=8)	96.8 ± 1.2

The reduction of the carbachol contracture by adenosine, was not mirrored by any effect of P1-specific compounds, except in part by CPA.

3.6.2 The effect of P1 compounds on carbachol-induced contractures of stable human detrusor

The addition of 1 mM adenosine to carbachol (n=9), caused a significant reduction of the contracture as compared to control, 58.1 ± 6.8% ($p<0.05$). The other, P1-specific, compounds did not cause significant reduction of the carbachol contracture in human stable detrusor. Results are shown in table 3.12.

Table 3.12: Effect of P1-compounds on the carbachol contracture in human stable bladder; *$p<0.05$ as compared to control (paired Student's t-test).

Compound	% of control carbachol contracture
1 mM adenosine (n=9)	58.1 ± 6.8*
10 μM CPA (n=9)	90.7 ± 3.7
10 μM CGS-21680 (n=8)	98.2 ± 2.1
1 μM alloxazine (n=8)	98.3 ± 1.1
10 μM IB-MECA (n=8)	99.9 ± 1.7

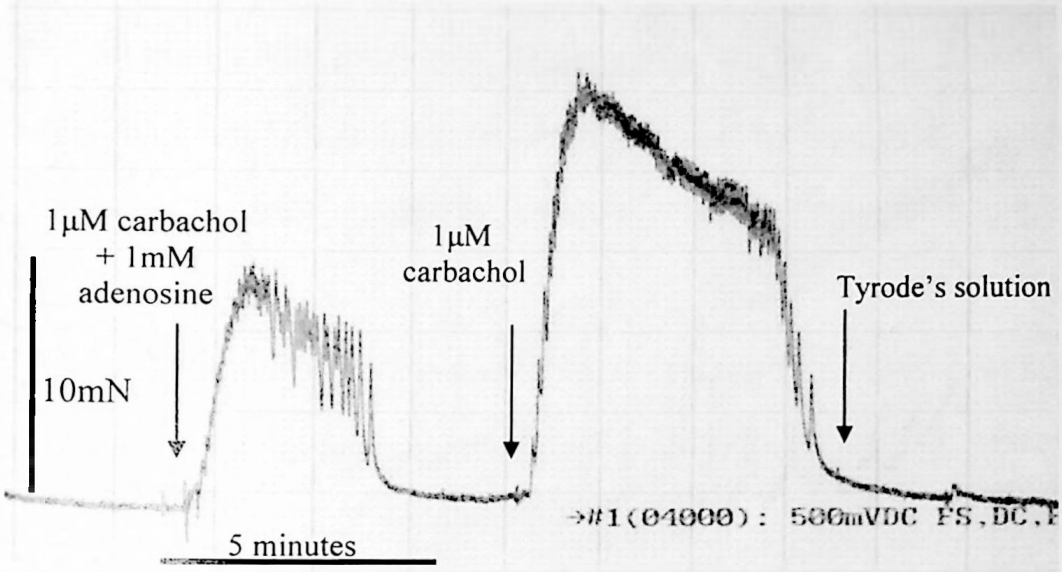


Figure 3.19: Effect of 1mM adenosine on carbachol contractures in detrusor from stable human bladder.

3.6.3 The effect of P1 compounds on carbachol-induced contractures of human samples with neurogenic detrusor overactivity

Adenosine caused a significant reduction in the carbachol contracture of human NDO specimens. The other, P1-specific, compounds had no significant effect (table 3.13).

Table 3.13: Effect of P1-compounds on the carbachol contracture in human NDO bladders; *p<0.05 as compared to control (paired Student's t-test).

Compound	% of control carbachol contracture (HNDO)
1 mM adenosine (n=6)	56.4 ± 19.9*
10 μM CPA (n=6)	93.3 ± 1.8
10 μM CGS-21680 (n=4)	98.4 ± 1.9
1 μM alloxazine (n=4)	89.1 ± 2.0
10 μM IB-MECA (n=4)	98.2 ± 8.5

3.6.4 Summary of tension data

These results indicate a difference in the force-frequency relationship between the guinea-pig and human bladder. The human samples (all three groups) required a higher stimulation frequency than the guinea-pigs, as indicated by the log $f_{1/2}$ values, which may indicate a difference in the neurotransmitter release between the two species.

Adenosine caused a significant increase in the log $f_{1/2}$ values from force-frequency plots in both guinea-pig and human detrusor samples. This right shift may be due to inhibition of neurotransmitter release or depression of contractile activity in the smooth muscle itself. Furthermore, adenosine caused a greater reduction of force at low stimulation frequencies than at high stimulation frequencies, in both guinea-pig and human samples. Experiments showed that ATP is released preferentially at low frequencies of stimulation so it may be hypothesised that adenosine attenuates ATP release more than acetylcholine.

The A_1 agonist, CPA reduced the force of contraction in the guinea-pig and NDO human samples, but had minimal effects on stable and IDO samples from human bladders. CPA did cause an increase in the T_{20}/T_2 ratio in guinea-pig samples, preferentially reducing force at lower frequencies of stimulation. CPA did not significantly affect log $f_{1/2}$ values in either species. This implies receptors other than the A_1 receptor contribute to adenosine-modulated reduction of nerve-mediated contractions.

The A_{2A} agonist CGS-21680 reduced the force of contraction in the guinea-pig but had minimal effect on human samples. CGS-21680 did not significantly affect log $f_{1/2}$ values in either species, but did cause an increase in the T_{20}/T_2 and T_{40}/T_4 ratios in guinea-pig and stable human samples, respectively.

The A_{2B} antagonist itself, alloxazine significantly reduced nerve-mediated contractions, increased the log $f_{1/2}$ value and increased the T_{20}/T_2 and T_{40}/T_4 ratios, in the guinea-pig and in NDO samples, respectively.

The A_3 agonist, IB-MECA had no significant effect on the force, log $f_{1/2}$ value or T_{20}/T_2 and T_{40}/T_4 ratios, in either species.

These results indicate that ATP-mediated contractions are present in human detrusor, but are significantly greater in pathological overactive bladders as compared with stable ones.

As ABMA caused a greater reduction of force at lower frequencies (as shown by the increase in T_{20}/T_2 ratio in guinea-pig), this implies ATP has a proportionately greater effect at lower stimulation frequencies. Higher levels of ATP at low frequencies suggest higher levels of adenosine too. Conversely, atropine generated a significant force reduction at the higher stimulation frequencies, indicating that Ach is preferentially released at this higher range. These results are mirrored in samples from NDO bladders suggesting that differential release from motor nerves of the neurotransmitter ATP (and subsequently its breakdown product adenosine), at low stimulation frequencies have a role to play in the overactive bladder.

In both stable and NDO bladder samples the only significant reduction in the carbachol contracture was achieved by adenosine. The other P1-receptor agonists and antagonists had no effect in either of the bladder sub-groups. These results suggest that the adenosine mediated reduction of detrusor contractions which are stimulated by muscarinic agonists, are mediated through a non-P1-receptor route.

3.7 Adenosine receptor gene expression in human detrusor

After examining the effects of adenosine on detrusor contraction in guinea-pig and human bladders, the aim was to determine whether the levels of the different subtypes of adenosine receptor vary in bladders of differing pathology, both in terms of gene transcription (RT-PCR studies) and translation (Western blotting studies).

3.7.1 Quantification of RNA, RT-PCR and agarose gel electrophoresis

Once the total cellular RNA (tcRNA) portion had been extracted from the frozen detrusor samples, the concentration and purity were determined by recording the integrated optical density from the Biotech photometer (section 2.9.5). The results for the 20 detrusor samples are shown in table 3.14.

To produce cDNA from RT-PCR at least 4 μg of RNA is required and this quantity was used in all the experiments. The precise volume required for each specimen was calculated using the concentration in table 3.14. The amount of RNA extracted from samples in the stable, idiopathic and neurogenic groups were not significantly different from each other (ANOVA, $p>0.05$; table 3.15). Thus similar quantities of RNA were extracted from biopsy samples from the three groups of bladders.

The amplified PCR products were electrophoresed on an agarose gel. A negative control (NC) lacking any detrusor tissue, a positive control (PC) of ileal mRNA, and a

100 base pair (bp) ladder (L) were also run on the gel. The gels were visualised under UV light and a Polaroid photograph of each gel was taken. The photograph was analysed to determine the IOD of each band (section 2.15.2) and the level of gene transcription was estimated in a semi-quantitative manner as a ratio of the IOD of the gene compared to GAPDH-3, the internal control.

Table 3.14: Integrated optical density (IOD) and tcRNA concentration in each sample.

Specimen Number	Bladder Pathology	Gender	Age (Years)	IOD (Absorbance)	RNA concentration (µg/µl)
1	Stable	M	54	0.358	1.432
2	Stable	M	50	0.542	2.168
3	Stable	M	75	0.245	0.980
4	Stable	M	72	0.335	1.34
5	Stable	F	73	0.223	0.892
6	Stable	M	70	0.394	1.576
7	Idiopathic	M	71	0.293	1.172
8	Idiopathic	M	65	0.402	1.608
9	Idiopathic	M	66	0.414	1.656
10	Idiopathic	F	65	0.533	2.132
11	Idiopathic	F	56	0.475	1.900
12	Idiopathic	M	39	0.615	2.460
13	Idiopathic	M	56	0.390	1.560
14	Neurogenic	M	39	0.629	2.516
15	Neurogenic	F	46	0.240	0.960
16	Neurogenic	M	27	0.283	1.120
17	Neurogenic	M	17	0.489	1.956
18	Neurogenic	F	43	0.595	2.380
19	Neurogenic	F	37	0.437	1.748
20	Neurogenic	F	37	0.365	1.460

Table 3.15: Group data for RNA concentrations according to pathology. Medians, 25, 75% quartiles.

Group	Gender	Age	RNA conc. (μg/ml)
Stable	5M, 1F	65.7 ± 10.7	0.69 (0.54, 0.77)
IDO	5M, 2F	59.7 ± 10.6	0.83 (0.79, 1.01)
NDO	3M, 4F	35.1 ± 10.0	0.87 (0.65, 1.08)

3.7.2 Analysis of data

The levels of gene transcription were analysed for the relative levels of adenosine receptor subtypes in the bladder specimens of differing pathology.

3.7.2.1 A_1 receptor gene expression

All samples were prepared and run on two separate occasions. Polaroid photographs of A_1 gels for samples 1-10 and samples 11-20, each with positive and negative controls, as well as a calibration ladders, are shown in figure 3.20. Corresponding bands for GAPDH-3 are also shown. The average IOD from both runs was used in the final analysis (table 3.16). The A_1 receptor bands were weak in all samples analysed, especially when compared to the corresponding GAPDH-3 bands.

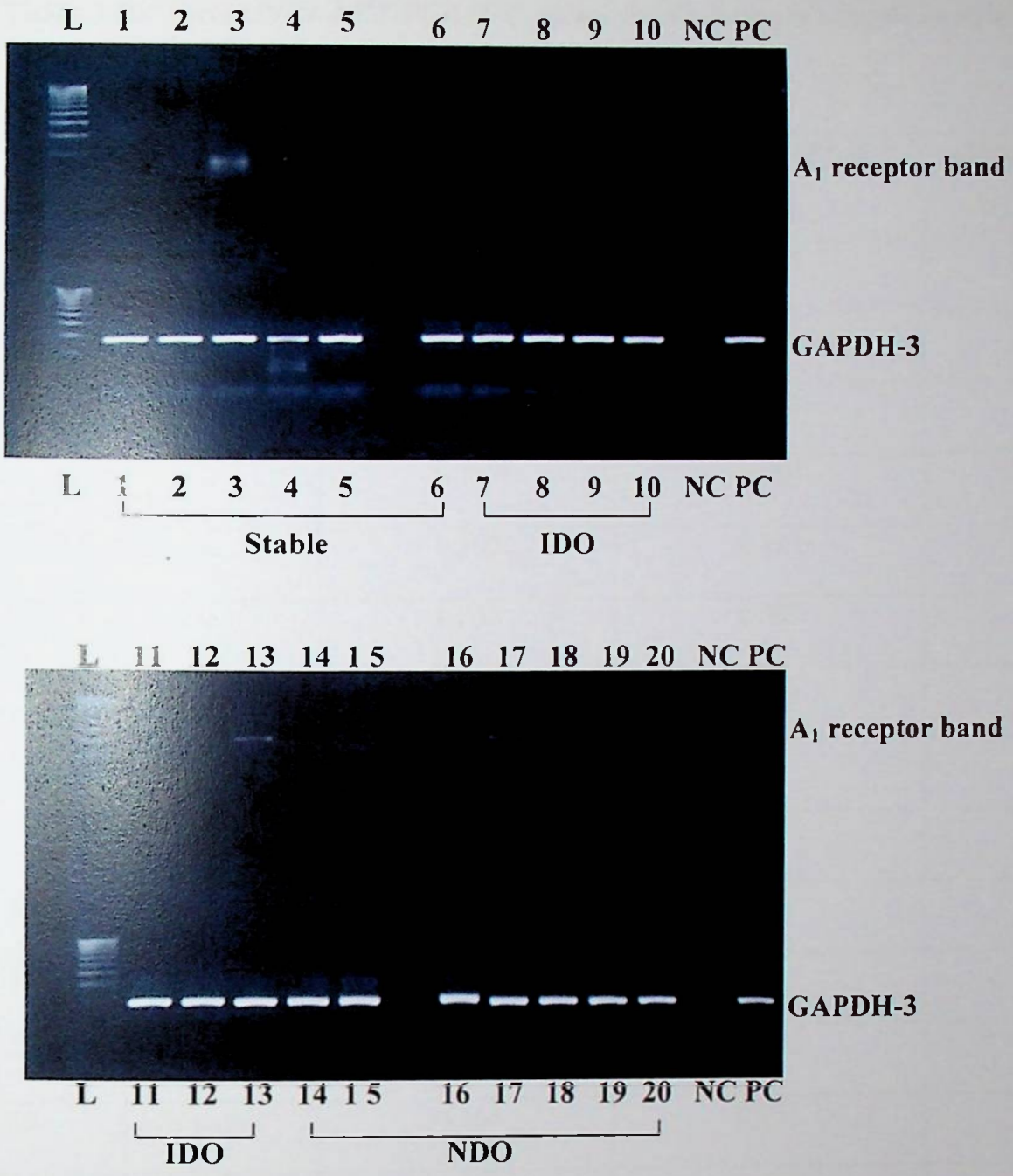


Figure 3.20: Photographs of agarose gels for A_1 receptor and GAPDH-3 gene products. *Top: samples 1-10,* ***bottom:*** *samples 11-20.* ***In each gel a base-pair calibration ladder (L) and negative (NC) and positive (PC) controls were also run.***

Table 3.16: A_1:GAPDH-3 RT-PCR IOD values for the detrusor samples analysed in this study.

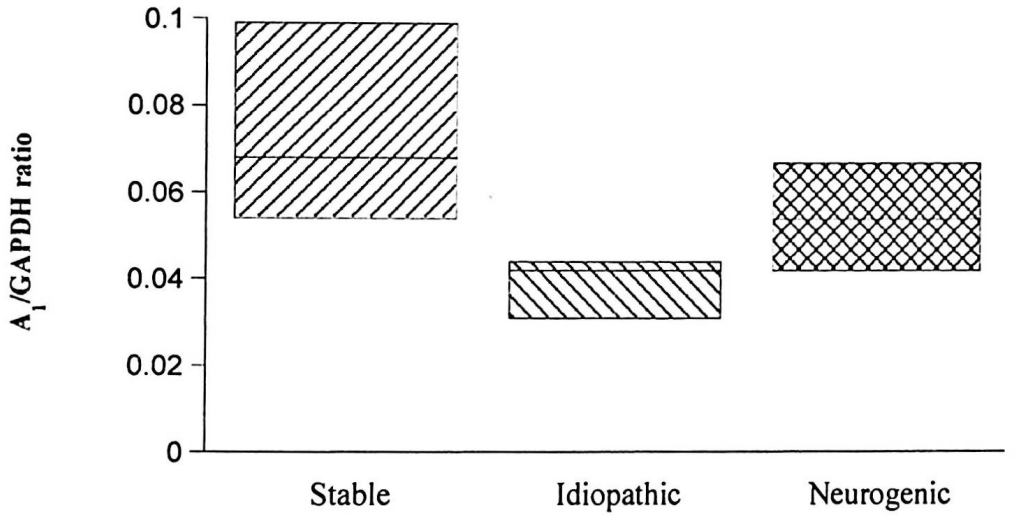
Sample	Pathology	Ratio A_1:GAPDH-3 IOD_1	Ratio A_1:GAPDH-3 IOD_2	Mean IOD Ratio A_1: GAPDH-3
1	Stable	0.041	0.034	0.038
2	Stable	0.054	0.050	0.052
3	Stable	0.073	0.045	0.059
4	Stable	0.117	0.097	0.107
5	Stable	0.108	0.121	0.114
6	Stable	0.055	0.097	0.076
7	Idiopathic	0.018	0.135	0.077
8	Idiopathic	0.017	0.073	0.045
9	Idiopathic	0.023	0.061	0.042
10	Idiopathic	0.041	0.046	0.043
11	Idiopathic	0.030	0.049	0.040
12	Idiopathic	0.002	0.042	0.022
13	Idiopathic	0.165	0.061	0.011
14	Neurogenic	0.009	0.049	0.029
15	Neurogenic	0.021	0.090	0.056
16	Neurogenic	0.051	0.057	0.054
17	Neurogenic	0.138	0.077	0.108
18	Neurogenic	0.000	0.095	0.048
19	Neurogenic	0.071	0.083	0.077
20	Neurogenic	0.028	0.044	0.036

The results were analysed to determine if there were any differences in the expression of the A_1 receptor gene in the three pathological groups (stable, idiopathic overactivity and neurogenic overactivity). Table 3.17 shows the median values (25%, 75% interquartiles) of the A_1/GAPDH-3 ratio in the three groups. There were no significant differences between the values in these groups. Figure 3.21 shows a plot of these data in the form of a bar chart (upper) and a dot-plot of individual values (bottom).

Table 3.17: Median (25%, 75% interquartiles) values for the A_1/GAPDH-3 IOD ratio for RT-PCR determinations using samples from stable, idiopathic overactive and neurogenic overactive bladders.

Pathology	**Median IOD A_1 receptor: GAPDH-3**
Stable	0.068 (0.054, 0.099)
Idiopathic	0.042 (0.031, 0.044)
Neurogenic	0.054 (0.042, 0.067)

A



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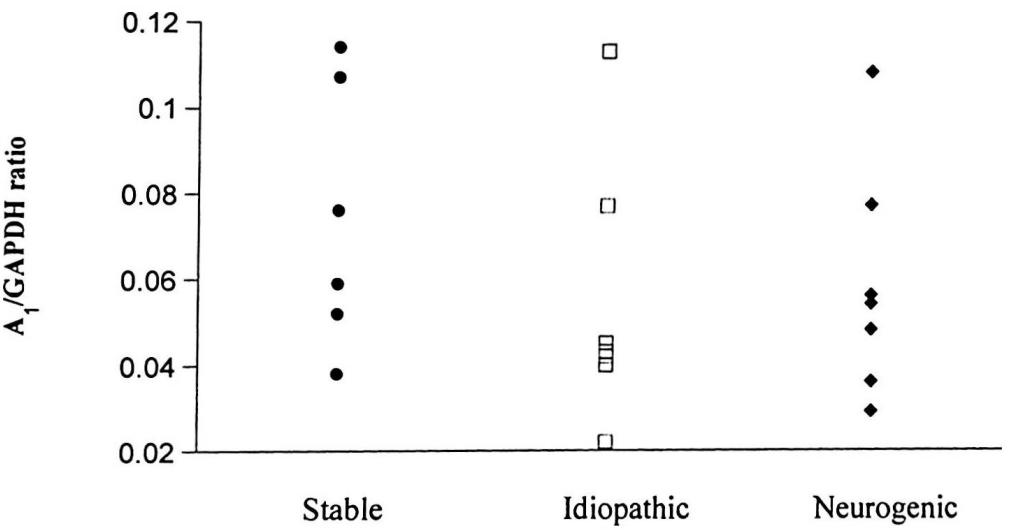


Figure 3.21: Values for the A1/GAPDH-3 IOD ratio for RT-PCR determinations using samples from stable, idiopathic overactive and neurogenic overactive bladders.

A: *median (25%, 75% interquartiles),* ***B:*** *individual values from the three groups.*

3.7.2.2. A_{2A} receptor gene expression

As before, all samples were prepared and run on two separate occasions. Polaroid photographs of A_{2A} gels for samples 1-10 and samples 11-20, each with positive and negative controls, as well as a calibration ladders, are shown in figure 3.22. Corresponding bands for GAPDH-3 are also shown. The average IOD from both runs was used in the final analysis (table 3.18).

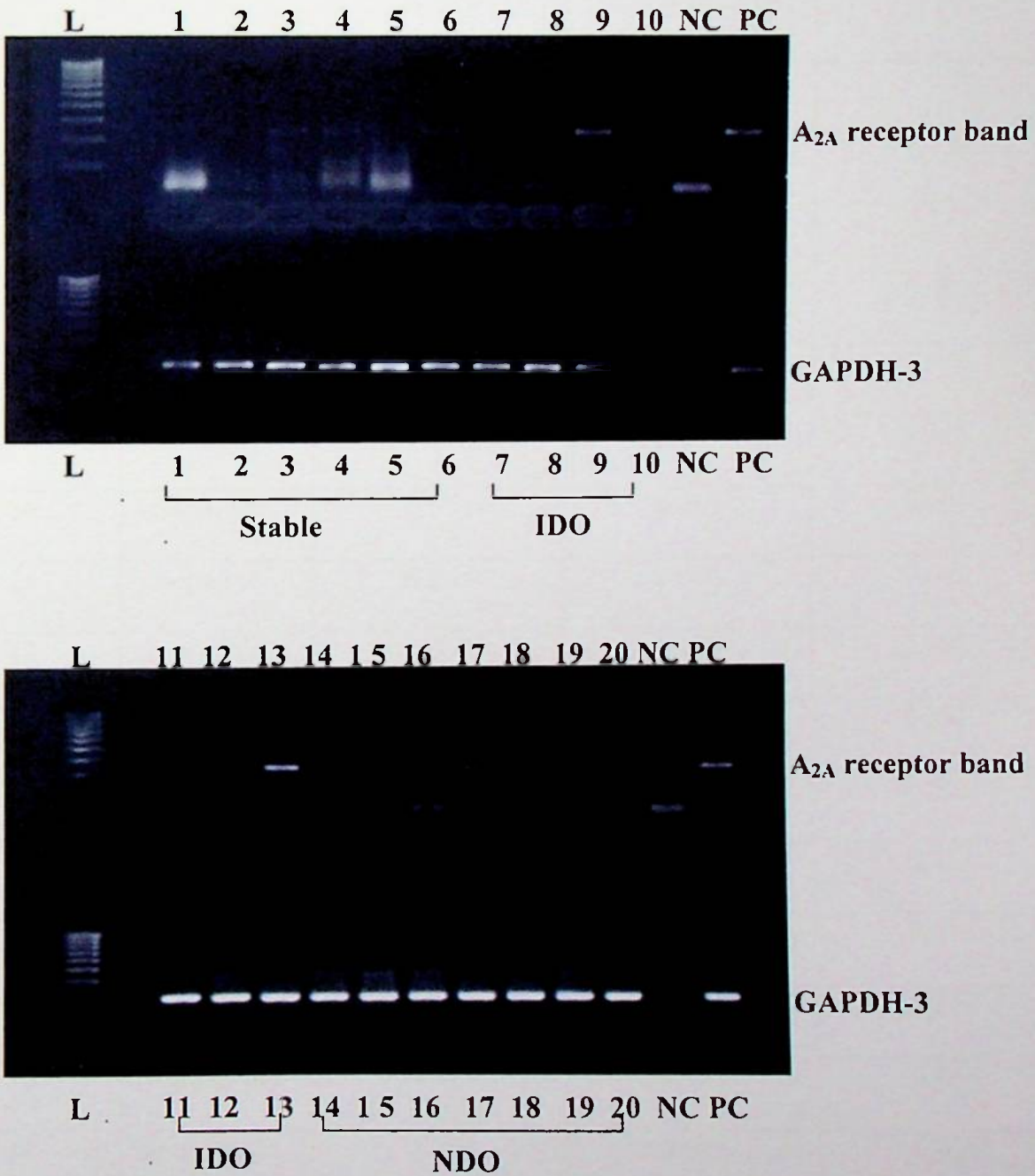


Figure 3.22: Photographs of agarose gels for A_{2A} receptor and GAPDH-3 gene products. Top:* samples 1-10, *bottom: samples 11-20. In each gel a base-pair calibration ladder (L) and negative (NC) and positive (PC) controls were also run.

Table 3.18: Lists the IOD ratios for A_{2A}/GAPDH-3 in all samples, for each separate determination and the average of the two runs.

Sample	Pathology	Ratio A_{2A}:GAPDH-3 IOD_1	Ratio A_{2A}:GAPDH-3 IOD_2	Mean IOD A_{2A}: GAPDH-3
1	Stable	0.102	0.145	0.124
2	Stable	0.173	0.276	0.225
3	Stable	0.192	0.204	0.198
4	Stable	0.252	0.203	0.228
5	Stable	0.119	0.258	0.188
6	Stable	0.143	0.151	0.147
7	Idiopathic	0.034	0.139	0.087
8	Idiopathic	0.091	0.109	0.099
9	Idiopathic	0.496	0.134	0.315
10	Idiopathic	0.151	0.061	0.106
11	Idiopathic	0.065	0.058	0.062
12	Idiopathic	0.058	0.125	0.092
13	Idiopathic	0.488	0.303	0.395
14	Neurogenic	0.024	0.040	0.0320
15	Neurogenic	0.089	0.025	0.057
16	Neurogenic	0.036	0.033	0.034
17	Neurogenic	0.230	0.062	0.146
18	Neurogenic	0.045	0.025	0.035
19	Neurogenic	0.069	0.003	0.036
20	Neurogenic	0.122	0.024	0.073

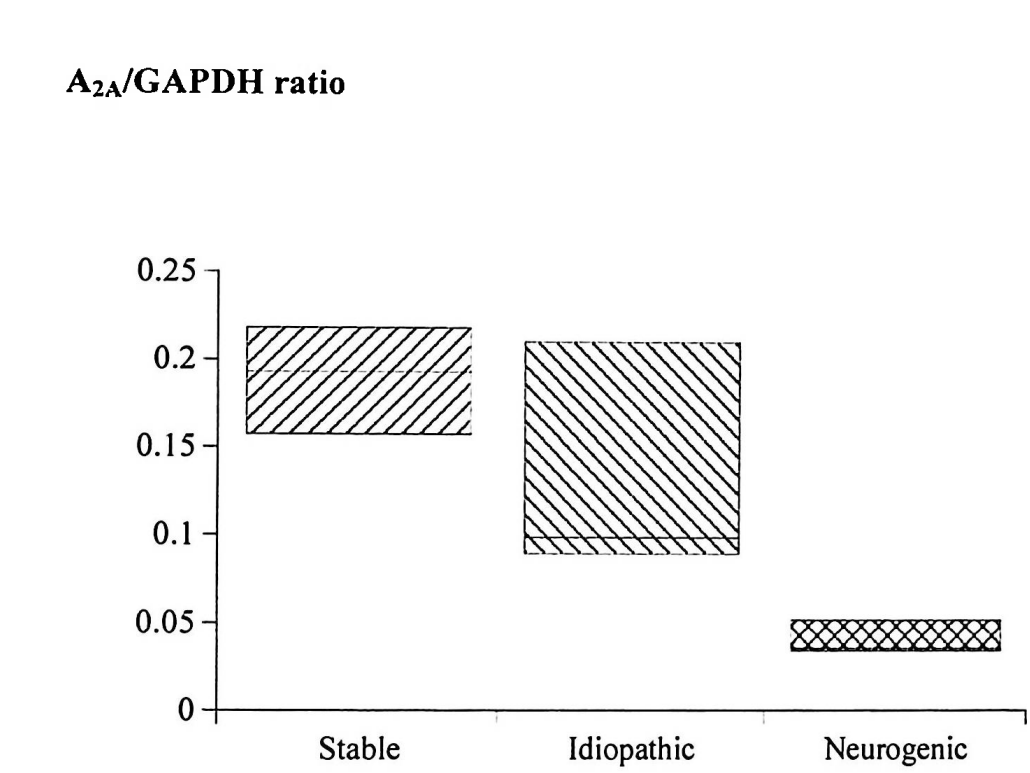
The results were analysed to determine if there were any differences in the expression of the A_{2A} receptor gene in the three pathological groups (stable, idiopathic overactivity and neurogenic overactivity). Table 3.19 shows the median values (25%, 75% interquartiles) of the A_{2A}/GAPDH-3 ratio in the three groups. There was a significant difference between the values of the stable and neurogenic groups. Figure 3.23 shows a plot of these data in the form of a bar chart (upper) and a dot-plot of individual values (bottom).

Table 3.19: Median (25%, 75% interquartiles) values for the A_{2A}/GAPDH-3 for RT-PCR determinations using samples from stable, idiopathic overactive and neurogenic overactive bladders.

Pathology	**Median IOD A_{2A}:GAPDH-3**
Stable	0.193 (0.157, 0.218)
Idiopathic	0.099 (0.090, 0.211)
Neurogenic	0.036 (0.034, 0.052)

The A_{2A} receptor mRNA expression was down-regulated in neurogenic as compared with stable bladders, ($p<0.05$), and neurogenic vs. idiopathic bladders. However, there was no significant difference between the idiopathic and stable groups.

A



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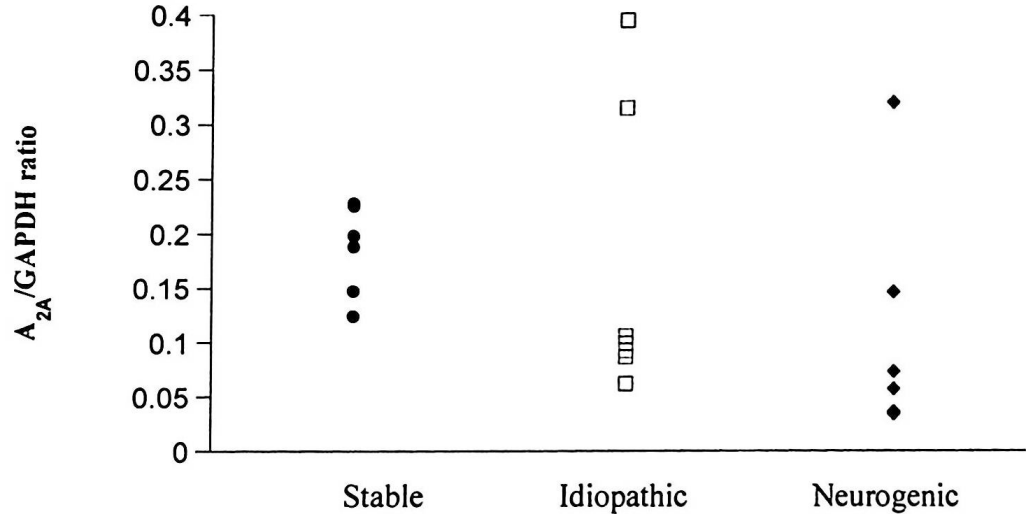


Figure 3.23: Values for the A_{2A}/GAPDH-3 IOD ratio for RT-PCR determinations using samples from stable, idiopathic overactive and neurogenic overactive bladders.

A: *median (25%, 75% interquartiles),* ***B:*** *individual values from the three groups.*

3.7.2.3 A_{2B} receptor gene expression

All samples were prepared and run on two separate occasions. Polaroid photographs of A_{2B} gels for samples 1-10 and samples 11-20, each with positive and negative controls, as well as a calibration ladders, are shown in figure 3.24. Corresponding bands for GAPDH-3 are also shown. The average IOD from both runs was used in the final analysis (table 3.20).

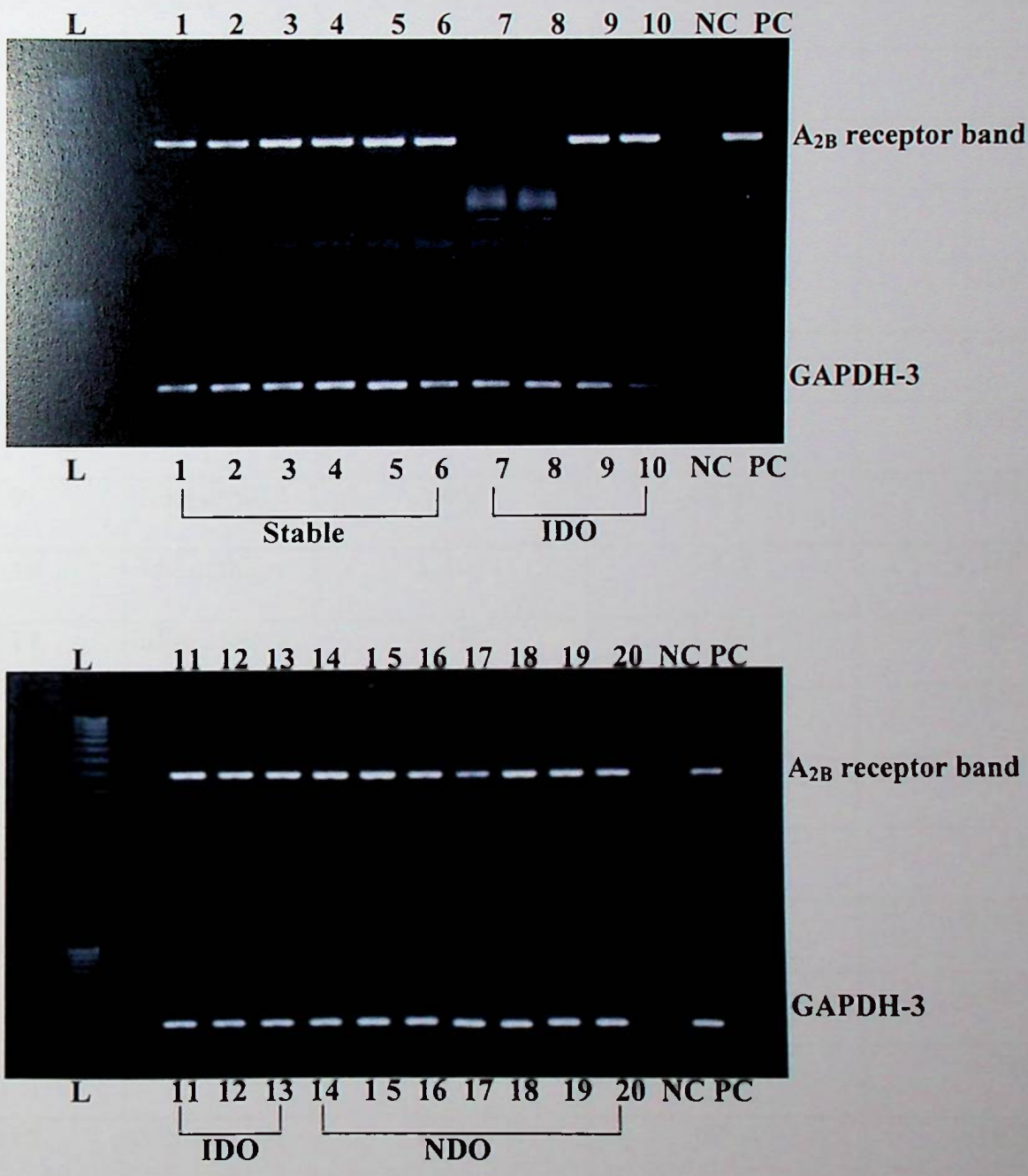


Figure 3.24: Photographs of agarose gels for A_{2B} receptor and GAPDH-3 gene products. Top: samples 1-10, bottom: samples 11-20. In each gel a base-pair calibration ladder (L) and negative (NC) and positive (PC) controls were also run.

Table 3.20: Lists the IOD ratios for A_{2B}/GAPDH-3 in all samples, for each separate determination and the average of the two runs.

Sample	Pathology	Ratio A_{2B}:GAPDH-3 IOD_1	Ratio A_{2B}:GAPDH-3 IOD_2	Mean IOD A_{2B}: GAPDH-3
1	Stable	1.000	0.480	0.740
2	Stable	0.862	0.880	0.871
3	Stable	1.126	0.339	0.732
4	Stable	0.974	0.211	0.592
5	Stable	0.979	1.074	1.027
6	Stable	1.337	1.051	1.194
7	Idiopathic	0.232	1.202	0.717
8	Idiopathic	0.206	1.217	0.712
9	Idiopathic	1.403	1.005	1.204
10	Idiopathic	2.197	0.828	1.512
11	Idiopathic	1.048	1.048	1.048
12	Idiopathic	0.979	0.979	0.979
13	Idiopathic	1.055	1.055	1.055
14	Neurogenic	1.114	1.116	1.115
15	Neurogenic	0.930	0.930	0.930
16	Neurogenic	0.848	0.850	0.849
17	Neurogenic	0.708	0.724	0.716
18	Neurogenic	0.913	0.941	0.927
19	Neurogenic	1.007	1.010	1.009
20	Neurogenic	0.846	1.154	0.619

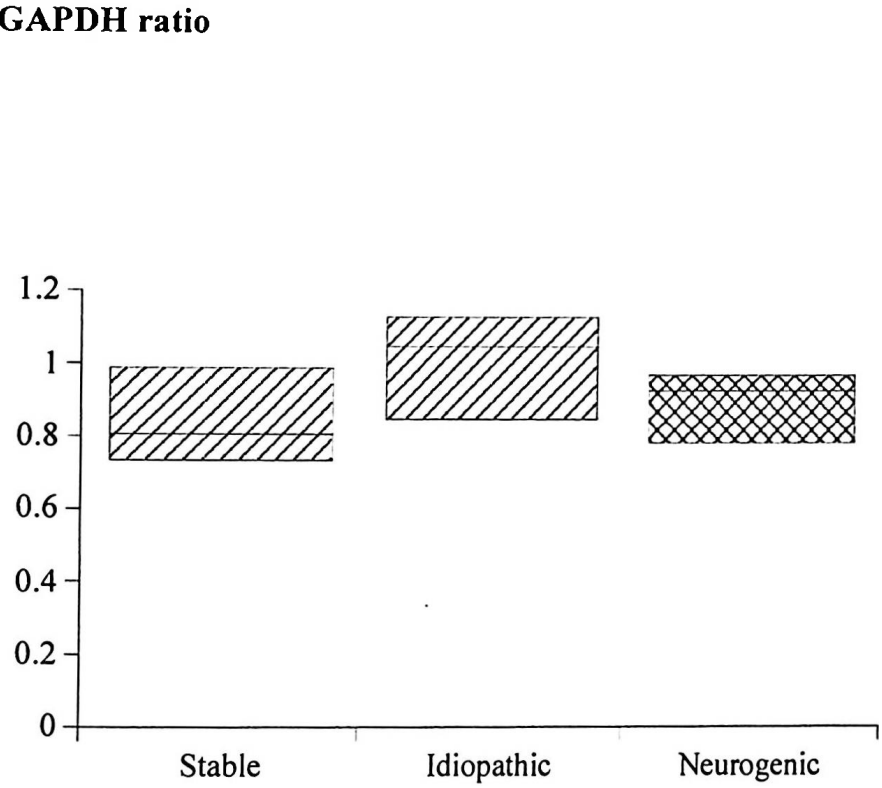
The results were analysed to determine if there were any differences in the expression of the A_{2B} receptor gene in the three pathological groups (stable, idiopathic overactivity and neurogenic overactivity). Table 3.21 shows the median values (25%, 75% interquartiles) of the A_{2B}/GAPDH-3 ratio in the three groups. There was no significant difference between the values of any of the three groups. Figure 3.25 shows a plot of these data in the form of a bar chart (upper) and a dot-plot of individual values (bottom).

Table 3.21: Median (25%, 75% interquartiles) values for the A_{2B}/GAPDH-3 IOD ratios for RT-PCR determinations using samples from stable, idiopathic overactive and neurogenic overactive bladders.

Pathology	**Median IOD A_{2B}:GAPDH-3**
Stable	0.806 (0.734, 0.988)
Idiopathic	1.048 (0.848, 1.130)
Neurogenic	0.927 (0.783, 0.970)

The A_{2B} receptor mRNA was universally and equally expressed in all bladder samples, with no statistically significant difference between the pathological groups

A



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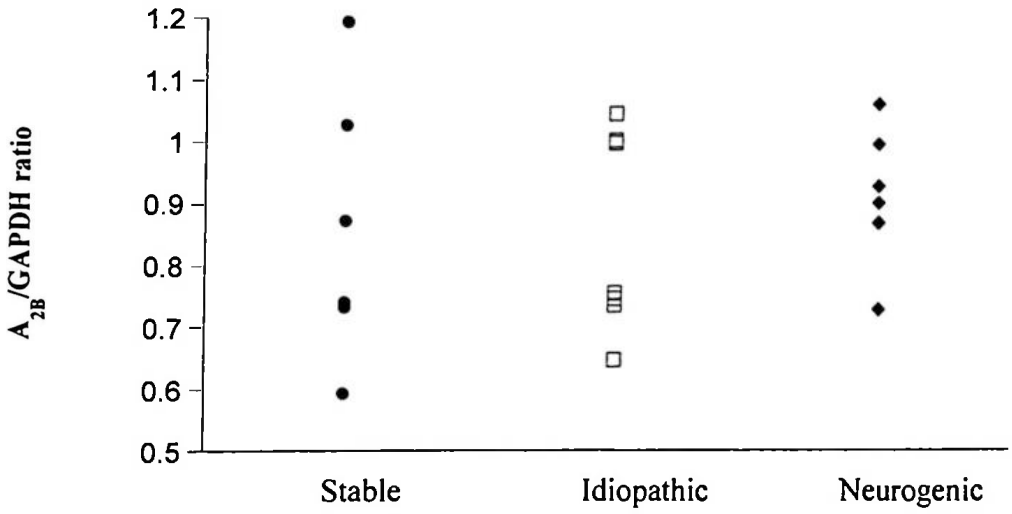


Figure 3.25: Values for the A_{2B}/GAPDH-3 IOD ratio for RT-PCR determinations using samples from stable, idiopathic overactive and neurogenic overactive bladders.

A: median (25%, 75% interquartiles), B: individual values from the three groups.

3.7.2.4 A_3 receptor gene expression

All samples were prepared and run on two separate occasions. Polaroid photographs of A_3 gels for samples 1-10 and samples 11-20, each with positive and negative controls, as well as a calibration ladders, are shown in figure 3.26. Corresponding bands for GAPDH-3 are also shown. The average IOD from both runs was used in the final analysis (table 3.22).

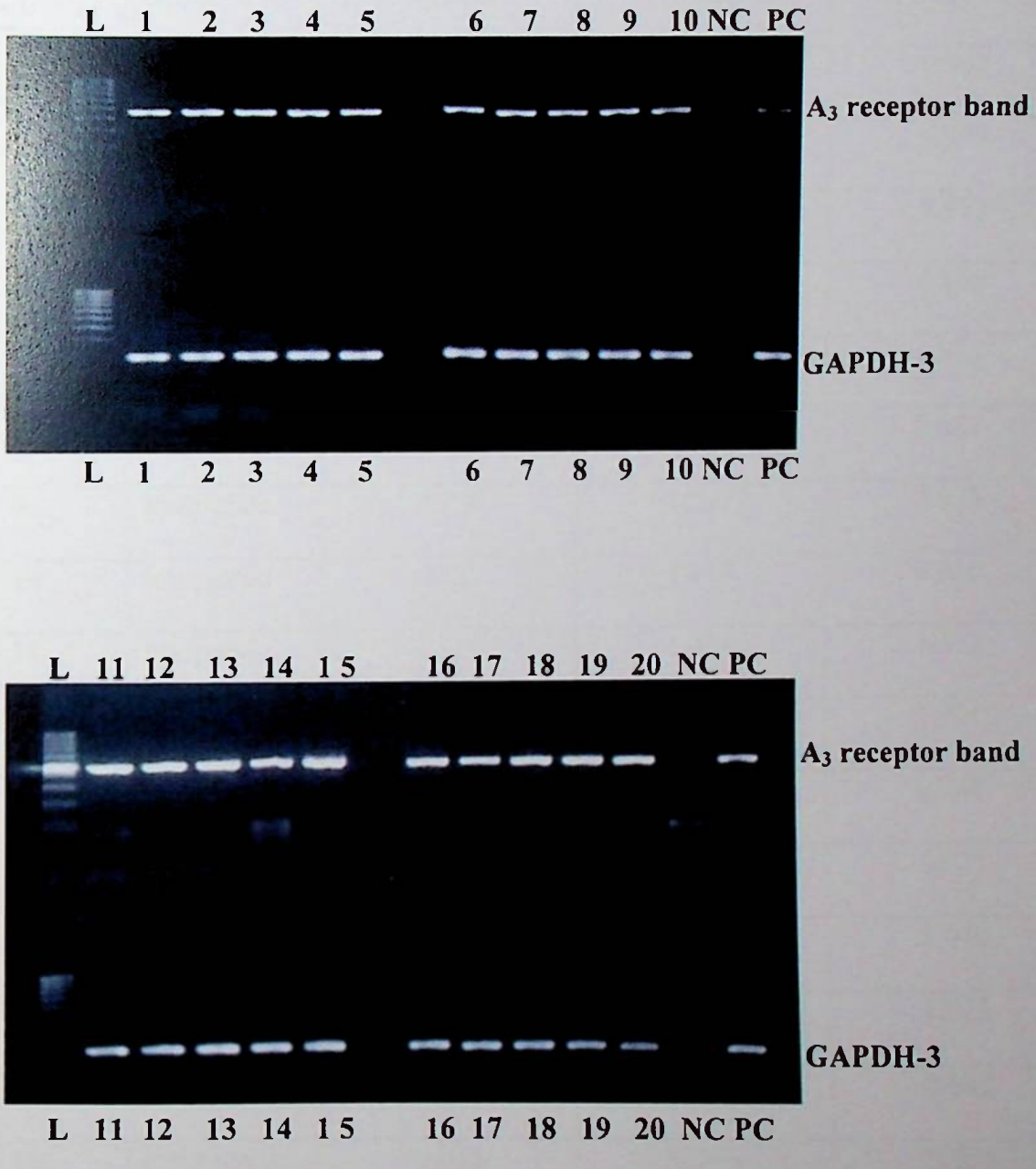


Figure 3.26: Photographs of agarose gels for A_3 receptor and GAPDH-3 gene products. Top: samples 1-10, bottom: samples 11-20. In each gel a base-pair calibration ladder (L) and negative (NC) and positive (PC) controls were also run.

Table 3.22: Lists the IOD ratios for A_3/GAPDH-3 in all samples, for each separate determination and the average of the two runs.

Sample	Pathology	Ratio A3:GAPDH-3 IOD_1	Ratio A3:GAPDH-3 IOD_2	Mean IOD A3: GAPDH-3
1	Stable	0.906	1.295	1.101
2	Stable	0.989	0.868	0.928
3	Stable	0.915	0.900	0.908
4	Stable	0.928	1.069	0.999
5	Stable	0.844	1.090	0.967
6	Stable	0.670	1.061	0.865
7	Idiopathic	0.757	1.012	0.885
8	Idiopathic	0.691	1.083	0.887
9	Idiopathic	0.730	1.217	0.973
10	Idiopathic	0.733	1.168	0.950
11	Idiopathic	1.310	1.434	1.372
12	Idiopathic	1.117	1.525	1.321
13	Idiopathic	0.991	0.904	0.948
14	Neurogenic	0.876	1.174	1.025
15	Neurogenic	1.193	0.887	1.040
16	Neurogenic	1.013	1.013	1.013
17	Neurogenic	0.806	0.890	0.849
18	Neurogenic	1.055	0.848	0.951
19	Neurogenic	1.184	1.033	1.108
20	Neurogenic	1.256	0.773	1.014

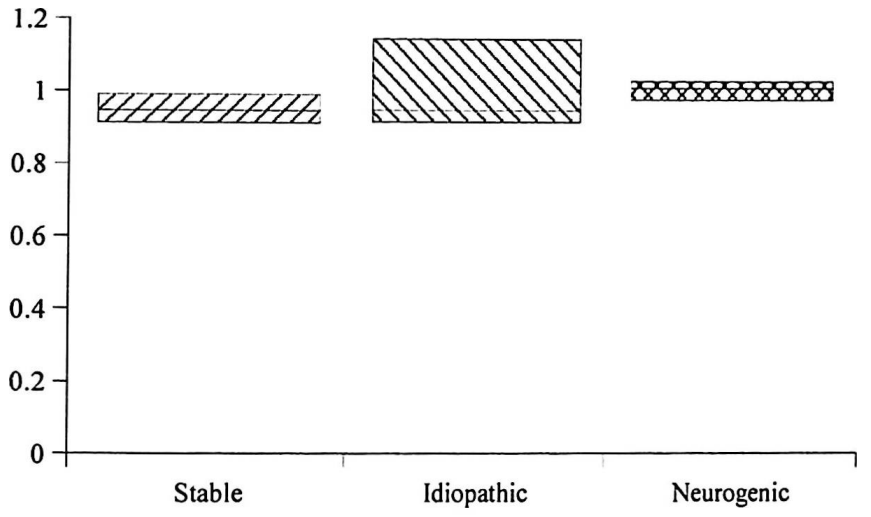
The results were analysed to determine if there were any differences in the expression of the A_3 receptor gene in the three pathological groups (stable, idiopathic overactivity and neurogenic overactivity). Table 3.23 shows the median values (25%, 75% interquartiles) of the A_3/GAPDH-3 ratio in the three groups. Figure 3.27 shows a plot of these data in the form of a bar chart (upper) and a dot-plot of individual values (bottom).

Table 3.23: Median (25%, 75% interquartiles) values for the A_3/GAPDH-3 IOD ratios for RT-PCR determinations using samples from stable, idiopathic overactive and neurogenic overactive bladders.

Pathology	**Median IOD A_3:GAPDH-3**
Stable	0.948 (0.913, 0.991)
Idiopathic	0.949 (0.918, 1.147)
Neurogenic	1.014 (0.982, 1.033)

A_3 receptor mRNA was universally and equally expressed in all bladder samples with no differences between the three groups studied.

A



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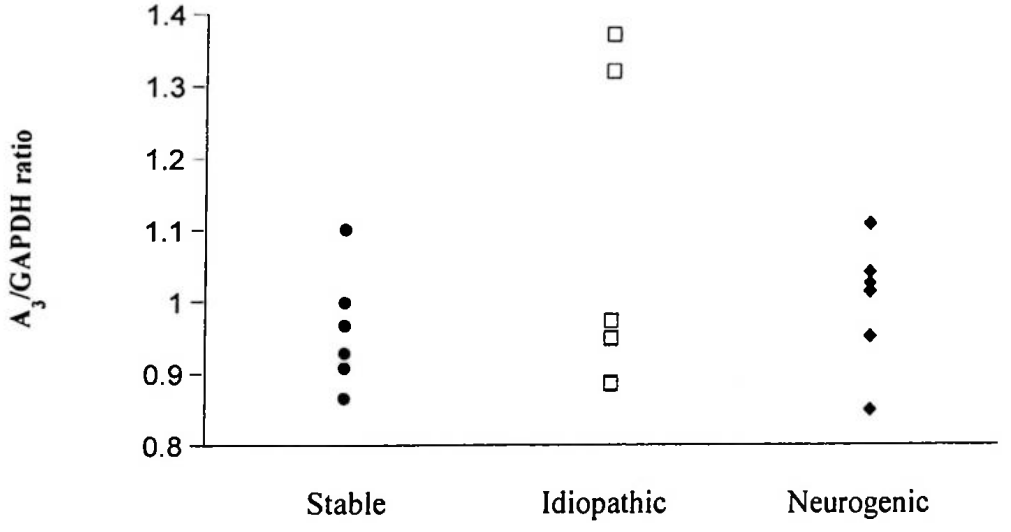


Figure 3.27: ***Values for the A_3/GAPDH-3 IOD ratio for RT-PCR determinations using samples from stable, idiopathic overactive and neurogenic overactive bladders.*** ***A:*** *median (25%, 75% interquartiles),* ***B:*** *individual values from the three groups.*

3.7.3 Differences in receptor expression as a function of age

Because of the similarities between the three groups, the influence of patient age from which the biopsies were taken was also examined. The mean (±SD) age of the patients was 52.9±16.8 years. The age of the stable and idiopathic groups was significantly older than the neurogenic group (65.7±10.7; 59.7±10.6; 35.1±10.0 years respectively). However, there were several patients below 60 years in the stable and idiopathic groups and so a comparison was made of the A_1 receptor expression in biopsies from patients aged < 60 (n=12) and ≥60 (n=8). For samples from patients below 60 years the median ratio was 0.044 (0.04, 0.055); this was significantly different to the median ratio for samples from patients above 60 years (0.068; 0.045, 0.085). The data are also shown in figure 3.28.

Although A_{2A} receptor mRNA expression was down regulated in NDO as compared with stable bladders. ($p<0.05$), these samples were taken from the youngest sub-group of patients (table 3.14). Hence it cannot be determined whether this down-regulation was an effect of age or an effect of pathology. Had a functional effect of the A_{2A} receptor been identified earlier, one would have been more determined to investigate this possible age dependency.

3.8 Adenosine receptor protein expression in human detrusor

After measuring the levels of adenosine receptor transcription in human bladder tissue of varying pathology, levels of adenosine receptor protein expression were measured using Western blotting techniques. The aim was to define the relative expression of adenosine receptors in human detrusor according to pathology.

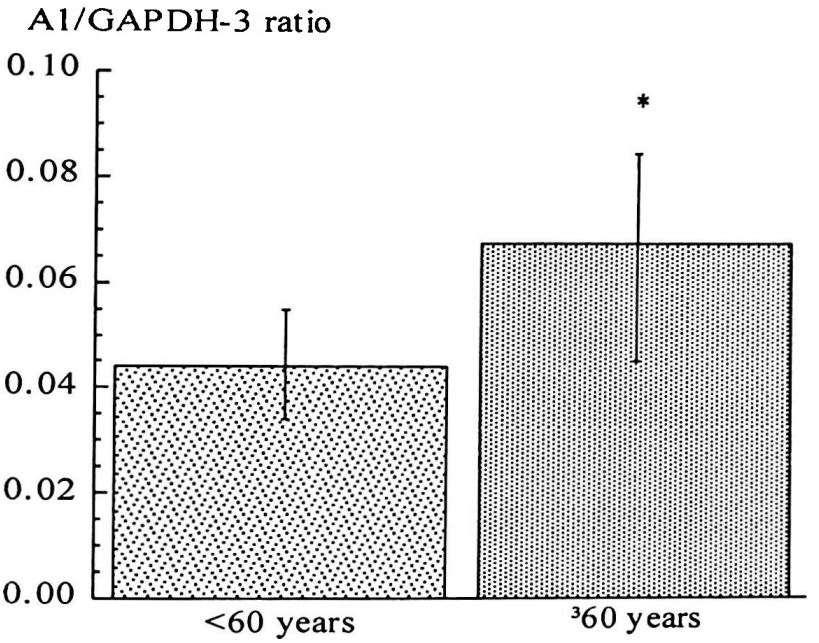


Figure 3.28: The values of the A_1/GAPDH-3 ratio in samples from patients younger or older than 60 years. Median (25%, 75% interquartiles). *p<0.05.

3.8.1 Protein Extraction

Protein from each of the 20 samples was extracted as described in section 2.9.3. A protein standard curve was constructed (figure 2.7) and the protein concentration in each sample was determined using the Bio-Rad Bradford micro-assay (section 2.17 and Appendix 5 for raw data).

3.8.2 Western blotting

Samples (20 μg/well) were loaded onto the 7.5 – 10% acrylamide gel and were subjected to SDS-PAGE. Proteins were then transferred to a PVDF membrane overnight. Following transfer the membrane was stained with ponceau red to confirm transfer of the proteins and photographs were taken (figure 3.29).

The membrane was blocked with 5% milk for 1 hour and then incubated with the primary antibody (anti-A1 receptor, anti-$A2_A$ receptor, anti-$A2_B$ receptor, anti-A3 receptor antibodies, respectively). The membranes were washed and then secondary antibody hybridisation was allowed to occur following the addition of anti-biotin HRP-linked antibody and goat-Anti-Rabbit IgG antibody-HRP–linked. HRP-labelled streptavidin was used to detect the presence of the secondary antibodies. The membranes were then exposed to film.

3.8.3 Analysis of Western blots

The x-ray films were scanned and the protein band densities were analysed with Bio-Rad densitometry software (Molecular Analyst, windows software for Bio-Rad image analysis system, version 1.5, U.S.A.).Background signals were obtained in each lane and subtracted from the band densities to correct for background signal.

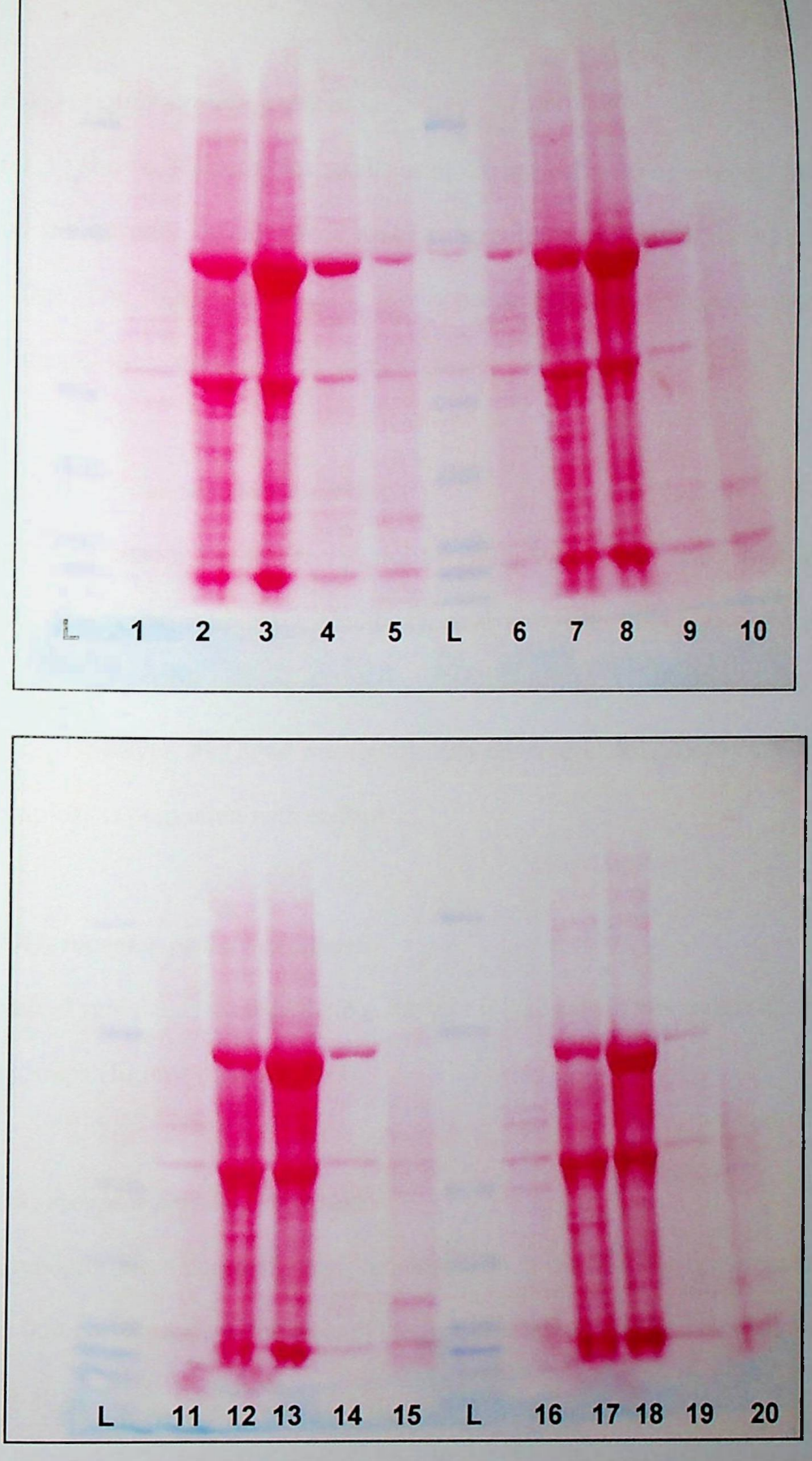


Figure 3.29: Ponceau red stain of PVDF membrane. L=ladder. Top; Human detrusor protein samples 1-10 are labeled. Bottom; samples 11-20 are labeled.

3.8.3.1 A_1 receptor protein expression

Figure 3.30 shows Western blot results using the anti-A1 receptor antibody. Analysis revealed the absence of any bands which correspond to the mass of the A1 receptor (36.5 kDa). The signal was small in all the human detrusor specimens compared to control tissue – see section 4.9 for discussion.

3.8.3.2 A_{2A} receptor protein expression

The nominal mass of the A_{2A} receptor is 44.7 kDa. Figure 3.31 shows Western blot results using the anti-A_{2A} receptor antibody. Analysis reveals the presence of a single, dense band at ~45kDa that corresponds to the mass of the A_{2A} receptor, in all three subgroups. However, this band was significantly down regulated in both the NDO and IDO samples, as compared with control.

3.8.3.3 A_{2B} receptor protein expression

The nominal molecular mass of the A_{2B} receptor is 36.2 kDa. It was present in all 3 patient groups (figures 3.32 and 3.33).

3.8.3.4 A_3 receptor protein expression

The A_3 receptor has a nominal molecular mass of 36.2kDa. A single, dense band at ~36kDa that corresponds to the mass of the A_3 receptor was identified in all samples (figure 3.34).

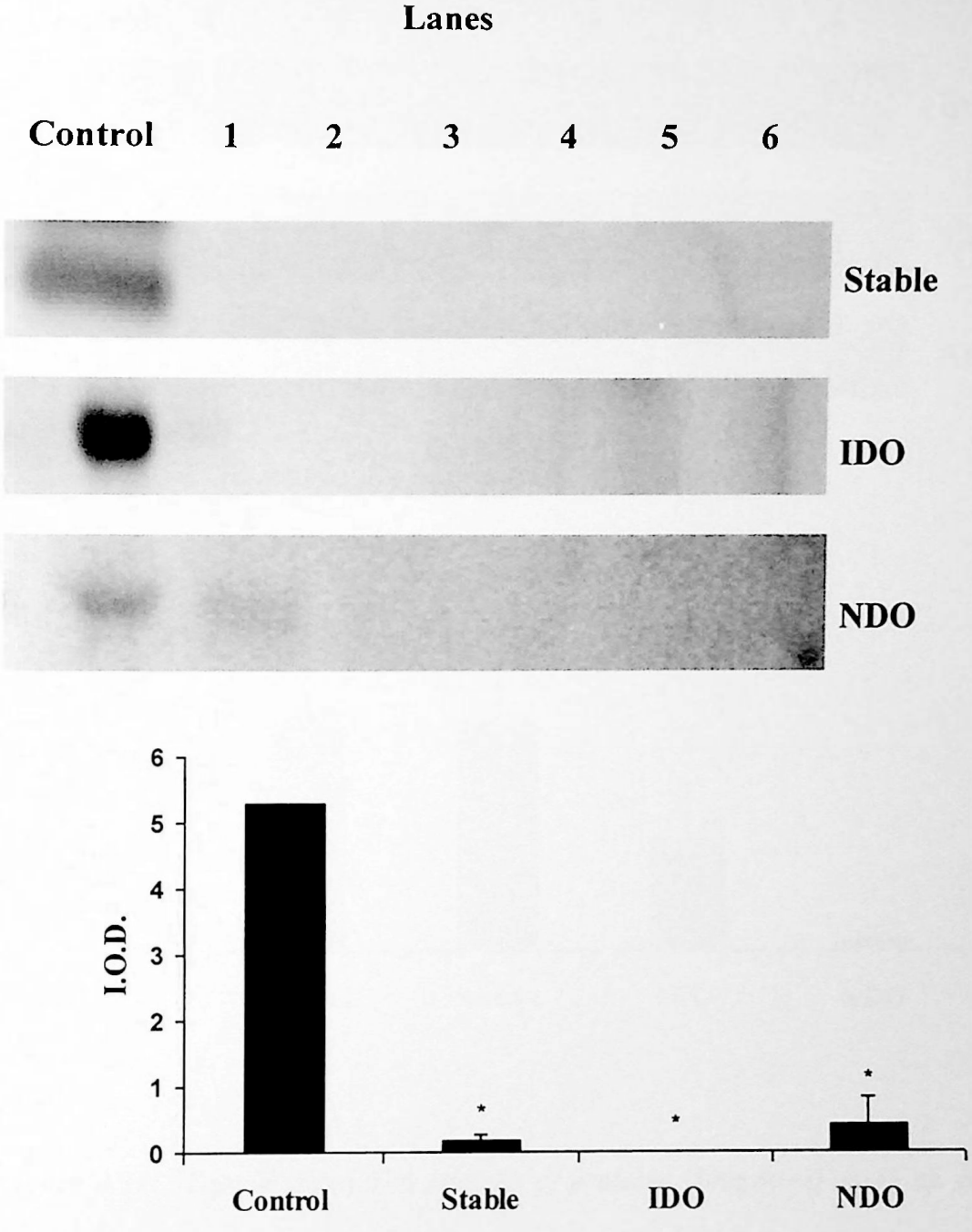
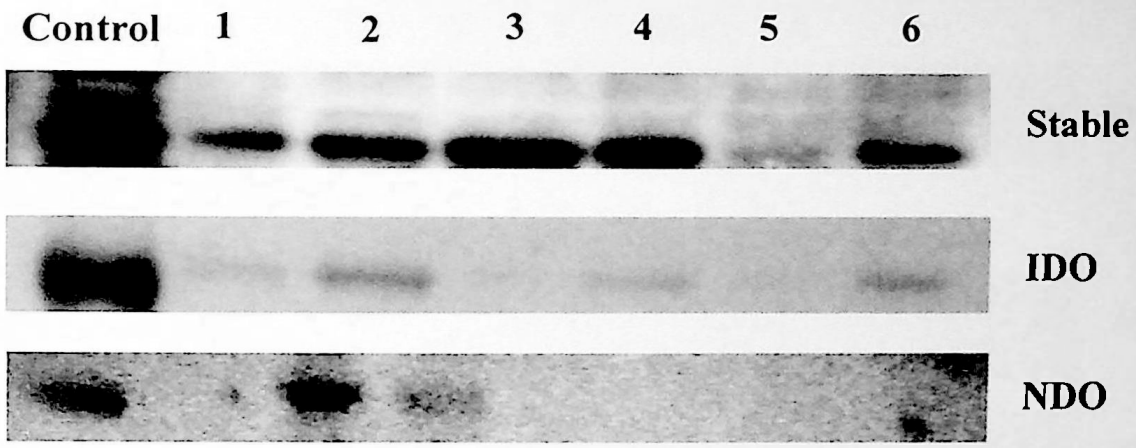


Figure 3.30: Top; Western blot analysis of proteins (30µg/lane) using an anti-A_1 receptor antibody. *Samples from stable, IDO and NDO bladders are shown in lanes 1-6. The nominal mass of the major protein species detected in the control and some of the test detrusor samples was ~36 kDa, which corresponds to the A_1 receptor.* ***Bottom; IOD values for western blots of those samples shown above.*** *Mean ± SD; n=6 per group.*



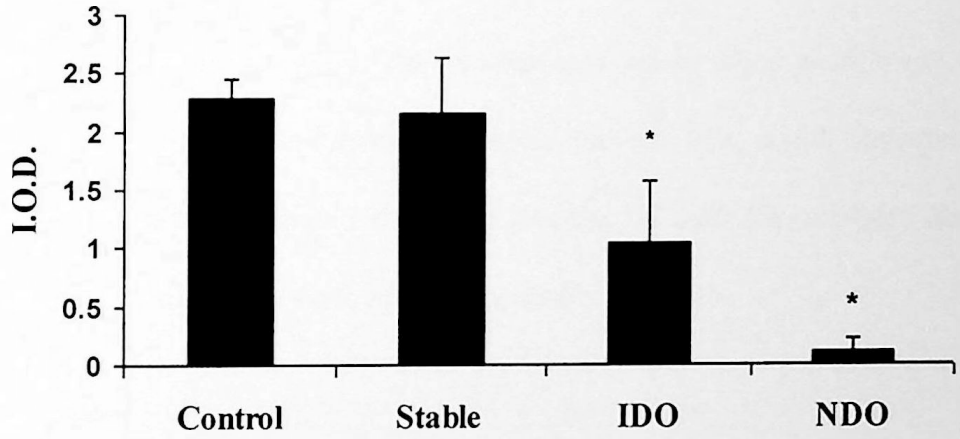


Figure 3.31: Top; Western blot analysis of proteins (30µg/lane) using an anti-A_{2A} receptor antibody. *Samples from stable, IDO and NDO bladders are shown in lanes 1-6. The nominal mass of the major protein species detected in the control and test detrusor samples was ~45kDa, which corresponds to the A_{2A} receptor.*

Bottom; IOD values for western blots of those samples shown above. *Mean ± SD; n=6 per group.*

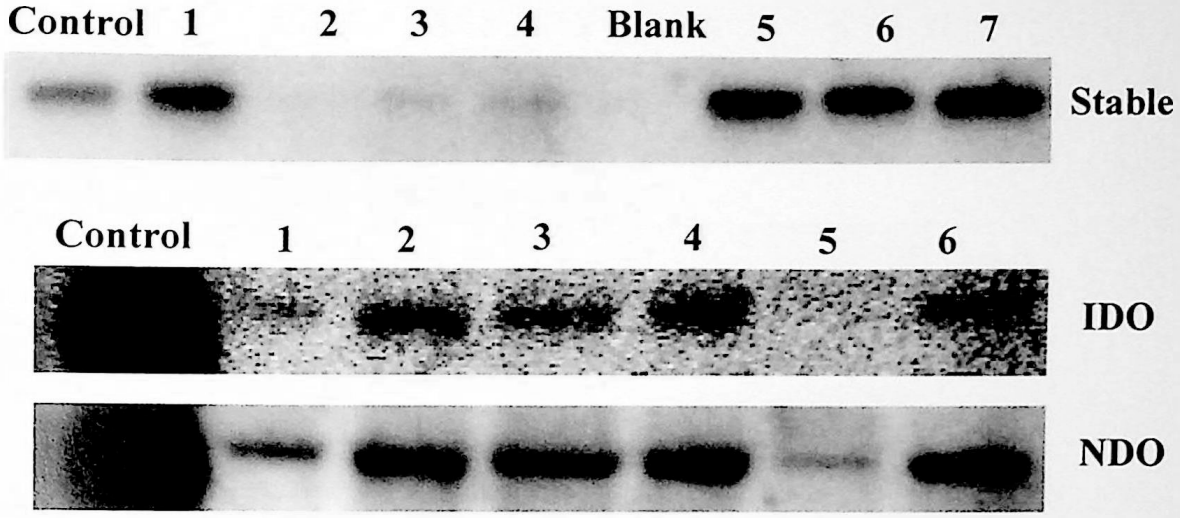


Figure 3.32: Western blot analysis of proteins (30 µg/lane) using an anti-A_{2B} receptor antibody. ***Top;*** *Lanes 1-6 contain human stable detrusor samples 1-6. Lane 7 contains human stable sample 1. The nominal mass of the major protein species detected in the control and test detrusor samples was ~36 kDa, which corresponds to the A_{2B} receptor.* ***Middle;*** *Lanes 1-6 contain samples 7-12, all IDO samples.* ***Bottom;*** *Lanes 1-6 contain samples 14-19, all NDO samples.*

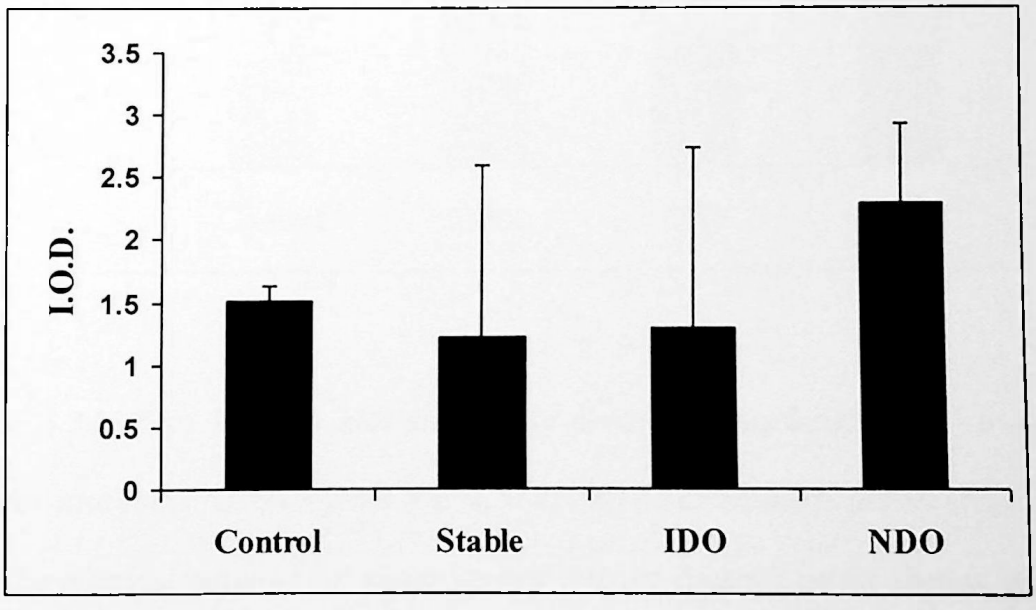
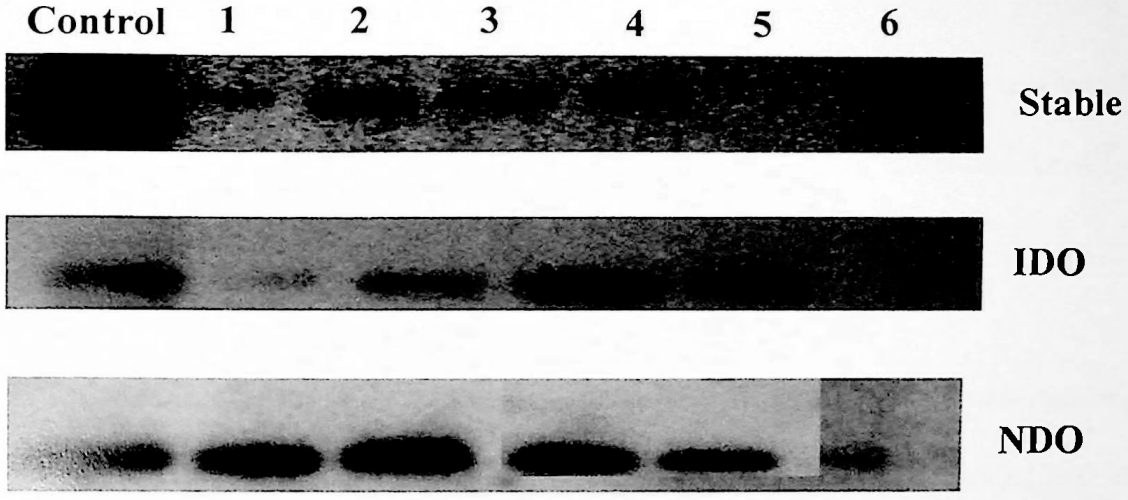


Figure 3.33: IOD values for western blots of those samples shown in figure 3.31. Mean ± SD; n=6 per group.



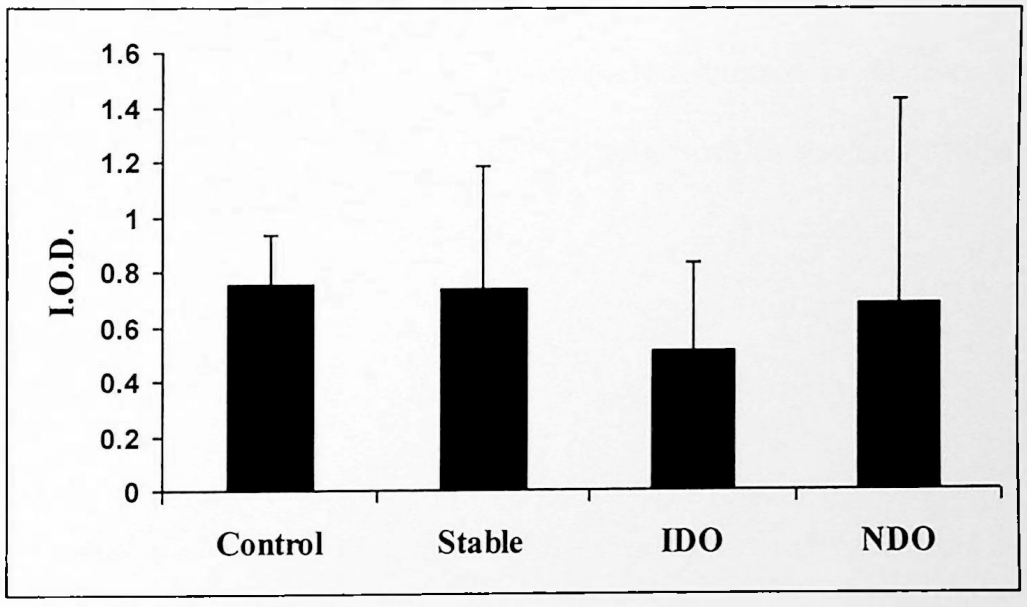


Figure 3.34: Top; Western blot analysis of proteins (30µg/lane) using an anti-A_3 receptor antibody. *Samples from stable, IDO and NDO bladders are shown in lanes 1-6. The nominal mass of the major protein species detected in the control and test detrusor samples was ~36kDa, which corresponds to the A_3 receptor.*

Bottom; IOD values for western blots of those samples shown above. *Mean ± SD; n=6 per group.*

3.8.4 Summary of findings

To summarise, protein bands corresponding to the A1 receptor were at very low levels in all three subgroups. A1 receptor mRNA was however identified in all three subgroups following PCR.

The protein corresponding to the A_{2A} receptor was significantly down-regulated in both the overactive (IDO and NDO) subgroups, compared with control and stable samples. PCR also identified a significant decrease of A_{2A} receptor mRNA in NDO versus stable detrusor.

The A_{2B} and A_3 receptor proteins were similarly expressed in all three detrusor subgroups, which correlates well with the PCR results which also identified A_{2B} and A_3 receptor mRNA in all three subgroups.

CHAPTER FOUR

Discussion

4.1 Experimental limitations

The role of P1-receptors in the modulation of detrusor smooth muscle from both guinea-pig and human sources was investigated using several techniques; isometric tension was measured following contraction initiated by either electrical field stimulation or through direct-muscle activation with agonists. The expression of the P1-receptors subtypes was investigated using molecular biological techniques to measure either mRNA or protein levels.

The supply of human detrusor samples was limited as it depended on whether the surgeons were performing cystectomy or ileocystoplasty operations during the thesis research period. The actual quantity of each sample was variable; part of the sample was used for tension experiments; any tissue remaining was frozen for molecular biology experiments.

4.1.1 Tension experiments

Although the detrusor strips dissected were normalised for cross-sectional area, it was impossible to control the connective tissue content of each strip. This shows inter-species variation, when strips have been examined with light microscopy; the content was higher in human as compared with guinea-pig specimens (M Bayliss and CH Fry, unpublished observations). Connective tissue is not contractile and may therefore interfere with contraction of a detrusor sample. An increased proportion would reduce normalised contraction amplitude.

Some detrusor samples from human overactive bladders required a higher stimulation voltage, as the response to electrical field stimulation with the voltage parameters used for samples from stable bladders was poor. This may be due to denervation of these samples either as a consequence of the disease or as a result of dissection technique, particularly as the serosa and urothelium were removed for separate experimentation. Such variability was minimised by determining the voltage required for maximum tension generation prior to experimentation. The increase of stimulus voltage might also increase the amount of direct muscle stimulation, but all contractions were abolished with 1 μM TTX at the end of the experiment, and so variations of stimulus voltage were not considered to influence significantly normalised force between different preparations.

4.1.2 RT-PCR experiments

Adenosine receptor subtype mRNA expression in human detrusor was determined using RT-PCR which is associated with several potential problems. The first issue is that of RNA degradation. RNA is an inherently unstable molecule and to prevent degradation by RNases (section 2.9.4), the sample should be frozen in liquid nitrogen as soon as it has been extracted from whole bladder. This was not always possible due to the physical distance between the operating theatre and the laboratory.

There are numerous factors which contribute to the success or failure of PCR reactions. These include primer design, magnesium concentration, enzyme choice, enzyme concentration, template, number of cycles used and choice/availability of thermocycler (section 2.12). Consequently, it required at least 15 experiments, including repeats, to perfect the conditions required for the final RT-PCR

experiments, reported here. To ensure accuracy, each of the 20 detrusor samples were run in triplicate and an internal control (housekeeping gene) was included in each sample. The final results are shown in section 3.7.2.

4.1.3 Western blotting experiments

Many steps required perfection before an adequate Western blot was achieved. It was only after several repeats and variation in technique and conditions that a satisfactory result was obtained. Listed below are explanations of how the various problems encountered in Western blotting were overcome:

- **Smearing of film/blot:** To avoid double bands appearing on either the blot or the film, it was important to position the gel precisely on the film and only once.
- **Blurred bands:** This was usually due to uneven contact between the gel and the membrane, hence the sandwich was weighed down. Blurred bands on the film meant that the cassette had not been fastened correctly, allowing poor contact between the membrane and the film.
- **Bald spots on the membrane:** Bubbles between the gel and the membrane creates areas of high resistance, reducing the efficiency of the transfer. Care was taken to try and remove all bubbles prior to transfer.
- **Incomplete transfer:** The power supply, electrode connections and orientation of transfer stack were checked. Higher molecular weight proteins needed longer blotting times or higher currents, and low molecular weight proteins can pass right through the membrane if the transfer time is too long. To avoid losing protein, a double layer of membrane was used. The transfer buffer was made fresh as impurities within it could inhibit efficient transfer.

- **High background noise:** If the concentration of antibody was too high or the incubation time too long there was a higher likelihood of non-specific staining. Several repeats were required before the ideal protocol was found e.g. by varying the number of wash steps. The concentration of the primary and secondary antibodies were also adjusted one at a time, to achieve the final values reported in this thesis.
- **Low signal:** The species reactivity of the antibodies were checked. Sometimes the antigen was not recognized by the primary antibody, especially if the antibody was monoclonal and was raised against a native protein. Alternative antibodies were then tried ie. polyclonals. Finally, low signal maybe due to the fact that the target protein is not actually expressed in the test samples - hence the importance of a reliable positive control (section 2.18.2).

4.2 Contractile properties of human and guinea pig detrusor during electrical field-stimulation

Electrical field-stimulation of both guinea-pig and human detrusor specimens was performed and their contractile properties were assessed. The magnitude of force generated when nerve-mediated contractions were elicited (normalized per unit cross-sectional area) in guinea-pig and all human specimens (stable and pathological), was similar at their half-maximum ($f_{1/2}$) stimulation frequencies (8 and 20 Hz respectively). This implies that guinea-pig and human detrusor in vitro preparations exhibit a similar unit contractility. The values for maximal tension (T_{max}) in both species were comparable to those previously reported (Bayliss *et al,* 1999).

There was however a difference in the frequency of stimulation required for half-maximal contraction, $f_{1/2}$, between the two species. The mean $f_{1/2}$ for guinea-pig was 8.9 Hz, and was significantly lower than the mean $f_{1/2}$ from human specimens from stable bladders, 18.8 Hz: concurring with results from a previous study (Ikeda, 2006). This inter-species variation could be due to a difference in the neurotransmitters released by each species. Guinea-pig detrusor demonstrated significant atropine-resistant contractions, implying that a significant proportion of its contraction is due to a neurotransmitter other than Ach. The application of ABMA desensitized $P2X_1$ receptors and then abolished nerve-mediated tension, particularly at the low frequencies of stimulation. This implies that ATP is the second neurotransmitter involved in guinea-pig detrusor contraction and also makes guinea-pig an excellent model for to study DO in humans, especially as both guinea-pig and human bladders from patients with DO both demonstrate atropine-resistant contractions.

The atropine-resistant, ABMA-dependent contractions in human specimens were not as significant as in the guinea-pig, implying that ATP has less of a role to play in human detrusor contraction. The greater dependence on ATP (for contractile activation) that guinea-pig detrusor shows, compared to that from stable human bladders may account for the significantly lower $f_{1/2}$ value and is compatible with the hypothesis that ATP is released at lower stimulations frequencies than Ach. Although stable human bladder did not demonstrate atropine-resistance, specimens from the overactive groups did. This implies that Ach is the sole neurotransmitter in the stable bladder, but that ATP has a functional role in detrusor contraction of overactive bladders. In addition, the partial dependence on ATP may explain the difference in $f_{1/2}$ values of the different human groups. The mean $f_{1/2}$ of the idiopathic (10 Hz) and

NDO (11.7 Hz) groups was higher than that from human stable bladders, but not significantly different from guinea-pig values.

The extracellular actions of purine nucleotides and nucleosides were first described in a seminal paper by Drury and Szent-Györgyi in 1929 in the cardiovascular system. Recognition for a physiological role for ATP at the neuromuscular junction followed as Buchthal and Folkow (1948) found that ACh-evoked contraction of skeletal muscle fibres was potentiated by exposure to ATP. The first hypothesis that ATP might be a neurotransmitter in the peripheral nervous system arose when Holton and Holten (1954) proposed that ATP released from sensory nerves during antidromic nerve stimulation of the great auricular nerve caused vasodilatation in the rabbit ear artery, and it was later shown that rabbit ear vessel dilatation was accompanied by ATP release (Holton, 1959). Non-adrenergic non-cholinergic (NANC) responses were blocked by tetrodotoxin, indicating the neurogenic origin of inhibitory junction potentials (Bülbring and Tomita, 1967). The excitatory response of the mammalian urinary bladder to parasympathetic nerve stimulation was also shown in the last century to be only partially antagonized by antimuscarinic agents (Langley and Anderson, 1895). It was postulated that the atropine-resistant response was due to the release of a noncholinergic excitatory transmitter (Ambache and Zar, 1970). Evidence for NANC nerves is now firmly established (Burnstock *et al*, 1966) and ATP is established as the neurotransmitter at these nerves.

Nerve-mediated contractions in rat (Yu *et al*, 1996) and rabbit detrusor (Zhao *et al*, 1993) are biphasic. The initial phase is due to ATP activating $P2X_1$ ligand-gated receptors allowing influx of Ca^{2+}. The second phase is due to the stimulation of M_3

receptors by acetylcholine. This results in the slow release of intracellular Ca^{2+} via second messenger pathways. This bi-phasic contraction is not easily observed in human and guinea-pig specimens, possibly because the visco-elastic properties of the extracellular matrix dampen the wave of contraction, and partly due to the relative unimportance of the ATP-dependent fraction in many human detrusor samples.

The overall duration of contraction was longer in human preparations compared to those of the guinea-pig and may reflect the greater amount of connective tissue in these samples.

Apart from these differences in neurotransmitter release in guinea-pig and human preparations, there are also variations in the extent of innervation of the bladder wall. One study showed that in NDO samples there was patchy denervation as compared to samples from stable bladders, which may reflect a deficit at the level of the peripheral ganglia. The result is a contraction in the areas of denervation that occurs out of synchrony with the rest of the detrusor bundles, or is co-ordinated by means of non-neural structures (Drake *et al*, 2003). Interstitial cells of Kajal have been proposed as important components involved in the modulation of the micturition reflex. They form a "myovesical plexus" which integrates inhibitory and excitatory inputs to the bladder with contraction occurring when the excitatory input exceeds the inhibitory input. An imbalance may result in OAB.

In the isolated whole bladder of the guinea-pig, stimulation of the bladder nerve at high frequencies (20-30 Hz) caused a rapid rise in intra-vesical pressure. Low-frequency nerve stimulation (1-10 Hz) activated phasic rises in pressure. This

suggests that frequency-dependent nerve stimulation generates phasic activity in the bladder (Gillespie *et al*, 2003).

Another explanation for the differences in $f_{1/2}$ values between the stable and overactive groups may be due to the activity of suburothelial myofibroblasts, which form a distinct layer below the urothelium and are connected to each other through connexin-43 gap junctions. When this suburothelial layer is stimulated, Ca^{2+} waves result, which propagate across the suburothelium first, and then to the detrusor layer (Kanai *et al*, 2007). It is proposed that the suburothelial myofibroblasts may amplify the sensory response to bladder wall stretch (Fry *et al*, 2007). Furthermore, there is evidence that connexin-43 expression is up-regulated in detrusor of patients with symptoms of urgency (Neuhaus *et al*, 2005, Ikeda et al, 2007). This altered connexin gap junction concentration in the suburothelial layer of overactive bladders, could cause an increase in propagation of the Ca^{2+} wave, leading to un-coordinated detrusor contractions.

4.3 Atropine-resistance

This study demonstrated atropine-resistant contractions in all guinea-pig specimens, in accordance with previous data (Creed *et al*, 1991); i.e. these contractions were driven by non-cholinergic neurotransmitters. In detrusor, it is well established that the neurotransmitter responsible is ATP (Burnstock *et al*, 1978). Atropine-resistant contractions, mediated by ATP, are present in most mammalian species except humans and old world monkeys (Kinder & Mundy, 1985). In stable human bladders, contraction is mediated solely by Ach, evidenced by the absence of atropine resistant contractions (Sjogren *et al*, 1982), confirmed in this thesis. In contrast, the idiopathic

and NDO groups demonstrated atropine resistance in 9/12 and 16/18 of the specimens, respectively. Overall, the idiopathic and NDO groups demonstrated similar extents of atropine-resistance. Previous studies have identified atropine-resistance to be greatest in idiopathic overactivity, especially in those with bladder outlet obstruction (Ikeda Y, 2006), but in only 3/10 samples from NDO bladders (Bayliss *et al*, 1999). In this thesis a greater number of NDO samples were studied. In female IDO, the purinergic component accounts for 50% of the unstable contraction (O'Reilly *et al*, 2002). This study found that 44.6% of the contraction was purinergic in IDO samples of mixed sex. These findings imply that ATP has a functional role to play in detrusor contraction of the overactive bladder. This could be explained by a reduction in ectonucleotidase activity which would lead to a persistence of undegraded ATP in the synaptic cleft, allowing it to bind to post-synaptic P2X receptors (Harvey *et al*, 2002).

Overactive bladder syndrome becomes more prevalent with age, and there is evidence that atropine -resistance increases with age (Yoshida *et al*, 2001). In this thesis, pathology and the age of the patient were not correlated with the presence of atropine-resistance. This may explain the significant degree of atropine-resistance seen in NDO specimens taken from significantly younger patients.

4.4 Guinea-pig detrusor

4.4.1 The effect of P1 receptor agonists and antagonists on nerve mediated and carbachol induced contraction

The selective A_1 receptor agonist, CPA caused inhibition of nerve-mediated contractions. However previous studies (Ikeda Y, 2006) have shown that NECA and

adenosine evoked a greater inhibitory response, implying that some, but not all, of adenosine's inhibitory activity is mediated via A_1 receptors. Adenosine and CPA also caused a shift to larger $f_{1/2}$ values which has been interpreted as these agents selectively reducing low-frequency contractions.

This phenomenon was investigated further using the selective A_{2A} receptor agonist, CGS-21680, the selective A_3 agonist, IB-MECA, and the A_{2B} antagonist, alloxazine (selective A_{2B} agonists were not available). As CGS-21680 also reduced nerve-mediated contractions, although not to the degree of adenosine or even CPA, it may be hypothesised that the inhibitory actions of adenosine are in part mediated through the A_{2A} receptor. In other animal species, such as the rat, northern blotting has revealed high levels of A_{2A} receptor mRNA in the detrusor (Stehle *et al*, 1992). My own RT-PCR studies suggest that the A_{2A} receptor is present, although not ubiquitous in human stable bladders. Both alloxazine and IB-MECA had no significant effect on nerve-mediated contractions.

When the muscle was directly stimulated by the application of the muscarinic agonist, carbachol, the effects of adenosine and various P1 compounds on detrusor muscle receptors were examined. Adenosine caused a significant reduction in carbachol-induced contraction; as did the A_1-selective agonist CPA, although to a lesser degree. CGS-21680, alloxazine and IB-MECA had no effect. This implies that adenosine does not act via A_{2A}, A_{2B} or A_3 post-synaptic receptors to modulate detrusor contraction in guinea-pig detrusor. However, alloxazine is not a particularly specific A_{2B} receptor modulator, and this makes assumptions about A_{2B} receptor post-synaptic activity difficult. In fact some experiments (see section 4.7.1) showed that alloxazine

augmented the effects of adenosine. Adenosine and NECA reduced the magnitude of carbachol-induced Ca^{2+} transients in the human and guinea-pig indicating there are functional P1 receptors on the detrusor cell itself. It has been proposed that the A_{2B} receptor post-synaptically modulates detrusor contraction, but this is based on the fact that other modulators, that show increased selectivity for other P1 receptor subtypes, were without significant effect (Ikeda Y, 2006, unpublished data). However in other smooth muscles, such as guinea-pig taenia-coli, there is some evidence that A_{2B} receptors cause relaxation (Prentice & Hourani, 1997).

Carbachol-induced 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 was not mediated by an adenylate cyclase pathway (Y Ikeda unpublished data).

4.4.2 Frequency-dependent inhibition by P1 compounds

α,β-methylene ATP (ABMA, a non-hydrolysable analogue of ATP) had a greater effect in reducing tension at 2 Hz than at 20 Hz. In contrast, atropine reduced force more at 20 Hz compared to that at 2 Hz. We have interpreted this to mean that ATP is preferentially reduced at low stimulation frequencies and Ach at higher frequencies. Studies in mouse detrusor have shown similar effects where the stimulation frequency required to obtain a half-maximal force was significantly lower for the purinergic than the cholinergic pathway, whereas the maximum force was similar (Werner *et al*, 2007).

Adenosine also reduced force relatively more at low stimulation frequencies (below frequency, $f_{1/2}$ for half-maximal activation) than at high frequencies (above $f_{1/2}$). 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. Both CPA and CGS-21680 reduced the force of contraction comparatively more at lower (2 Hz) stimulation frequencies than higher ones (20 Hz). However, the effect was most marked with adenosine. Conversely, alloxazine and IB-MECA displayed no evidence of frequency dependent inhibition.

It may be hypothesisied therefore that adenosine limits more ATP release from motor nerves than acetylcholine, through A_1, and to a lesser extent A_{2A}, receptors – see also Nicholls *et al,* 1992.

4.5 Stable human detrusor

4.5.1 The effect of P1 receptor agonists and antagonists on nerve mediated and carbachol induced contractions

Adenosine reduced the force of nerve-mediated contraction and increased the T_{40}/T_4 ratio (shifted the $f_{1/2}$ to the right). CPA had a small but insignificant effect on reducing the force of contraction. CGS-21680, alloxazine and IB-MECA also did not display any effects. One may therefore hypothesise that pre-junctional P1 receptors have a functional role in modulating detrusor contraction in the stable human bladder.

The addition of P1-specific compounds did not cause significant reduction of the carbachol contracture in human stable detrusor. However, adenosine did cause a significant reduction of the contraction as compared to control. This raises the

possibility that the post-synaptic effects of adenosine are mediated through receptors other than P1 receptors (see section 4.7.2).

4.5.2 Frequency-dependent inhibition by P1 compounds

The ratio of tension at low vs. high frequency was deemed to be a more reliable means of analyzing the data rather than comparing $f_{1/2}$ and T_{max} values. The frequencies chosen for calculation of the ratio of tension were chosen as they lie either side of the $f_{1/2}$, where the values are less prone to error, as they lie on the points of inflection of the force-frequency curve. Adenosine caused a significant reduction in force at 4 Hz as compared with 40 Hz; CPA had no significant effect. Of interest was that CGS-21680 caused the greatest mean reduction of nerve-mediated contraction of any of the more selective compounds, although this was not statistically significant, and generated a small increase of the T_{40}/T_4 ratio. None of the other P1-specific compounds exhibited any frequency-dependent inhibition or any significant reduction in nerve-mediated contractions. In view of the lack of atropine-resistance in these preparations a frequency-dependent effect of adenosine may not be anticipated and so the effect may be non-selective.

4.6 Human bladder with idiopathic detrusor overactivity

4.6.1 The effect of P1 receptor agonists and anatagonists on nerve-mediated contractions

After desensitization of P2X receptors with ABMA, the reduction in tension in IDO specimens was significantly greater at lower than at higher stimulation frequencies. These findings support previous studies which found that the purinergic component of contraction in IDO specimens was significant and particularly prominent at stimulation frequencies of 2 to 16 Hz (O'Reilly *et al*, 2002). Furthermore, atropine

reduced tension relatively more at higher frequencies of stimulation. This supports the hypothesis that there is differential release of neurotransmitters which varies according to the frequency of stimulation.

Adenosine generated a small but significant reduction of the force of contraction compared with control at 20 Hz, but had no effect on the frequency-dependent properties. The other P1 specific compounds, including CPA had no real effect on reducing the tension of nerve-mediated contractions. It may be concluded that, in contrast to detrusor from stable and (NDO (see below) bladders adenosine exerted only small effects.

Due to a paucity in availability of IDO specimens it was not possible to perform carbachol experiments. These experiments would answer the question as to whether P1 compounds have any post-synaptic effect in patients with IDO.

4.7 Human bladder with neurogenic detrusor overactivity

4.7.1 The effect of P1 receptor agonists and anatagonists on nerve-mediated contractions

Adenosine caused a much greater reduction in tension of nerve-mediated contractions in NDO compared with IDO and stable specimens. CPA and alloxazine also had a depressant effect on detrusor contraction, whereas they had no real effect in IDO specimens. Alloxazine in the presence of 1 mM adenosine reduced tension further. This suggests that in the overactive bladder, adenosine has an action on pre-junctional, A_1, and possibly A_{2B} receptors. At the time of this study, the only available A_{2B} receptor modulator was alloxazine. However, recently new potent and selective

antagonists of the human A_{2B} adenosine receptor have been discovered. These include 1-, 3- and 8-substituted-9-deazaxanthines (Stefanachi *et al*, 2008). It would be interesting to examine the effect of these on nerve-mediated contractions in human detrusor.

In the human overactive bladder, A_{2B} receptor antagonism dampens detrusor contraction. A similar situation is seen in in vitro preparations of human lung tissue where activation of the A_{2B} receptor results in adenosine-dependent pulmonary inflammation. Antagonising the receptor reduces proinflammatory cytokines and chemokines and dampens the effects of disease (Sun *et al*, 2006). This receptor could indeed represent a therapeutic target in the treatment of OAB, however further studies are necessary. CGS-21680 and IB-MECA had no significant effect on nerve-mediated contraction in NDO.

There was a significant increase of the T_{40}/T_4 ratio in the presence of adenosine as compared with control, an effect absent in IDO specimens, as noted above.

4.7.2 The effect of P1 receptor agonists and anatagonists on carbachol-induced contractions

Adenosine had a significant post-synaptic effect in reducing by almost half, the force of detrusor contraction in NDO when compared with control. None of the more subtype-selective modulators had any significant effects

Adenosine caused a relatively greater reduction in the carbachol contracture in NDO as compared with stable bladder. This may be due to the fact that adenosine has a

greater post-synaptic effect in pathological bladders and further experiments are required to see if this phenomenon exists in patients with idiopathic detrusor overactivity.

The addition of P1-specific compounds did not cause significant reduction of the carbachol contracture in human NDO samples. Therefore it may be hypothesized that adenosine modulated detrusor contraction via a non-P1 route on the muscle itself. Studies on rat urothelium and detrusor indicate an increase in Western blotting detected expression of M2, M3 and P2X3 receptors, and M3 receptors, respectively, in rats with bladder outflow obstruction-induced detrusor overactivity compared with the control group (Kim *et al,* 2008). It is therefore possible that the post-synaptic effects of adenosine are via these receptors, which are upregulated in the overactive bladder.

4.8 Frequency –dependent release of different neurotransmitters

Table 4.1 summarises the effect of ABMA and atropine on the T_{20}/T_2 and T_{40}/T_4 ratios in the various experimental groups investigated in this thesis. The guinea-pig data was consistent with the hypothesis that ATP is released preferentially at low stimulation frequencies and acetylcholine at higher frequencies. This was corroborated by data from NDO human detrusor. Data from stable human bladder could not test the hypothesis due to the lack of atropine-resistant contractions, and the human IDO data failed to reach statistical significance, although the mean T_{40}/T_4 ratio was greater in the presence of ABMA. This is the first indication for the frequency-dependent release of activator neurotransmitters in human detrusor.

Table 4.1: Summary of T_{20}/T_2 and T_{40}/T_4 ratios with ABMA and atropine in all 4 detrusor subtypes.

T_{20}/T_2 ratio (guinea-pig) and T_{40}/T_4 ratio (human)				
Detrusor	**Control**	**ABMA**	**Control**	**Atropine**
Guinea-pig	4.4 ± 0.8	18.3 ± 10.9* (n=8)	5.3 ± 1.0	2.7 ± 0.8 * (n=10)
Stable	4.4 ± 0.7	13.9 ± 5.7 (n=11)	5.1 ± 1.2	13.5 ± 7.1 (n=7)
IDO	5.7 ± 2.9	9.4 ± 6.6 (n=7)	4.9 ± 2.1	4.7 ± 1.9 (n=9)
NDO	5.1 ± 2.5	21.1 ± 9.7* (n=6)	14.2 ± 7.8	9.6 ± 7.1* (n=16)

These results imply that ATP and Ach are not co-released from the same vesicle. When ATP release was measured using biosensors, ATP release peaked at 10 Hz in guinea-pig specimens however, tension continued to rise and peaked at a higher frequency (25 Hz) (McCarthy, 2007).

The inference that can be made from these results is that the purinergic pathway is more active at lower stimulation frequencies, particularly in the overactive bladder. Support for this observation arises from studies on diabetic rabbits. Diabetes mellitus can cause neuropathy which may affect the nerve supply of the bladder and result in NDO. Studies have found that there is enhanced purinergic and a reduction of

cholinergic neurotransmission in the detrusor muscle of the diabetic rabbit (Mumtaz *et al*, 2006). The fact that adenosine attenuated nerve-mediated contractions more at low frequencies suggests that it may be used to target preferentially ATP release. As ATP is a functional transmitter only in pathological human bladders such a target might be used to manage preferentially this pathological mechanism.

4.9 Adenosine receptor mRNA expression in human detrusor

RT-PCR studies were used to investigate whether the different subtypes of adenosine receptor vary in bladders of differing pathology. Studies on whole rat bladder identified message for all four adenosine receptor subtypes by RT-PCR analysis (Dixon *et al*, 1996), however, no studies have analysed differences in adenosine receptor expression between stable and overactive bladders.

Results indicated that there was no significant difference in A_1 receptor mRNA expression in any patient group. Compared with the other adenosine receptor subtypes, A_1 mRNA expression was low in all samples, regardless of pathology. This may be because during preparation of the detrusor the nervous tissue was stripped from it. Certainly the functional data suggests that it has a major role to play in detrusor contraction predominantly via pre-synaptic actions. In addition, if the A_1 receptor is limited to nerve endings then the low relative proportion of nerve tissue in a detrusor sample means that the amount of DNA for the receptor would be very small, so that differences in expression would be difficult to measure. The small absolute signal confirms this interpretation. Other methods, such as in situ hybridisation, may be required in future studies to identify different extent of A_1 receptor expression in samples from stable and pathological bladders.

RT-PCR revealed varied levels of amplification of the A_{2A} receptor transcript. Although expression was not ubiquitious, A_{2A} receptor mRNA expression was down-regulated in neurogenic as compared with stable and idiopathic bladders. The interpretation of this is unclear as the A_{2A} receptor agonist CGS-21680 had no real effect on nerve-mediated or carbachol induced contraction in NDO specimens. Thus, alterations in the expression of the A_{2A} receptor did not correlate with functional studies and indicates that the receptor subtype may not have a significant effect in modulating contractile function.

Examination of the tissue distribution in rats of the A_{2B} receptor mRNA by Northern blot analysis showed a restricted distribution with the highest levels expressed in large intestine, caecum, and detrusor muscle (Stehle *et al*, 1992). The results in this thesis concur with previous findings in that A_{2B} receptor mRNA was universally and equally expressed in all human bladder samples, with no difference between the pathological groups.

A_3 receptor mRNA was universally and equally expressed in all human bladder samples. High levels of amplification product were identified in each patient group. Previously it was thought that the A_3 receptor demonstrates a species-specific tissue distribution (Linden *et al,* 1993). More recent work suggests that this is due to quantitative differences in the abundance of transcript between species and tissues and not due to qualitative variation (Dixon *et al*, 1996). However, due to the lack of action of A_3-selective agonists on contractile function the data suggest that the receptors, although present, do not modulate significantly detrusor contractile function.

4.10 Adenosine receptor protein expression

Analysis of the Western blot for the A_1 receptor revealed low levels of any bands which corresponded to the mass of the A_1 receptor (36.5 kDa). This correlated with the presence, although at a low level, of A_1 receptor message in the RT-PCR experiments. The small protein expression may also be attributed to post-transcriptional or post-translational changes. However, A_1 receptor protein was identified in the control lane i.e. commercially bought human bladder lysate.

The low signal levels may be due to the fact the tissue may have been denervated either due to the disease process or during tissue retrieval. However, equally likely is the fact that the total protein level would be expected to be very low if located only on nerve endings. In addition, A_1 receptors may be located on the urothelium, as in rat tissue where they have been localised to the apical or outermost plasma membrane of the umbrella cells (Yu *et al,* 2006). If A_1 receptors are also located on human urothelium, the lack of signal in these experiments demonstrates the effectiveness with which the urothelium was removed from the human detrusor samples used in this study. Of interest however, is that A_1 receptor presence on detrusor may be species-dependent as on cat detrusor receptors were present, but within mouse and rat, staining of the muscle did not identify A_1 receptors (Yang *et al,* 2000).

In this thesis atropine resistance was demonstrated in both IDO and NDO specimens. In the latter there was a down regulation of A_{2A} mRNA compared with stable specimens. This correlates well with the Western blot findings in which the protein corresponding to the A_{2A} receptor was significantly down-regulated in both overactive groups, compared with control and stable samples, and in particular in the NDO

group. The functional significance of this is unclear, as CGS-21680 did not demonstrate any effect on nerve or carbachol-mediated contractions in the NDO bladder. However, an alteration in purinergic receptor expression may result in an increase in atropine-resistant contractions, and it has been shown that patients with idiopathic detrusor overactivity do not express P2X(3) and P2X(5) receptors (O'Reilly *et al*, 2002).

The A_{2B} receptor was expressed in all three patient groups, consistent with further data reporting intense A_{2B} receptor staining in rat detrusor (Yu *et al,* 1996). Functional studies using alloxazine revealed a depressant effect on nerve-mediated contractions in the NDO but not the IDO group. The addition of adenosine depressed nerve-mediated contractions even further, suggesting that in NDO, adenosine may act upon on pre-junctional A_{2B} receptors. Functional studies in this thesis do not suggest that the A_{2B} receptor acts post-synaptically to modulate detrusor contraction. However, as previously mentioned, the lack of specific high affinity A_{2B} receptor compounds means that the mere measurement of tension generated on carbachol-induced contraction is not a sensitive enough technique of assessing the post-synaptic effects of P1-receptor compounds. The more specific technique of measuring carbachol-induced Ca^{2+}-transients in isolated detrusor myocytes addresses this issue. Results have shown the A_{2B} receptor does indeed have a role to play in modulating detrusor contraction at the post-synaptic site, as demonstrated by the reduction of magnitude of carbachol-induced Ca^{2+}-transients in human by adenosine and alloxazine alike (Ikeda, 2006).

The A_3 receptor proteins were ubiquitously expressed in all three detrusor subgroups, which correlates well with the PCR results which also identified A_3 receptor mRNA

in all three subgroups. The functional significance of the A_3 receptor in the detrusor remains unclear. In this thesis the A_3 agonist, IB-MECA did not elicit any effect on either nerve- or carbachol-mediated contraction. However, in other organs, the A_3 receptor has been shown to have important effects. There is an abundance of A_3 receptor mRNA found in the lung of several species, and it has a role in mediating allergic responses in the respiratory tract (Olah & Stiles, 1995).

A_3 receptors also protect the lung against ischaemia-reperfusion injury, by reducing apoptosis. In the cat model the protective effects of IB-MECA were completely blocked by pretreatment with the selective A_3 receptor antagonist MRS-1191 (Rivo *et al*, 2004).

Studies on A_3 receptor gene-deficient knockout mice that were exposed to transverse aortic constriction (TAC), resulted in left ventricular hypertrophy, fibrosis, and a reduced left ventricular ejection fraction. It is proposed that antagonism of the A_3 receptor may be a novel treatment of pressure overload-induced left ventricular hypertrophy and dysfunction (Lu *et al,* 2008).

Studies on the uroepithelium have shown that $A_{2A} > A_1 > A_{2B}$ receptors contribute to adenosine-induced changes in exocytosis. However, it was not possible to assign such a role to the A_3 receptor.

4.11 Summary

Adenosine and its receptors play an integral role in modulating detrusor function in the guinea-pig and human bladder. Adenosine depresses the magnitude of both nerve-

and agonist-mediated detrusor contraction, indicating that there are both pre- and post-junctional adenosine receptors involved.

The pre-junctional A_1 receptor plays a major role as is evidenced by the profound depressant effect of CPA on nerve-mediated contractions of both guinea-pig and human detrusor with neurogenic overactivity. In the stable and idiopathic overactive bladders, the A_1 receptor plays less of a role in modulating detrusor contraction. Furthermore CPA caused frequency-dependent inhibition, preferentially at lower frequencies ie. frequencies at which ATP is released. RT-PCR and Western blotting analysis indicated a paucity in both A_1 receptor message and protein in all detrusor sub-groups. This is a result of either low endogenous DNA/RNA and protein levels, or inadvertent dennervation of the detrusor during transport, storage or preparation. Further studies need to be performed in order to perfect the techniques involved in A_1 receptor analysis.

There is evidence for an A_2 receptor-mediated post-synaptic inhibition by adenosine. However, as neither CGS nor alloxazine demonstrated any significant effect on carbachol-induced contractions, conclusions about the A_2 receptors and their role in the modulation of detrusor contraction remain unanswered. As alloxazine is a non-specific A_{2B} antagonist (with possible agonist activities as well), further research using more specific A_2 compounds is needed.

In NDO bladders, it seems that a pre-junctional A_{2B} receptor may play a role in modulating overactive detrusor contraction, as antagonism of this receptor led to a reduction in the tension generated. This may represent a future therapeutic target in

the management of OAB caused by neurogenic detrusor overactivity. Results indicate a decrease in A_{2A} receptor mRNA expression in neurogenic as compared with stable and idiopathic bladders. However protein expression was relatively reduced in both the overactive sub-groups. The functional significance of these findings is unclear as the A_{2A} receptor agonist CGS-21680 had no real effect on nerve- or agonist-mediated contraction in this sub-group.

A_{2B} receptor mRNA and protein was universally and equally expressed in all human bladder samples, and although this receptor does play a role in modulating detrusor contraction, the significance of this role in stable human detrusor is questioned as the receptor has a low affinity for adenosine. In the stable bladder it is doubtful whether the physiological extracellular concentration of adenosine would be sufficient to activate the A_{2B} receptor. However in bladders with neurogenic detrusor overactivity the A_{2B} receptor may become more sensitive to adenosine and activation may result in uncontrolled detrusor contraction.

A_3 receptor mRNA and protein was also universally and equally expressed in all human bladder samples. However the role of this receptor in modulating detrusor contraction remains unclear. More studies are required in particular to examine the effects of specific A_3 antagonists on both nerve- and agonist-induced detrusor contraction.

Adenosine attenuates nerve-mediated contractions at all frequencies, and the results indicate it has a greater proportional effect at lower stimulation frequencies, confirming the hypothesis that adenosine preferentially affects ATP release.

Furthermore, purinergic neurotransmission at lower stimulation frequencies appears to be more active, particularly in human bladders with neurogenic detrusor overactivity and the guinea-pig, implying that it is an imbalance in the purinergic and cholinergic pathways which causes detrusor overactivity in the human bladder.

Adenosine causes a quantitatively greater reduction in force of contraction in overactive as compared with stable bladders, and more so in NDO as compared with IDO bladders.

4.12 Future directions

The development of improved and more specific adenosine receptor agonists and antagonists is required in order to define firmly the functional effects of the four adenosine receptor subtypes in the human detrusor.

In hindsight, the technique of PCR was not sensitive enough to isolate the adenosine receptors in the bladder specimens. Techniques such as in situ hybridisation (ISH) may give a higher yield of receptor mRNA. This technique localizes mRNA in morphologically preserved tissue by hybridizing an oligonucleotide probe to the sequence of interest without prior RNA isolation as is required by RT-PCR. Cost aside, this would answer the question of differences in concentration of adenosine receptors according to bladder pathology.

This leads to the question of why the $A2_A$ receptor is down regulated in the overactive bladder, in particular those with NDO. Brading's dennervation hypothesis may be relevant here as the paucity of $A2_A$ receptor pick-up in OAB samples may be due to their dennervation.

It would also be interesting to investigate the role of adenosine in the synaptic cleft and whether a change in concentration either by increase or a decrease in production has an effect on detrusor contraction.

In summary, although this thesis attempts to categorise patients into specific groups ie. Stable, IDO and NDO bladders, in the clinical setting this is difficult to do as patients often experience a wide range of symptoms, sometimes including both storage and voiding LUTS. It is important to stress the necessity of treating each patient as an individual case as each may have a number of different pathophysiological mechanisms involved.

CHAPTER FIVE

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