PURINERGIC SIGNALLING IN THE GENITO-URINARY TRACT

A thesis presented for the degree of Doctor of Medicine to the Faculty of Medicine of the University of London

By

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Abstract

The main objective of this thesis was to examine the role of purinergic signalling in the contraction of the smooth muscle of the genito-urinary tract of laboratory animals and compare it to that of man. It also examined purinergic signalling in the maturation of sperm within the epididymis.

The main methodology involved organ bath studies on the functional physiology of smooth muscle contraction, in conjunction with immunohistochemical examination of smooth muscle P2X receptor expression.

In Chapter 3, a comparative study of the smooth muscle cells of the testicular capsule or tunica albuginea of the testis from man, mouse, rat and rabbit was made. The smooth muscle cell arrangement was demonstrated by electron microscopy, and the role of purinergic co-transmission in the contraction of this smooth muscle was investigated.

Chapter 4 examined purinergic signalling in the contraction of the human vas deferens smooth muscle.

P2X receptors are involved in cell-to-cell signalling. Chapter 5 was a comparative study of the expression of P2X receptors on sperm contained within the head and tail of the epididymides of mice, rats, hamsters and man. This study demonstrated changing expression with maturity.

Alterations in the relative purinergic and cholinergic components of detrusor contraction have been demonstrated in the over active bladder. Chapter 6 details the partial bladder outlet obstruction model that was developed in the rat. This model demonstrated an up–regulation of the cholinergic component of detrusor contraction with no significant change in the purinergic component, which implied the rat detrusor adapts to outflow obstruction in a different manner to the human detrusor.

In Chapter 7, a general discussion of the role of purinergic signalling in the genito-urinary tract is given. The extent of how well the hypothesis was tested is considered in this chapter and future directions are suggested.

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Abbreviations

ABC	avidin-biotin complex
ACh	acetylcholine
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ANOVA	analysis of variance
ASO	antisense oligonucleotides
ATP	adenosine 5'-triphosphate
$\alpha\beta$-meATP	α,β-methylene ATP
$\beta\gamma$-meATP	β,γ-methylene ATP
BOO	bladder outlet obstruction
BPH	benign prostatic hyperplasia
CGRP	calcitonin gene-related polypeptide
CNS	central nervous system
DAB	diaminobenzide
DNA	deoxyribonucleic acid
EJP	excitatory junction potential
EM	electron microscopy
FITC	fluoroscein isothiocyanate
H&E	haematoxylin and eosin
5-HT	5 hydroxytriptamine
ICSI	intra cytoplasmic sperm injection
IP_3	inositol triphosphate
mRNA	messenger ribonucleic acid

N	number
NANC	non-adrenergic, non-cholinergic
NHS	normal horse serum
NPY	neuropeptide Y
OAB	over active bladder
P	probability
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PPADS	pyridoxal-phosphate-6-azophenyl-2',5'-disulphonic acid
PPNDS	pyridoxal-5'-phosphate-6-(2'-napthylazo-6'-nitro-4',8' disulfonate)
RNA	ribonucleic acid
siRNA	double stranded, short interfering RNAs
RT-PCR	reverse transcriptase-polymerase chain reaction
s.e.m	standard error of the mean
SP	substance P

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Publications arising from this work

Papers

Alterations in purinergic and cholinergic components of contractile responses of isolated detrusor contraction in a rat model of partial bladder outflow obstruction

F.C.L. Banks, G.E. Knight, R.C. Calvert, C.S. Thompson, R.J. Morgan, G. Burnstock, British Journal of Urology International 2006 Feb;97(2):372-8

The purinergic component of human vas deferens contraction

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Smooth muscle and purinergic contraction of the human, rabbit, rat and mouse testicular capsule

F.C.L. Banks, G.E. Knight, R.C. Calvert, M. Turmaine, C.S. Thompson, D.P. Mikhailidis, R.J. Morgan, G. Burnstock, Biology of Reproduction 2006 Mar;74(3):473-80

Changing P2X receptor expression on maturing sperm in the epididymides of mice, hamsters, rats and man

F.C.L. Banks, R.C. Calvert, R.J. Morgan, G. Burnstock, (Submitted Reproduction October 2005)

Abstracts

Purinergic co-transmission in the contraction of human vas deferens smooth muscle

F.C.L. Banks, A. Crump, R.C. Calvert, G.E Knight, C.S. Thompson,

D.P Mikhailidis, R.J. Morgan, G. Burnstock, Journal of Urology (2003) 169 (4):P412

(abstract 1541)

The purinergic component of human vas deferens contraction

F.C.L. Banks, A. Crump, R.C. Calvert, G. E. Knight, C.S. Thompson,

D.P Mikhailidis, R.J. Morgan, G. Burnstock, European Urology (2003) Suppl 2 (1):

P120 (abstract 472)

Capsule contractility in the testes of humans, rats and mice

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Journal of Urology (2002) 167 (4):P316 (abstract 1250)

Is ATP signalling involved in the development of sperm motility in the epididymis?

F.C.L. Banks, R.C. Calvert, C.S. Thompson, D.P Mikhailidis, R.J. Morgan, G. Burnstock, Journal of Urology (2002) 167 (4):P314 (abstract 1242)

Ultrastructure and pharmacology of the human testicular capsule contraction

F.C.L. Banks, R.C. Calvert, M. Turmaine, G.E. Knight, R.J. Morgan,

G. Burnstock, European Urology (2002) Suppl 1 (1):P183 (abstract 723)

ATP: more than just rocket fuel for sperm?

F.C.L. Banks, R.C. Calvert, C.S. Thompson, D.P Mikhailidis, R.J. Morgan, G. Burnstock, European Urology (2002) Suppl 1(1):P183 (abstract 722)

Preface and hypothesis

ATP, as an extracellular signalling molecule, under the broad umbrella of purinergic signalling, probably represents a primitive signalling system, being present throughout animal phyla. However, research into purinergic signalling remains a relatively new field, and it is only recently that the field has rapidly advanced. The cloning of P2 receptors and modern techniques of gene or protein recognition have identified P2 receptor expression in a huge variety of tissues. The field has therefore jumped from the co-transmission explanation of non-adrenergic, non-cholinergic (NANC) smooth muscle contraction, to include a rapidly increasing range of biological functions. P2 receptors are now thought to mediate endocrine and epithelial cell secretion, nociception, cell differentiation, maturation and apoptosis to name only a few functions (Burnstock and Knight 2004).

An observation of purinergic signalling is that animal studies are not necessarily replicated in man, and in healthy tissues the significance of purinergic signalling may be different to than that demonstrated in animals. However, in certain human pathological states, the significance of purinergic signalling is altered, which potentially leads to the exciting prospect of pharmacological therapy. In this study, I attempted to include human tissue to identify similarities and differences between animal and human tissue.

The therapies for male infertility are extremely limited, other than surgical bypass for tubular blockage. The success of intra cytoplasmic sperm injection (ICSI) in achieving fertilisation, even with immature testicular sperm, has focussed research away from the factors causing male factor infertility into the mechanism of embryo

implantation and the maintenance of pregnancy. The very basis of normal sperm production, maturation and transportation through the genital tract is incompletely understood, and much 'evidence' is implied from anatomical studies. Objective evidence for how a sperm is propelled from the seminiferous tubule into the epididymis, through the genital tract and subsequently ejaculated, is extremely limited. There is very limited evidence for contractions of the seminiferous tubules, and, as non-ejaculated sperm are quiescent, the passage of sperm from the testis into the epididymis must be down a pressure gradient. I therefore examined the contractile properties of the tunica albuginea of the testis, which is generally thought to be an acontractile fibrous sac, despite its obvious natural tone, demonstrable by the immediate extrusion of seminiferous tubules on puncture. The smooth muscle cell phenotype and distribution was examined, and the role of purinergic signalling in the contraction of this smooth muscle was investigated. The natural progression from this was to examine the role of purinergic signalling in the vas deferens. Despite the fact that the most convincing evidence for purinergic co-transmission was undertaken in the vas deferens of animals, and $P2X_1$ receptors were cloned from the rat vas deferens, few reports of purinergic function in human vas deferens are published. The purinergic component of human vas deferens was therefore characterised.

Extracellular ATP is increasingly being shown to have trophic effects on cellular differentiation and maturation. The presence of ATP in ejaculated seminal fluid is well known, but ATP concentration does not correlate with fertility or sperm motility. This is suggestive of a further function for ATP beyond being an energy substrate for ejaculated sperm. A chance observation demonstrated $P2X_1$ receptor expression on sperm contained in the head of the epididymis, which was subsequently

lost on sperm in the tail of the epididymis. The possibility that P2X receptors may be involved in the maturation of epididymal sperm was investigated.

The pathological, overactive bladder is a huge clinical problem with poorly correlating symptoms and no unifying cause. The mainstay of treatment remains anticholinergic medication, which is poorly tolerated, and trials show minimal benefit over placebo. The worldwide anticholinergic market is worth over $6 billion a year. An up regulation of the normally minimal purinergic component of human detrusor contraction has been shown in the over active bladder. Further evidence for purinergic up regulation was demonstrated in a rabbit model of partial bladder outflow obstruction within this department. The recent development of polyclonal antibodies to the seven P2X receptor subtypes, invited the opportunity to examine the P2X receptor subtypes involved in the observed purinergic up-regulation of detrusor contraction. As the antibodies were prepared in a rabbit, a rat model of partial bladder outlet obstruction was developed, and the purinergic component of the obstructed detrusor was characterised.

In this thesis I tested the hypothesis that:

"Purinergic signalling in the genito-urinary tract of man may differ from that in laboratory animals".

CHAPTER 1

GENERAL INTRODUCTION

1.1 Historical perspective

1.1.1. Extracellular functions of purines

The purine nucleotide adenosine 5'-triphosphate (ATP) can be considered the energy molecule of life. It is widely known as the fundamental intracellular energy source, and there are few students who do not have some vague recollection of its production via the Kreb's cycle. The role of ATP as an extracellular signalling molecule is less well known, and its intracellular role was probably the cause for resistance in accepting its function as an extracellular signalling molecule as it seemed intuitively unlikely that such an essential compound should have such diverse roles both in and outside the cell. The other point of view is that as it is such an evolutionary essential molecule, it is not surprising that it has functions beyond being an intracellular energy substrate.

The history of ATP as an extracellular signalling compound really began in 1929 when Drury and Szent-Gyorgi found that a purified white powder extract from cardiac muscle, and thought to be adenylic acid, produced heart block, dilated the coronary vasculature, caused hypotension and reduced the contractions of isolated intestinal strips (Drury et al., 1929). Subsequent studies demonstrated that the pharmacological activity of such extracts was largely due to adenosine 5'-monophosphate. ATP was subsequently isolated from skeletal muscle by Lohman in 1931 (Lohman, 1931). At around this time, it became apparent that different nucleotides and nucleosides had differing potencies and activities, which appeared to relate to the number of phosphate groups (Deuticke, 1932). It was not until the latter part of the century that different receptor subtypes were discovered to explain this observation. It was likely that early extracts were of mixed purity, and it is probable,

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that this was the reason for the contrasting observations noted in some early experiments. The conversion of ATP to active metabolites would also explain some opposing observations. For instance in 1933 it was found that ATP injected at a low concentration into perfused cat lungs caused vasodilation, but at a high concentration caused vasoconstriction (Gaddum and Holtz, 1933). Research interest was directed into the actions of purines and principally adenosine on the heart. Clinical studies were developed to examine the possibility of adenosine as an anti-arrythmic agent (Honey et al., 1930; Jezer et al.; 1933). Richards (1934) found that normal human heart rate increased on injection of both adenosine and AMP, but large boluses were found to temporarily arrest the heart (Honey et al., 1930). Its short half life limited its role but it is still utilised in clinical practice in the diagnosis and treatment of supra-ventricular tachycardias. The next big advance in demonstrating the extracellular action of ATP was made by Holton (1959) who showed that antidromic stimulation of sensory nerves caused ATP release, sufficient to cause vasodilation of rabbit ear arteries.

Berne (1963) suggested that adenosine mediated coronary vasodilation in response to hypoxia. This was based on the demonstration of adenosine metabolites in the effluent of hypoxic isolated perfused hearts. He suggested that this was indicative of low intracellular ATP stores, and adenosine crossed the sarcolemma to relax coronary vascular smooth muscle. More recently, Burnstock (1999) has suggested that ATP released from endothelial cells during hypoxic and shear stress, acts on endothelial receptors causing the release of the potent vasodilator nitric oxide (NO).

1.2 Receptors for Adenosine and Purine Nucleotides

The first demonstration that multiple receptors for adenosine and adenine nucleotides existed came from Gillespie in 1934. He demonstrated that deamination of adenine compounds greatly reduced their pharmacological activity and removal of phosphates influenced not only potency, but also the type of response. The ability of adenine to cause vasodilation increased following the removal of phosphates, and ATP was shown to be more potent than AMP and adenosine in causing contraction of guinea-pig ileum and uterus (Gillespie, 1934). This was the first demonstration of different actions of adenosine and ATP, and with subsequent hindsight the demonstration of the two main families of purine receptors.

This fundamental division in the classification of purine receptors persists today. The huge advances in genetics, receptor cloning, and the use recombinant receptors in the last few years have rapidly changed and clarified the receptor classification. The main division was formally recognised by Burnstock in 1978. He proposed that "purinergic" receptors be divided into P1-purinoceptors, at which adenosine was the principal ligand, and P2-purinoceptors at which ATP was the principal ligand (Burnstock, 1978). This division was based on the relative potencies of ATP, ADP, AMP and adenosine, the selective antagonism of adenosine effects by methylxanthines (caffeine and theophylline), activation of adenylate cyclase by adenosine and stimulation of prostaglandin synthesis by ATP and ADP.

On the basis of receptor cloning the P1 receptor has been further divided into four subtypes: A_1, A_{2A}, A_{2B}, and A_3, all of which couple to G proteins. Molecular studies have shown them to be structurally distinct and pharmacological profiles support this.

Evidence suggested two different types of P2 receptor distinguishable on pharmacological grounds (Burnstock and Kennedy, 1985). P2 receptors were shown to be ligand-gated cation channels (Benham and Tsien, 1987), or involved in G protein activation (Dubyak, 1993). In the early 1990's it became apparent that different subtypes of receptors existed, there then followed what has been described as a "random walk through the alphabet" in the classification of these receptors, which included P_{2X}, P_{2Y}, P_{2U}, P_{2T}. Abbrachio and Burnstock revised this unsatisfactory classification in 1994. They proposed the division of the P2 receptors into an ion-gated P2X family and a G protein-coupled P2Y family (Abbrachio and Burnstock, 1994). Currently, in mammals, seven subtypes of P2X receptors ($P2X_{1,2,3,4,5,6 \text{ and } 7}$) receptors and eight subtypes of P2Y receptors ($P2Y_{1,2,4,6,11,12,13 \text{ and } 14}$) are recognised. Their tissue distribution and pharmacological properties have been defined. (Ralevic and Burnstock, 1998; Burnstock, 2003a,b; Burnstock and Knight, 2004)

The classification of purinoceptors has been difficult for a number of reasons. It has been shown that often several types of receptor are present within one tissue. Agonists are rapidly metabolised by ecto-nucleotidases to active metabolites, for instance, ATP is rapidly metabolised to adenosine, whereupon the P1 effect may mask or counteract the P2 effect, with the result that only the dominant receptor effect is seen. Receptor subtypes have differing affinities for agonists. The purinergic field has been hampered by a paucity of truly selective subtype antagonists, furthermore many antagonists have been to found to inhibit ecto-nucleotidases, and so distort pharmacological profiles. The development of subtype receptor gene-deleted mice has helped circumvent this problem. It is becoming apparent that receptors form both homomultimers, and heteromultimers, and there is also evidence for splice variants of some receptors.

Major advances have been made following the cloning of the receptors. Subsequent expression of recombinant receptors in *Xenopus* oocytes or transfected cells has allowed pharmacological characterisation of the specific receptor subtypes. Subsequent development of antibodies to the C-terminus of the different receptor subtypes has allowed the investigation of tissue distribution, which has revealed a wealth of receptor expression in a wide variety of tissues.

1.2.1 P2X Topology

The molecular structure of the P2X receptor is now established. The subtype structures show overlap by 26-47% (North, 2002). They are characterised by two transmembrane domains that cross the plasma membrane, linked by a cystine rich extracellular loop. The majority of the receptor is extracellular (Brake et al., 1994; Valera et al. 1994; Rettinger et al., 2000) with each subunit being between 379 and 595 amino acids in length. The ATP binding site is contained within this extracellular loop (Ennion et al., 2000; Jiang et al., 2000) and it is the site for antagonists and modulators (Garcia-Guzman et al., 1997; Clarke et al., 2000). There is a short N-terminal and a C-terminal of variable length (see Figure 1.1). Three or four units combine to form an ionic pore channel as either homomultimers or heteromultimers e.g. $P2X_{2/3}$ (Lewis et al., 1995). Receptor subtypes have been shown to have variable affinities to different agonists and pharmacological profiles have been established (Khakh et al., 1999).

Electrophysiological studies have shown that all P2X receptors are cation-selective channels. It is thought that Na^+ and K^+ have almost equal permeability, and that Ca^{2+} also has significant permeability. The influx of calcium through the P2X receptors may be important in the physiological functions seen. Calcium has also been

shown to modulate ATP-evoked currents at expressed P2X receptors (Evans et al., 1996; Khakh et al., 1999).

1.2.2 P2Y Topology

These receptors are G-protein coupled which are 328-532 amino acids in length and have seven transmembrane domains, with an extracellular N-terminus and an intracellular C-terminus (Figure 1.2). The conservation between different subtypes is greatest in the transmembrane domains with the C-terminus confering the greatest diversity between subtypes (Ralevic and Burnstock, 1998). The classification of P2Y receptors is less clear than that of P2X receptors with up to 16 heptahelical proteins identified that may be associated with the P2Y family, although at present only 8 subtypes are established in mammals (P2Y$_{1,2,4,6,11,12,13}$ and $_{14}$). The difference has occurred because subtypes have been redesignated, such as the p2y3 receptor which is regarded as an an orthologue of the P2Y$_6$ receptor, P2Y$_{5,9}$ and $_{10}$ are considered 'orphan' receptors with no functional role and have been removed from the classification. The P2Y$_6$ receptor was found to be UTP selective, the P2Y$_7$ receptor was reclassified as a leukotrienne receptor, and finally the p2y$_8$ receptor, which was cloned from the frog has not had a mammalian counterpart identified, and so is not included in the classification (King et al., 2000; Burnstock, 2001b; King and Burnstock, 2002).

The P2Y family can be further subdivided into those receptors that couple to G$_q$ and consequently activate phospholipase C-β (P2Y$_{1,2,4,6}$ and $_{11}$) and those that predominantly couple to G$_i$ that inhibit adenyl cyclase and regulate ion channels (P2Y$_{12,13}$ and $_{14}$) (Abbracchio et al., 2003; Lazarowski et al., 2003). A list of tissue distribution and functions of P2Y receptors is given in Table 1.1.

Figure 1.1
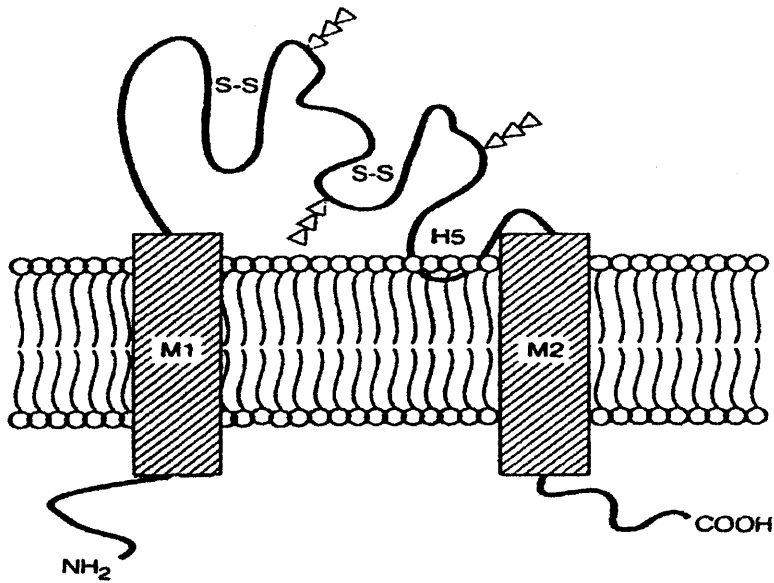
Schematic diagram of a P2X receptor subunit demonstrating 2 transmembrane domains (from Brake et al., 1994).

Figure 1.2
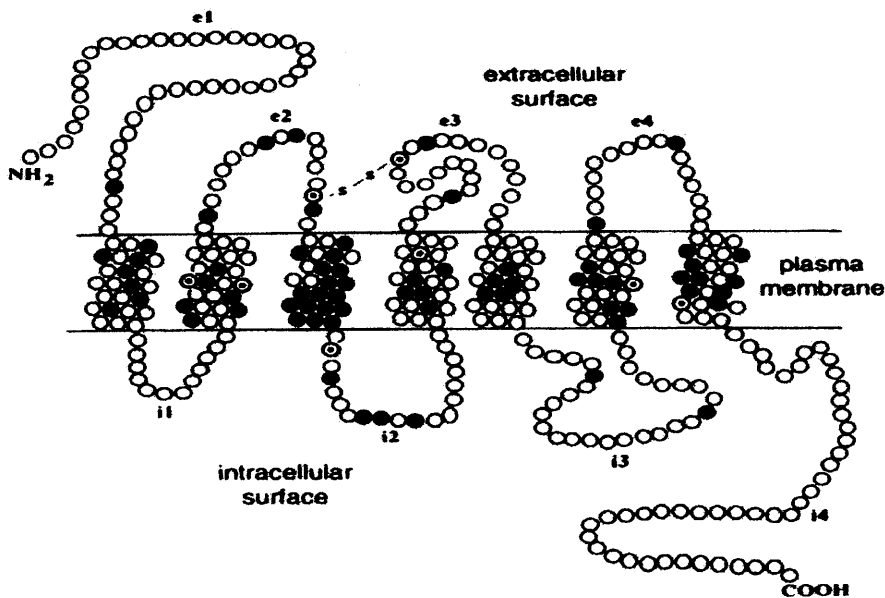
Schematic diagram of a P2Y receptor subunit demonstrating 7 transmembrane domains (from Barnard et al., 1994).

1.3   Purinergic transmission and co-transmission

It was observed more than 100 years ago that stimulation of the vagus nerve induced relaxation of the stomach, even when the cholinergic component was blocked by atropine (Langley, 1898; May, 1904; McSwiney and Robson, 1929). It was thought that this relaxation was adrenergic in origin, although it was only blocked by adrenergic antagonists at sufficiently high concentrations to inhibit ganglionic transmission (Ambache, 1951; Ambache and Edwards, 1951). Later studies demonstrated that relaxations and inhibitory junction potentials of guinea-pig taenia coli were resistant to both atropine and guanethidine antagonism (Burnstock et al., 1963; 1964; 1966), but tetrodotoxin (TTX) sensitive (Bulbring and Tomita, 1967), confirming that they were nerve-mediated. These observations were the basis of non-adrenergic, non-cholinergic (NANC) transmission. At this stage in time "Dale's Principle" dominanted neurotransmission research, with the principle that single neurons utilize a single neurotransmitter. It was clear that other neurotransmitters were involved, however, confirmation of their identities was elusive. In a classical paper in 1970, ATP was shown to be a NANC inhibitory transmitter in the gut, and the concept of purinergic nerves and transmission was developed (Burnstock et al., 1970; Burnstock, 1972).

Evidence for NANC transmission in other tissues was also observed. Atropine-resistant contractions of the bladder had been observed in 1895 (Langley and Anderson, 1895). Subsequently it was proposed that there was a separate NANC innervation of the bladder (Henderson and Roepke, 1934; Chesher and Thorp, 1965; Chesher and James, 1966; Ambache and Zar, 1970). Subsequently, these atropine-resistant contractions would be shown to be mediated by ATP (Burnstock et al., 1972a; 1978a; 1978b).

During this period it became apparent that there were numerous 'transmission systems' involving transmitters other than ATP, such as glutamate, dopamine, 5-hydroxytryptamine (5-HT), as well as a number of peptides. The recognition of several neurotransmitters led to a challenge to "Dale's Principle" of a neuron utilising a single neurotransmitter. This longstanding concept was questioned in 1976 in a seminal review of the evidence that some nerve fibres synthesise, store and release more than one transmitter, with effects induced via postjunctional receptors (Burnstock, 1976). This review was the basis of cotransmission from which it has become accepted that nerves release multiple neurotransmitters and neuromodulators.

Nerve stimulated contraction of the vas deferens illustrates the story of cotransmission whereby excitatory junction potentials (EJPs) were shown to be blocked by the sympathetic neurone blocking agents, bretylium and guanethedine, but not by the postsynaptic adrenoceptor antagonists prazosin and phentolamine (Burnstock and Holman, 1960; 1961). Subsequently, it was shown that ATP induced the EJPs and the initial fast phase of the the biphasic contraction of the vas deferens to nerve stimulation, with co-released NA inducing the slower, tonic phase of the contraction (Sneddon and Westfall, 1983; 1984; Sneddon and Burnstock, 1984).

The expression of purinoceptors in most tissues in conjunction with non-neuronal release of ATP has led to an explosion of interest in the role of ATP as a signalling molecule. Ever increasingly, ATP is being shown to have fast signalling and slow trophic and regulatory roles in a wide range of tissues. Key areas of clinical interest in purinergic signalling include, pain, nociception, inflammation, bone development, anti-neoplastic activity and insulin secretion (Burnstock, 2002). It is beyond this thesis to review this topic but excellent reviews are available (Burnstock, 2003a;b; Burnstock and Knight, 2004).

1.4 ATP release and metabolism

Inherent with the the demonstration of P2 receptors is that they require ATP as a ligand. ATP has been demonstrated in the neuronal vesicles that store NA in sympathetic nerves (Langerkrantz, 1976), and also in conjuction with ACh in parasympathetic nerves (Hoyes et al., 1975). ATP is therefore released from vesicles along with other neurotransmitters following axonal stimulation. The extent of ATP release appears to be modulated at a prejunctional level, particularly by adenosine (Parija et al., 1991; Palea et al., 1995). Experimentally, the extent of ATP release is also affected by the degree of nerve stimulation demonstrating a variable purinergic component at differing frequencies (Calvert et al., 2000).

The demonstration of P2 receptors in an ever-increasing range of tissues required the demonstration of non-neurogenic release of ATP. Convincing evidence for ATP release from vascular endothelial cells and from epithelial cells lining hollow viscera on distension and shear stress has now been demonstrated (Figure 1.3), (Bodin and Burnstock, 1996; Burnstock, 1999; Vlaskovska et al., 2001; Knight et al., 2002). P2X$_3$ receptors have been demonstrated on small diameter sensory neurones and it is suggested epithelially-released ATP acts on these receptors during nociception (Burnstock, 1999; 2001b).

ATP is rapidly broken down to adenosine and other active metabolites by extracellular ecto-ATPases (Zimmermann, 1996; 2001; Burnstock, 1999; Burnstock, 2001a). These are powerful enzymes, which rapidly metabolise ATP. The efficacy of these enzymes is demonstrated by the relatively greater potency of non-hydrolysable ATP analogues such as α,β-methylene ATP (α,β-meATP) in organ bath experiments. Furthermore, tissue analysis has shown that the 'bulk phase' ATP concentration is typically much smaller than the cell surface ATP concentration (Lazarowski, 2003).

The ubiquitous distribution of these enzymes which catalyze nucleotide breakdown and interconversion is further evidence for the wider roles of purinergic signalling.

The mechanisms of ATP transport and release from cells is currently much debated, and it is thought many tissues such as platelets and secretory cells, utilise exocytotic release of specialised vesicular granules which resembles neurotrasmitter release. Non-lytic release of ATP is thought to occur in response to mechanical stimulation or shear stress from epithlial cells as well as glial cells (see Fig 1.3). Furthermore, ATP release from cells may occur in response to pharmacological stimuli such as hypoxia or acidosis, or occur without a known stimulus such as the secretion of ATP by seminiferous tubules (Gelain et al., 2003).

In addition to vesicular exocytosis other mechanisms for ATP release have been suggested, these include ATP binding cassette transporters (ABC), maxi ion channels, connexin hemichannels, mitochondrial porins and stretch-activated channels (Bodin and Burnstock, 2001; Burnstock, 2003a; Lazarowski et al., 2003).

Figure 1.3
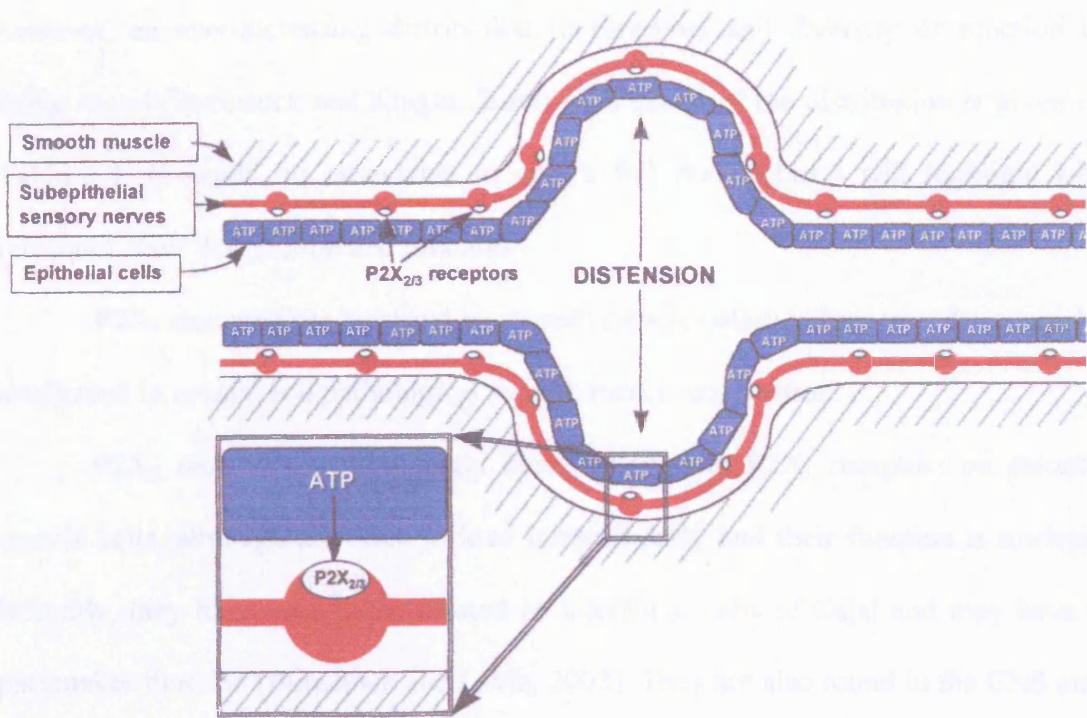
Figure 1.3

Hypothesis that ATP is released from the urothelium of the ureter on distension and acts on P2X₃ nociceptors on the suburothelial sensory nerve plexus to relay message to the CNS pain centres. (from Burnstock, 1999b).

1.5 Distribution and function of P2X receptors

A major role of purinergic signalling is in the contraction of smooth muscle, however, an ever-increasing distribution of receptors and diversity of function is being found (Burnstock and Knight, 2004). The extent of the distribution is given in Table 1.1. It would be exhaustive to give a full review but I will highlight key receptors, their distribution and function.

$P2X_1$ receptors are localised on smooth muscle cell membranes and are widely implicated in normal and pathological smooth muscle contraction.

$P2X_2$ receptors are frequently coexpressed with $P2X_1$ receptors on smooth muscle cells, although are often located intracellularly and their function is unclear. Recently, they have been demonstrated on interstitial cells of Cajal and may have a pacemaker function (Burnstock and Lavin, 2002). They are also found in the CNS and autonomic ganglia and commonly form heteromultimers with $P2X_3$ receptors on sensory nerve endings.

$P2X_3$ receptors are principally located on sensory neurones, nodose, trigeminal and dorsal root ganglia. It is thought they are predominantly involved with nociception as indicated by $P2X_3$ receptor deficient mice demonstrating bladder hyporeflexia (Cockayne et al., 2000).

$P2X_4$ receptors have been located within the CNS and testis, however, their function remains elusive. They also form heteromultimers with $P2X_6$ receptors.

$P2X_5$ receptors have been shown to be associated with cellular differentiation.

$P2X_6$ receptors are slightly unusual in that that they seem unable to form a functional homomultimer, but form functional heteromultimers with $P2X_2$ and/or $P2X_4$ receptors. Such heteromultimers have been demonstrated in the CNS.

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P2X$_7$ receptors have been demonstrated on immune cells and may play a role in apoptosis in many other cell types.

Table 1.1

Receptor		Main Distribution	Transduction Mechanisms
P2X	**P2X$_1$**	smooth muscle, platelets, cerebellum, dorsal horn spinal neurones	intrinsic cation channel (Ca^{2+} and Na^+)
	P2X$_2$	smooth muscle, CNS, retina, chromaffin cells, autonomic and sensory ganglia	intrinsic ion channel (particularly Ca^{2+})
	P2X$_3$	sensory neurones, NTS, some sympathetic neurones	intrinsic cation channel
	P2X$_4$	CNS, testis, colon	intrinsic ion channel
	P2X$_5$	proliferating cells in skin, gut, bladder, thymus, spinal cord	intrinsic ion channel
	P2X$_6$	CNS, *motor neurones in spinal cord*	intrinsic ion channel
	P2X$_7$	apoptotic cells in immune cells, pancreas, skin etc.	intrinsic cation channel and a large pore with prolonged activation
P2Y	**P2Y$_1$**	Epithelial and endothelial cells, platelets, immune cells	G_q/G_{11}; PLCβ activation
	P2Y$_2$	Immune cells, epithelial and endothelial cells, kidney tubules, osteoblasts	G_q/G_{11} and possibly G_i; PLCβ activation
	P2Y$_4$	Endothelial cells	G_q/G_{11} and possibly G_i; PLCβ activation
	P2Y$_6$	Some epithelial cells, placenta, T-cells, thymus	G_q/G_{11}; PLCβ activation
	P2Y$_{11}$	Spleen, intestine, granulocytes	G_q/G_{11} and Gs; PLCβ activation
	P2Y$_{12}$	Platelets, glial cells	G_i (2); inhibition of adenylate cyclase
	P2Y$_{13}$	Spleen, brain, lymph nodes, bone marrow	G_i
	P2Y$_{14}$	Placenta, adipose tissue, stomach, intestine,discrete brain regions	$G_{i/o}$

Tissue distribution and transduction mechanisms of purinoceptors (modified from Burnstock, 2003).

1.6 Sperm production, maturation and transport in the genito-urinary system

1.6.1.1 Sperm production

The daily spermatozoal production from the human testes is estimated to be between 45 and 207 million sperm per day with an average in the order of 100 million per day (Freund, 1962; Amann and Howard, 1980). Exact estimates are difficult due to variations in the frequency of ejaculation, and the assumption that spermatogenesis occurs at a constant rate (Freund, 1963). In comparison to other animals, man has one of the lowest daily sperm productions, and human sperm production is estimated at 4.45×10^6 sperm per gm tissue compared to the rat and the ram, which produce 23.7 and 19×10^6 sperm per gm tissue respectively. However, the quail produces the most recorded at 92×10^6 per gm testis (Amann and Howard, 1980; Clulow and Jones, 1982). Sperm leaving the testis are immature and lack both forward motility and zona pellucida binding capability. These functions develop during their passage through the epididymis, however, the extrinsic and intrinsic mechanisms controlling maturation are poorly understood. The epididymis contains the majority of the extragonadal sperm reserve, being 364×10^6 sperm in man, but this is far greater in other species being more than 165000×10^6 sperm in the ram. One consistency between animals is that the majority of epididymal sperm is stored in the cauda. In man, 25% is stored in the caput, 24% in the corpus and 52% in the cauda, which approximates to other animals (Jones, 1999). The proximal vas deferens sperm reserve in man is estimated at 18×10^6 and consequently represents less than 5% of the extragonadal reserve. This figure is in keeping with other species, which range from 3-15% of total spermatozoa storage in the vas deferens (Jones, 1999). Post vasectomy ejaculation studies suggest that sperm counts fall to 40% of pre-vasectomy levels within 10 ejaculations

receptors were also demonstrated on Sertoli cells throughout all stages of development. $P2X_1$ and $P2X_2$ receptors were expressed on blood vessels within the testis, but no $P2X_1$ receptors were observed on spermatids (Glass et al., 2001). Sertoli cells have been demonstrated to release ATP and adenosine into the extracellular medium at a calculated rate of 0.3 and 0.1nmol/mg protein respectively (Gelain, 2003). To date the physiological function of the expressed P2X receptors remains unclear.

1.6.2   Maturation of sperm in the epididymis

1.6.2.1   Structure of epididymis

The epididymis is an internal structure common to all mammals, birds, amphibians and some fish (chondrichthyes (sharks and rays)). The common anatomical arrangement remains similar across classes. In essence, the seminiferous tubules of the testis progressively converge to pass to the rete testis and immediately into the ducti efferentes, which converge to become the single convoluted tubule of the epididymis. The epididymis length is variable, being approximately 4 metres in man (Turner, 1978) and in excess of 40 metres in bovines (Hoskins, 1978). The epididymal transit time mirrors this length variation, being between 2 and 16 days pending on species and is 4 days in man (Amann et al., 1976; 1980).

The epididymis is divided into 3 histologically distinct regions, the caput, corpus and cauda, although a proximal fourth area, the ducti efferentes is usually included. In the dog, the ducti efferentes are characterised by short columnar epithelia with either cilia or microvilli, which would suggest they are involved with the movement of sperm and absorption of testicular fluid. The caput epididymidis is characterised by an increased epithelial height, and the presence of stereocilia

suggesting the ampulla of the vas deferens stores about 40×10^6 sperm (Freund, 1969).

Spermatids develop from stem spermatogonia at the basal membrane of the seminiferous tubules, progressively developing before release into the lumen. In the rat this process has been shown to have 14 identifiable stages with germ cell development having 19 different steps (Leblond and Clermont, 1952; Hess, 1990). These steps are not always seen in man and it is questioned whether all stages occur in man. Studies have shown that the human seminiferous tubule has a complex helical arrangement, which may explain why tubular cross sections do not show all the classical stages of spermatogenesis (Schulze and Rehder, 1984).

Subfertility affects one in 20 men and 15% of couples trying to conceive fail to do so within 1 year and are deemed infertile. Male factor infertility is directly responsible in 25% of cases and a contributory factor in a further 25%. In 50% of cases there is no identifiable cause and any pharmaceutical intervention is empirical with no randomised evidence supporting any treatment (Hirsh, 2003; Nicopoullis, 2004). Surgery is appropriate for obstructive cases and hypogonadal causes can be treated with hormonal manipulation. Consequently, expensive, invasive and time-consuming assisted conception remains the only line of treatment for the vast majority of men with male factor infertility.

1.6.1.2 Purinergic signalling and sperm production

The developing rat spermatid, within the testis, has been shown to differentially express P2X receptors. $P2X_2$ and $P2X_3$ receptors were demonstrated together on developing spermatids in stages I to VIII and were thought to be on the developing acrosome. $P2X_5$ receptors were demonstrated in stages X to XIII. $P2X_7$

(microvilli with a filamentous core) as well as extensive Golgi activity. Micropinocytotic activity was evident at the apical membrane suggestive of an absorptive function. The corpus epididymidis was characterised by the presence of long stereocilia, often engulfing spermatozoa as well as extensive and organised Golgi vesicles. The cauda epididymidis was characterised by a much lower epithelial height and the continued presence of stereocilia. There were large numbers of breakdown products at the apex of the cells as well as seminal debris within the lumen. There were less Golgi vesicles suggesting less active absorption or secretion but prominent rough endoplasmic reticulum suggesting greater protein synthesis. The smooth muscle cells were arranged in layers, which increased from 2 to 6 layers from the caput to the cauda (Chandler et al., 1981).

In summary, the epithelium of the proximal and mid epididymidis is high with stereocilia and has a small diameter lumen with minimal surrounding smooth muscle. There is a sharp reduction in epithelial thickness with a corresponding increase in lumen diameter and smooth muscle coat as the corpus continues into the cauda epididymis. The proximal epididymidis has an epithelium suggestive of an absorptive and/or secretory function and the distal epididymal epithelium appears less functionally active, presumably more appropriate for the storage of mature sperm.

1.6.2.2   Properties of epididymal sperm

Mammalian sperm are immotile when they leave the testis, although can develop non-progressive movement if stimulated (Volgmayr et al., 1967). The anatomical and ultrastructural characteristics of the epididymis suggest an active role in sperm maturation, which is born out by functional studies on sperm taken from different regions of the epididymis. First evidence of this was demonstrated 80 years

ago, when it was shown that that only 33% of female guinea pigs became pregnant when artificially inseminated with sperm obtained from the proximal epididymis, whereas 68% became pregnant when inseminated with sperm from the distal epididymis (Young, 1929). Early studies suggested that the maturation process was intrinsic to the sperm rather than passage through the epididymis, as epididymal occlusion studies demonstrated increased fertilisation rates from sperm prevented from leaving the proximal epididymis (Young, 1931). Human studies involving fertility rates following epididymo-vasostomy suggest that only passage through the caput epididymis is required for fertility, although the success rate increased with more distal anastomoses (Schoysman, 1986). These studies must be interpreted with caution, as by definition they involve abnormal epididymis, and, as the anastomosis is proximal to the obstruction there may be a degree of reflux of epithelial fluids from downstream of the anastomosis. Spermatozoa may be motile as they have been exposed to distal maturing factors, despite their anatomically proximal position (Cooper, 1990). This is supported by the demonstration of forward motility and IVF potential in sperm taken from the caput epididymis from men with vas deferens agenesis but not from anatomically normal controls in whom motility was only observed in sperm from the corpus epididymis (Mathieu, 1992). In normal epididymides, it was found that 22.9% of sperm from the caput/corpus junction were motile in comparison to 68.3% from the mid to distal corpus with a slight reduction in the cauda (Yeung, 1993). Human micro-canulation studies suggest that the development of motility appears suddenly at the junction of the distal caput and proximal corpus epididymis (Dacheux, 1992). Similarly, it has been demonstrated that the ability of human epididymal sperm to bind to zona-free hamster oocytes increased

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with successive epididymal segments, and that only sperm from the cauda epididymis were able to penetrate the oocyte (Hinrichsen, 1980).

1.6.2.3 Membrane changes in epididymal sperm

Immature sperm entering the caput epididymis continue the process of spermiation and are morphologically different from those of the cauda although these observations are least distinct in man. The most obvious change is migration and loss of the cytoplasmic droplet. In testicular sperm this droplet is located at the anterior midpiece but migrates to the more distal annular region before dissociating completely by the time the spermatozoa reaches the cauda epididymis. The acrosome undergoes significant remodelling during the transit through the epididymis. It is thought that the development of spermatozoa in the testis is under the genomic regulation of the gamete, but once DNA condensation has occurred in elongated spermatids, the transcription process in the germinal DNA stops (Dacheux, 1998). It is thought the external milieu surrounding the gamete is responsible for further differentiation. It is the content and interaction of the the spermatozoa with the epididymal fluid, as well as spermatozoa interaction with the epididymal epithelium that is responsible for further spermatid development. Over 200 proteins have been identified in epididymal fluid, although most are secreted in very small amounts and gel electrophoresis suggests that 10 proteins make up 90%, and 2 proteins 52% of secreted protein. The pattern of protein secretion is specific to different regions of the epididymis. The epididymal secretions responsible for specific sperm modification appear to be discrete unidentified proteins (Dacheux, 1998).

The sperm membrane has an unusually high proportion of polyunsaturated phospholipids which give it special characteristics, furthermore, within the membrane

component proteins and lipids are compartmentalized into discrete domains on the head and tail. During maturation, the plasma membrane undergoes remodelling by the uptake of secreted glycoproteins, removal and utilization of phospholipids from the inner leaflet of the bilayer, and processing of existent glycoproteins (Jones, 1998). The ionic content of epididymal fluid is low which would allow most proteins to be in contact with the cell membrane (Jones, 1978). Changes in the charge on the cell membrane may facilitate the insertion of secreted integral membrane proteins. Changes in the charged integral proteins may lead to compartmentalization into a mosaic of specific domains. This may also be induced by the sterol content of the membrane, which in turn may be affected by the many lipid-binding proteins secreted by the epididymal epithelium. (Cooper, 1998). The sperm membrane is rich in glycoproteins, which are thought to be essential in the recognition and binding to the zona pellucida. The epididymal fluid is rich in both glycosyltransferases (synthetic) and glycosidases (hydrolytic), which have been demonstrated to modify sperm membrane glycoproteins during epididymal transit (Tulsiani, 1998).

1.6.2.4   Capacitation and the acrosome reaction

In order for a sperm to achieve successful fertilization of an egg, it must firstly undergo capacitation, which occurs in the female genital tract during the phase between ejaculation and fertilization. It involves a series of incompletely understood cellular and molecular alterations, which enable the spermatozoa to subsequently undergo the acrosome reaction immediately prior to fertilization. Modification and induction of capacitation may occur as a result of interaction with, or incorporation of proteins in fluid from both the male and female genital tracts. Proteins or molecules from within the spermatozoa itself may also be released for integration with the

membrane. Spermatozoa become coated with proteins as these pass through the epididymis, which may inhibit capacitation and prevent the acrosome reaction occurring prematurely. These proteins may stabilise the membrane and possibly block receptors (Mbivzo and Alexander, 1991; Rajalakshmi and Griffin, 1999). This process is reversible as capacitated sperm can be decapacitated by the supernatatant from centrifuged semen (Fraser, 1990). Capacitation is characterised by increased membrane fluidity, a decrease in plasma membrane cholesterol to phospholipid ratio, a reduction in surface charge, and an increase in cAMP and oxidative processes in conjunction with a change in swimming patterns (Guraya, 2000). The resultant membrane change may facilitate Ca^{2+} entry, which then chelates anionic phospholipid molecules and promotes fusion between the plasma membrane and outer acrosomal membrane (Mbivzo and Alexander, 1991; Cross, 1998). The precise trigger for capacitation is unidentified, and may not be a single agent as numerous media are able to induce capacitation and the acrosome reaction in vitro. One of the simplest stimulants used is serum and particularly albumin or the macromolecules associated with albumin (Guraya, 2000).

The acrosome reaction involves point fusions between the outer acrosomal membrane and the overlying plasmalemma. Calcium alone is required for this reaction to occur, providing capacitation is complete. The acrosome reaction results in the release of hydrolytic enzymes from the acrosome and the generation of greater thrust and hyperactivity to facilitate penetration of the of the egg investments by the spermatozoa. There is no clear biochemical marker for completion of the acrosome reaction.

ATP may have an important role in the acrosome reaction, as ATP rapidly induced the acrosome reaction in a Ca^{2+} independent manner, probably through the

activation of Na^+ channels, and ATP-activated sperm from patients with male factor infertility had a significantly higher fertilisation rate (Foresta et al., 1992; 1996; Rossato et al., 1999). Sperm in the cauda epididymis have been shown to have an ecto-ATPase present on the cell membrane which potentially maintains inactivity in the epididymis by hydrolysis of ATP (Majunder and Biswas, 1979).

1.6.2.5   Sperm motilty and ATP

The microscopic arrangement of mitochondria around the midpiece of sperm implies significant synthesis and utilisation of ATP for rapid swimming. Seminal fluid has a relatively high ATP content, which is generally viewed as an energy substrate (Singer et al., 1983). The presence of ATP in semen suggests a limited substrate or fuel source, that when consumed results in sperm immotility or death. It has been suggested that reduced seminal ATP levels are implicated in infertility, however, investigative studies have failed to find a correlation between seminal ATP and infertility (Irvine and Aitken, 1985; Mieussetet al., 1989; Vigue et al., 1992; WHO, 1992). This may be explained by the fact that sperm synthesise ATP, and so seminal fluid ATP is not the only ATP source. Quiescent rat sperm from the cauda epididymis were found to have a very high ATP content of about 100 $mM/10^8$ spermatozoa, which had presumably accumulated during epididymal transit. When sperm were placed into a substrate (acetate, lactate, pyruvate and glucose) containing medium they immediately became motile and the ATP content rapidly fell to a steady state of 50$mM/10^8$ spermatozoa, which was maintained for at least 2 hours. In the absence of substrates sperm motility reduced rapidly after 90 minutes. The pattern of sperm motility was significantly dependent on the presence of substrates. Initial head rotation was observed to be 2.3 rotations /second, straight-line velocity was 105 $\mu M/s$

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and flagella beat frequency was approximately 10Hz. After 120 minutes without substrates all parameters were significantly reduced and only 18% of spermatozoa were still progressively motile. These parameters were reversible on the addition of substrates (Jeulin and Soufir, 1992). It has been postulated that the very high ATP content of caudal epididymal sperm may actually inhibit ATP induced microtubule sliding and mitochondrial activity in order to maintain the spermatozoa in a quiescent state (Ishijima and Witman, 1987; Jeulin and Soufir, 1992).

1.7 Movement of sperm from the seminiferous tubules

The basic mechanism by which sperm undergo expulsion from the seminiferous tubules of the testis into the rete testis and pass through the epididymis and vas deferens is poorly understood. It is postulated that sperm move out of the seminiferous tubules due to peristaltic forces of the seminiferous tubules themselves, an effluent tide of fluid produced by the seminiferous tubules, the movement of cilia on the epithelium of the rete testis and finally contractions of the tunica albuginea of the testis (Hargrove et al., 1977). Evidence for cilia-induced movement is limited with calculations suggesting inadequate numbers of cilia for estimated flux and the presence of normal numbers of sperm in the epididymis in patients with Kartageners syndrome in which cilia are immotile (Winet, 1977; Afzelius, 1976). The effluent tide is significant, with the testes of boars producing 40 mls of fluid a day of which only 1 ml leaves the epididymis (Setchell, 1970). There are myoid cells, usually only 1 cell thick surrounding the seminiferous tubules and despite an absence of innervation, these have been shown to contract to noradrenaline (NA), acetylcholine (ACh), oxytocin, endothelin and prostaglandins (Ellis et al., 1977; Miyakye et al., 1986;

Barone et al., 2002). The functional significance of these contractions is unknown and there are no reports of contractions to purinergic agonists.

Smooth muscle contraction of the rabbit tunica albuginea was first shown in 1967 (Holstein, 1967), and contractions of the rat tunica albuginea to NA and ACh were demonstrated in 1969 (Davis and Langford, 1969). Smooth muscle has been demonstrated in the human, rat, rabbit, cat, dog, sheep, pig, cow and horse testicular capsules (Davis, 1969; 1970; Langford, 1973; Leeson, 1974; Ohanian et al., 1979; Chacon-Arellano et al., 1980). The extent and organisation of the smooth muscle varies between species and only in the rabbit does an organised muscle layer appear to exist, and indeed two layers perpendicular to each other are reported (Davis, 1970). In most other species examined, including man, no distinct muscle layer is present and no neuronal innervation demonstrated, although more recently Middendorf has claimed that there is a distinct inner smooth muscle layer (Middendorf et al., 2002). Developmental studies suggest that the smooth muscle develops postnatally at the time of sexual maturity (Leeson, 1981; Holt et al., 2004). The mechanisms controlling muscular contraction and propulsion are varied, but NA, ACh, oxytocin, prostaglandins and gonadal hormones have all been implicated (Davis and Langford, 1969; Hargrove et al., 1972; Seeley, 1972; Sanchez, 1991). Spontaneous contractions have been demonstrated in man, rabbits and rats. Contractions have typically been monophasic and slow (Davis et al., 1970). It is unknown whether contractions are continuous or occur in response to stimulation or ejaculation. To date no contraction to purinergic agonists has been reported, and contraction of the mouse tunica albuginea has not been demonstrated.

1.8 Purinergic co-transmission in the contraction of vas deferens smooth muscle

NA and ATP have been shown to mediate contraction of the rat and guinea pig vas deferens smooth muscle. The contraction, in response to electrical field stimulation (EFS) of sympathetic nerves has been shown to be biphasic (Fedan et al., 1981; Meldrum and Burnstock, 1983; Sneddon et al., 1984). ATP, released from sympathetic nerves, acts through ion-gated P2X receptors to stimulate the initial fast phase of the contraction, with co-released NA causing the more sustained, but slower contraction, acting through G protein-coupled α adrenoceptors. $P2X_1$ receptors have been demonstrated on vas deferens smooth muscle membranes, with one report suggesting expression on all 3 muscle layers and another suggesting expression on only the outer 2 layers, and not the inner layer (Lee et al., 2000a; Mulryan et al., 2000). Functional importance of $P2X_1$ receptors in contraction of the vas deferens was shown by the demonstration that $P2X_1$ receptor null mice had a 90% reduction in fertility, despite fertile sperm. The infertility was due to reduced nerve-induced contractions of the vas deferens smooth muscle. The smooth muscle was still contractile in response to NA, but resulting ejaculates had insufficient sperm for fertilisation. This study suggested that notional $P2X_1$ receptor antagonists could be successful in male contraception, despite a complete absence of knowledge of either the expression or function of $P2X_1$ receptors in human vas deferens (Mulryan et al., 2000).

Contraction of the human vas deferens smooth muscle is reported to be purely adrenergic stimulation, although modified by numerous substances (Anton and McGrath, 1977; Steers, 1994). Human nerve-induced responses are reported to be

about 10 times slower than those of the rat. Twitch responses to single shocks have been found to be TTX insensitive, but electrical-induced tetanic responses have been shown to be sensitive to prazosin and TTX, although up to 23% of the original response persists. Some sections of human vas deferens have been shown to relax in response to low voltage (40V) EFS, which was atropine sensitive. Higher voltages (90V) induced contractions with a time to peak of 3-7 S. Exogenous NA caused tonic contractions, induced spontaneous contractions and potentiated the response to electrical stimulation. Some short segments of human vas deferens relaxed in response to NA, which was explained by contraction of the circular smooth muscle. Adenosine and ATP were found to inhibit the EFS induced responses by about 30%, however inhibition was variable (Pryor and Smith, 1986; Smith and Bray, 1990). To date there are no studies into purinergic co-transmission or P2X receptor expression in human vas deferens.

1.9 Purinergic signalling in the bladder

1.9.1 Normal bladder physiology

The primary function of the bladder is low pressure urinary storage with periodic voluntary emptying. Failure of normal bladder function can be very debilitating and represents a huge socio-economic health problem. Normal bladder function involves progressive relaxation of the detrusor as it fills, whilst maintaining continence by contraction of the urinary sphincter. During voiding, the reverse occurs with sphincter relaxation and coordinated detrusor contraction. The autonomic nervous system is integral to both detrusor and sphincter components. Normal detrusor contraction is mediated by the release of ACh from parasympathetic nerves acting on G-protein coupled muscarinic (M) receptors to cause smooth muscle

contraction. Sympathetic neurons help maintain continence through α_1 adrenergic receptors at the bladder neck and somatic nerves innervate the voluntary external sphincter. Several muscarinic receptors are expressed and despite the predominance of M_2 receptor expression, it appears the M_3 receptor subtype is functionally most important (Wang et al., 1995; Chapple et al., 2002). In man atropine almost completely antagonises nerve-mediated detrusor contraction (Bayliss et al., 1999). The situation in animals is different, and over 100 years ago atropine was shown to incompletely inhibit bladder contraction, leaving a significant NANC component (Langley and Anderson, 1895). ATP was shown to mimic tetanic nerve stimulation, which was antagonised by quinidine and tachyphylaxis to ATP reduced nerve-mediated responses. The demonstration of excitatory junction potentials due to the neurogenic release of ATP in the bladder has been integral to purinergic co-transmission (Burnstock et al., 1972; Moss and Burnstock, 1985; Burnstock, 2001). Despite the lack of purinergic function in normal human detrusor, as assessed by antagonism of nerve stimulated responses, isolated strips of bladder demonstrate concentration-dependent contractions to α,β-me ATP, smooth muscle cells generate inward currents in response to ATP, and mRNA for $P2X_{1,2,4,5\ and\ 7}$ receptor subtypes is detectable (Hoyle et al., 1989; Inoue and Brading, 1991; Palea et al., 1995; O'Reilly et al., 2002). $P2X_1$ receptors are expressed on the smooth muscle membranes of rat detrusor and non-membrane-bound $P2X_{2,5\ and\ 6}$ receptors are also expressed in the bladder (Lee et al., 2000a; Vial and Evans, 2001). The detrusor smooth muscle from $P2X_1$ receptor deficient mice showed no inward current in response to purinergic agonists confirming that the $P2X_1$ receptor subtype is responsible for purinergic EJPs. There was no compensatory change in either the myogenic or cholinergic components, and the bladders appeared morphologically and functionally normal

(Vial and Evans, 2000). In awake rats undergoing cystometry, intra arterial administration of ATP and α,β-meATP close to the bladder caused rapid pressure rises and immediate micturition. Pre-treatment with α,β-meATP caused a subsequent reduction in micturition pressures and increased bladder capacity. Similarly, carbachol (CCH) caused rapid sustained pressure rises leading to micturition and spontaneous contractions continued after treatment with atropine. The combination of atropine and pre-treatment with α,β-meATP resulted in retention and dribbling incontinence (Igawa et al., 1993). This would suggest that both systems are functionally important in normal rat micturition.

1.9.2 Purinergic signalling in the pathophysiology of the overactive bladder

In the UK, it is estimated 9 million people over 40 years old are affected by urinary storage symptoms and 5 million require healthcare (McGrowther, 2004). Bladder overactivity is thought to be the cause in one third of female sufferers, and up to half of male sufferers. The condition of bladder overactivity is characterised by involuntary bladder contractions causing pressure rises during bladder filling, which result in a strong and uncontrollable urge to pass urine, which often leads to incontinence (Abrams et al., 1990). Prevalence increases with age and deteriorating health and it is twice as common in females as males. Presentation in women is most common in the 2nd to 4th decade (Thakar, 2000). There is significant associated morbidity in terms of increased falls or fractures, and it increases the risk of admission to hospital or nursing care (Thom et al., 1997; Brown et al., 2000).

The natural history of bladder overactivity is incompletely understood, however, in a subset of men it is thought to develop secondarily to progressive

bladder outflow obstruction (BOO). BOO is a common condition in the older male, usually resulting from benign prostatic hyperplasia (BPH). Nearly 50% of all men aged 60-69 suffer from symptoms of BPH (Garraway et al., 1991). BPH is histologically evident in up to 88% of prostates obtained at post mortem from patients aged over 80 years (Napalkov et al., 1995). Symptoms can arise from the physical obstruction to flow, such as hesitancy and reduced urinary stream, or from secondary damage to the detrusor muscle resulting in storage difficulties. This can manifest as detrusor overactivity, with symptoms of frequency, urgency and urge incontinence. Studies have shown that 50-75% of men with BOO due to BPH develop detrusor overactivity, but 62% will revert to normal cystometry following prostatectomy (Abrams et al., 1979; 1985). However, 19% of patients will continue to have symptoms of bladder overactivity post surgery (Gormley, 1993; Thomas and Abrams, 2000), and a large proportion of patients who had their overactivity reversed by prostatectomy will subsequently return to overactivity in the long-term (Leach et al., 1996). The mainstay of pharmacological management of detrusor overactivity are anti-cholinergic agents, of which oxybutynin is the most established. Studies have suggested that the frequency of micturition can be decreased by oxybutynin in 20 % of patients, versus 10% with placebo (Appell, 1997) and incontinence episodes reduced by 71% versus 19 % with placebo (Abrams et al., 1998). A Cochrane review collating 32 studies and 6800 people suggested that anticholinergic treatment reduced the number of micturitions in 24 hours by 2.5, however placebo reduced the number by 1.6 (Herbison, 2003). The incidence of anti-muscarinic side effects remains very high with 57-93% of patients experiencing some, and 23 % of patients stopping treatment as a result (Moisey et al., 1990; Appell, 1997). Long-term data is deficient, but it would seem that treatment is unsatisfactory. With surgical treatments reserved

for only intractable cases of bladder overactivity, the pharmaceutical market remains huge, grossing an estimated $6.11 billion per year (Madersbacher, 2005). Consequently, novel therapeutic targets offer exciting prospects.

Two main theories have been suggested as to the cause of detrusor overactivity, principally a myogenic cause, in which the smooth muscle excitability and electrical coupling are intrinsically altered, and a neurogenic cause, in which there is up or down regulation of the neuronal mechanisms responsible for detrusor contraction. The mainstay of the myogenic theory is that the spontaneous contractions that characterise detrusor overactivity are TTX resistant, and structural changes are evident at electron microscopic level (Brading, 1997; Elbadawi et al., 1993). The neurogenic theory suggests that peripheral or central pathways are altered, possibly from reduced central inhibition, enhancement of local pathways, increased afferent input from lower urinary tract or the development of resistant local bladder reflexes (De Groat, 1997).

These theories are not incompatible with each other as detrusor overactivity represents a spectrum of disease, depending on the underlying pathological cause, and the development of the condition. The human detrusor adapts to bladder outflow obstruction and undergoes a progression from initial hypertrophy, resulting in compensation, to a decompensation phase which results in either a small, fibrotic, low capacity, poorly compliant overactive bladder, or a grossly dilated, thin, high capacity bladder. It is therefore not surprising that a unified theory does not exist, as different theories may apply in different pathological settings. Animal models may imitate a particular phase or point on a progressive pathology.

A number of studies have suggested that the purinergic component of human bladder contraction is up-regulated in the pathological state. In the normal human

bladder the purinergic component of detrusor contraction is probably less than 5%, as atropine completely antagonises field stimulation responses (Bayliss et al., 1999). In the pathological human bladder the purinergic component accounted for 40 % of the contraction in interstitial cystitis, up to 50 % in idiopathic female detrusor instability, and in cases of proven bladder instability, significant up-regulation was demonstrated (Palea et al., 1983; Bayliss et al., 1999; O'Reilly et al., 2002). The purinergic component of human bladder contraction has been shown to be increased and the cholinergic component reduced with age (Yoshida et al., 2001). Other studies contradict this, suggesting that the purinergic component as assessed by antagonism is 5% in overactivity and 3% in controls, with a significant up-regulation of the myogenic or TTX resistant component (Mills et al., 2000).

The up-regulation of the purinergic component cannot be explained by altered sensitivity, as detrusor myocytes from stable and unstable bladders were equally sensitive to cholinergic and purinergic agonists (Wu et al., 1999). A reduction in ecto-ATPase enzyme activity has been demonstrated from unstable or obstructed bladders, and this may account for increased potency of ATP in these pathological states (Harvey et al., 2002). Increased expression of the P2X receptors has also been suggested, but only increased $P2X_2$ receptor expression has been demonstrated in detrusor overactivity (O'Reilly et al., 2002). The function of the $P2X_2$ receptor in smooth muscle contraction has yet to be elucidated.

Animal models of bladder outflow obstruction have demonstrated that bladder instability occurs following obstruction (Malmgren et al., 1987; Igawa et al., 1993; O'Connor et al., 1997; Lluel et al., 1998). A number of animal models have demonstrated changes in the purinergic and cholinergic components of detrusor neurogenic contractions. Findings are variable and probably represent species

differences, as well as variations in the degree and duration of bladder obstruction. For instance, Sjuve found that sensitivity to purinergic receptor agonists was reduced following 10 days of obstruction in a rat model (Sjuve et al., 1995). In a long-term rat obstruction model, Lluel found only the cholinergic element of EFS-induced detrusor contractions to be increased (Lluel et al., 2002). In contrast, Calvert found that the purinergic component of EFS bladder contraction in a rabbit following 3 weeks of obstruction was increased, with a reduction in the cholinergic component (Calvert et al., 2001). Animal models of bladder outlet obstruction must be interpreted with caution as they may represent the response to acute retention and subsequent detrusor failure, as indicated by bladder weight increases of several hundred percent and reduced contractile responses to potassium chloride (KCl).

In the present study, the effect of partial bladder outlet obstruction on the contraction of the detrusor in the rat was investigated. The hypothesis that up-regulation of the purinergic component occurred in response to bladder outlet obstruction was tested.

CHAPTER 2

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GENERAL METHODS

2.1   Organ-bath pharmacological studies

2.1.1   Apparatus

Glass organ-baths of 10ml capacity were used for all pharmacological experiments. Experiments were performed at temperatures that most approximated to their *in vivo* situation. Therefore, testes and vas deferens studies were carried out at 35° +/- 1° C to approximate to scrotal body temperature, and bladder studies at 37° +/- 1° C for bladder temperature. All experiments were carried out in Krebs solution continually gassed with a mixture of 95 % O_2 and 5 % CO_2.

Depending on the tissue size, either whole preparations, or strips of tissue were used. 3'O silk ligatures were attached to each end of the preparation. One end of the specimen was fixed to a rigid support and the other end was attached to a force displacement transducer (Grass FTOC3, Quincy, MA, USA). For Electrical Field Stimulation (EFS) studies, the support was modified to incorporate two platinum ring electrodes 2.5mm in diameter and placed vertically 1 cm apart. The tissue preparation was threaded through these ring electrodes. Isometric contractions were recorded using the software PowerLab Chart for Windows (version 4; AD Instruments, Australia).

EFS was produced by an electrical stimulator (Grass, Quincy, MA, USA). EFS parameters were 100 V, 0.3-0.5 ms, 0.5-64 Hz, for 15-60s every 3-5 min. The exact parameters were optimised for each tissue type, and subsequently maintained for that tissue.

An initial load of between 0.2 -1.0 g was applied to the preparations, following their set up. The Krebs solution in the organ bath was then changed and the initial load readjusted. No subsequent load adjustments were made despite universal

relaxation of the specimens. All tissue preparations were allowed to equilibrate for at least 45 minutes prior to start of experimentation.

2.1.2 Animals

Mice, rats, hamsters and rabbits were supplied by UCL animal laboratories and were kept in accordance with Home Office (UK) regulations. Mice, rats and hamsters were sacrificed according to Home Office (UK) regulations covering Schedule 1 procedures –following CO_2 asphyxiation and cervical dislocation. Rabbits were sacrificed following terminal anaesthetic, followed by cervical dislocation in accordance with Schedule 1 procedures. The number of animals sacrificed was minimalised by obtaining genital tissue from animals being sacrificed for different studies.

2.1.3 Tissue Preparation for Organ-Bath Studies

All tissues were collected into Krebs solution, and subsequent preparation was performed as rapidly as possible. Tissues were pinned into wax-based petri dishes containing Krebs solution and prepared under an operating microscope.

2.1.3.1 Testicular capsule tissue

The testis, epididymis and vas deferens were removed en bloc following a scrotal-pelvic incision. The preparation was placed immediately into Krebs solution. Subsequently the epididymis and vas deferens were removed and residual fat was cleaned from the testis.

For rat and mouse tissue, a small incision was made parallel to the long axis of the testis in the anterior (i.e. opposite rete testis) border of the testis. The testis capsule

was then everted extruding the seminiferous tubules. The seminiferous tubules were only adherent to the capsule at the vascular/epididymal hilum of the testis. This attachment was divided and the capsule was returned to its original orientation. Silk ligatures were then attached to the two polar regions of the whole capsule or tunica albuginea preparation. Rabbit testes were relatively larger and so strips of tunica albuginea approximately 2 x 20mm were cut in the capsule and freed of underlying seminiferous tubules.

Ethical approval was obtained from the hospital ethical committee. Human testes were taken from patients undergoing an orchidectomy for either gender reassignment, or for the treatment of prostatic carcinoma. Individual consent was obtained from all patients, who were fully informed of the type of experiment their tissue would be used for and subsequent storage. Each patient was given an information sheet and a copy of their consent form. Whole capsule preparations were not feasible, therefore a patch of tunica albuginea was taken from the mid portion of the testis near the rete testis. This area was chosen because reported histological studies suggested a greater concentration of smooth muscle fibres near the rete testis. Each patch was then cut longitudinally into strips approximately 2 x 20 mm. These could then be mounted in a similar way to the whole capsule preparations of the mouse and rat. The seminiferous tubules were more adherent in man and some septae extended into the body of the testis from the capsule.

2.1.3.2 Human vas deferens tissue

Human vas deferens specimens were taken from patients undergoing a vasectomy for sterility or orchidectomy for either testicular or prostatic carcinoma. All patients gave consent and the hospital ethics committee approved the study.

Specimens were collected directly into Krebs solution. Each specimen was divided longitudinally into quarters to give specimens approximately 2 x 20 mm in size. This was for four reasons: the first being to minimise diffusion distances, the second to allow all three muscle layers exposure to agonists and antagonists, the third to allow the specimen to be used in the available apparatus and the fourth was to allow multiple studies on a small vas deferens specimen. Whole specimens were tried, but offered no obvious advantage. The specimens were mounted longitudinally. Vas deferens specimens from orchidectomy patients were taken from the scrotal vas deferens approximately 4 cm from the epididymis so that they would be comparable to the vasectomy vas deferens specimens.

Two sets of EFS were carried out on human tissue. Initial stimulations were often variable and inconsistent but second stimulations were much more consistent and these were used for investigation. An initial load of 1 g was applied.

2.1.3.3 Obstructed rat bladder tissue

Female rats, which had undergone partial bladder outlet obstruction or sham operation (see chapter 6), were killed following CO_2 asphyxiation and cervical dislocation. The bladders were exposed via a lower abdominal midline incision. In the obstructed rats the bladder was significantly larger and the peritoneum overlying the dome of the bladder was usually thickened and more adherent to the bladder. The peritoneum was dissected off the bladder, the ureters divided and the urethra excised just distal to the bladder neck. The bladder was then excised and placed into Krebs solution. The bladder was weighed as a total specimen. The bladder neck was excised at the level of the ureters. The detrusor was then incised longitudinally to the dome. Subsequently, longitudinal strips measuring approximately 2 x 20 mm were cut and

mounted as previously described. For the bladder studies, the organ bath Krebs solution was modified to include indomethacin (1 μM). Initial pilot studies showed that the obstructed bladder preparations demonstrated marked spontaneous smooth muscle activity, therefore, indomethacin was added to reduce this. An initial load of 1 g was applied.

2.2   Presentation of data and statistical analysis

2.2 1 Agonists

Wherever possible full concentration-response curves were constructed and EC_{50} values calculated. Concentration-response curves to ACh were constructed with cumulative applications. Those to NA, ATP, α,β-MeATP and β,γ-methylene ATP ($\beta\gamma$ ATP) were applied in single boluses. The reason for this was that preparations were prone to desensitise, particularly with purinergic agonists. Consequently, single dose administration, with immediate washout on achieving maximal response was utilised. Furthermore, wherever possible, different agonists were applied alternatively to allow tissues greater recovery time. At the end of each experiment a standard dose of 120 mM KCl was added to the organ bath to determine a response. To allow for differences in specimen size, concentration-response curves were expressed as a mean % of the KCl response obtained for any preparation ± standard error of the mean (s.e.) for the number of animals used (n).

For relaxation responses of the mouse testicular capsule, an initial contraction was applied to the preparation by pre-contracting it with ACh at its EC_{50} concentration. Subsequent relaxation responses to NA were examined in a cumulative manner and expressed as the mean % relaxation ± s.e. (n).

2.2.2 Antagonists and desensitisation

Potential antagonists were introduced to the organ bath preparations at least 20 min prior to repeat EFS. Responses were expressed as either the % of the KCl response, or as the % of the maximum response in the absence of antagonists. Desensitisation of the P2X receptors was achieved by adding 2 or 3 100 μM boluses of α,β-MeATP, 5-10 min apart which were not washed out. Desensitisation was confirmed by an absent response to a single bolus of 100 μM ATP.

2.2.3 Statistical analysis

Statistical analysis was carried out using GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA).To test if the frequency-response and concentration-response curves were significantly different from each other statistical significance was tested by a 2-way analysis of variance (ANOVA), followed by Bonferroni's test. A probability of $P < 0.05$ was considered significant.

2.3 Physiological salines

Modified Krebs solution of the following composition was used in all organ bath studies; (mM): NaCl, 133; KCl, 4.7; $NaHCO_3$, 16.4; $MgSO_4$, 0.6; NaH_2PO_4, 1.4; glucose, 7.7 and $CaCl_2$, 2.5; pH 7.3. In studies on the obstructed rat bladder indomethacin 3μM was added to the Krebs solution.

2.4 Immunohistochemistry

2.4.1 Preparation of tissue for immunohistochemistry

Tissue samples were collected in Krebs solution, then embedded in Tissue Tek OCT compound (Sakura, Zoederwoude, Netherlands) and snap-frozen in isopentane pre-cooled in liquid nitrogen. Tissues were sectioned at 12 μm using a cryostat (Leica CM 3050, Nussloch, Switzerland), sections were then thaw mounted on gelatin-coated slides and air-dried at room temperature. The slides were stored at -20°C and allowed to return to room temperature for at least 10 min prior to use.

2.4.2 Immunohistochemistry protocol

The avidin-biotin technique was used, as described by Llewellyn-Smith et al (Llewellyn-Smith, 1992; 1993). Briefly, the slides were fixed in 4% formaldehyde and 0.2% of a saturated picric acid solution in 0.1 M phosphate buffer for 2 min. To inactivate endogenous peroxidase, the sections were then treated with 50% methanol containing 0.4 % hydrogen peroxide for 10 min. Non-specific binding sites were blocked by incubating with 10 % normal horse serum (NHS) in phosphate buffered saline containing 0.05 % thimerosal (Merthiolate) for 20 minutes. The P2X antibodies were diluted to 0.25-5 μg/ml (determined by prior titration) with normal horse serum and the sections were incubated with primary antibodies overnight at room temperature. They were subsequently incubated with a secondary antibody which was a biotinylated donkey anti-rabbit IgG (Jackson Immunoresearch, Luton, UK) used at 1:500 for 1hr. Sections underwent a further incubation with extravidin peroxidase (Sigma Chemical Co, Poole, UK) at 1:1000 for 1 hr. The reaction product was visualised using the nickel-intensified diaminobenzide (DAB) enhancement technique

or using streptavidin fluoroscein isothiocyanate (FITC) immunofluorescence. The specimens were dehydrated in xylene and mounted in Eukitt (BDH). Controls were performed with pre-immune IgG and antibodies pre-adsorbed with the homologous peptides and omission of the primary antibody; minimal staining was observed under such conditions.

2.4.3 Antibodies

The immunogens used were peptides corresponding to 15 receptor type specific amino acids in the C-terminal region of the receptor. The synthetic peptides were covalently linked to limpet haemocyanin and the conjugate was administered to rabbits at monthly intervals (performed by Research Genetics, Huntsville, Ala., USA). Immunoglobulin G (IgG) fractions were isolated from the immune and pre-immune sera for the seven P2X receptor subtypes, using the method of Harboe and Ingild (Harboe and Ingild, 1973). The specificity of the antibodies was verified by immunoblotting as previously described (Oglesby et al., 1999). Peanut agglutinin antibodies were used for identification of the spermatid acrosome.

2.4.4 Microscopy and photography

The results were documented using the Edge R400 high definition light microscope (Edge Scientific Instruments, Santa Monica, CA). Pictures were stored using digital camera technology (Leica 2000, Leica, Heerbrugg, Switzerland) and printed using Photoshop 6.0 edition.

2.5 Electron Microscopy

2.5.1 Preparation of tissues

Fresh tissue was fixed in 4% paraformaldehyde and 0.1% glutaraldehyde. Sections were cut on an Oxford vibratome (Lancer) and collected in serial order in 0.1 M phosphate buffer. They were subsequently dehydrated in ethanol, cleared in propylene oxide, and embedded in araldite between two sheets of Melanex (ICI, Middlesborough, UK). Semi-thin (1 μm) sections were cut with glass knives and stained with toludine blue adjacent to thin sections (70 nm), cut with a diamond knife on a Reichert Ultracut ultramicrotome (Leica CM 3050, Nussloch, Switzerland). The sections were collected on copper mesh grids coated with a thin Formvar film (Agar Scientific, Stansted, UK), counterstained with lead citrate.

2.5.2 Electron microscopy and photography

Sections were viewed in a JEOL 1010 transmission electron microscope (JEOL instruments, Akishima, Japan). Plate photographs were taken which were scanned using Imax Scanner and produced in Photoshop 6.0 edition.

2.6 Ethical approval and animal licence

Ethical approval was obtained for the collection and study of human tissue, reference RREC 2662. All patients were individually consented and given a copy of their consent form together with an information sheet regarding the study. Stored tissue was anonymously catalogued. Schedule 1 sacrifice was carried out after accreditation under the Animals (Scientific Procedures) Act 1986 reference RVC/00/183. Bladder outlet obtruction was performed in rats under the Animals

(Scientific Procedures) Act 1986, personal licence reference PIL 70/16636 in conjunction with project licence PPL 70/4678.

2.7 Drugs and solutions

Acetylcholine	Sigma
Adenosine 5'-triphosphate sodium salt	Sigma
α,β-Methylene adenosine 5'-triphosphate salt	Sigma
β,γ-Methylene adenosine 5'-triphosphate salt	Sigma
Ascorbic acid	Sigma
Atropine sulphate	Antigen Pharmaceuticals
Carbachol	Sigma
Biotinylated donkey anti-rabbit IgG	Jackson Immunoresearch
ExtrAvidin-horseradish peroxidase	Sigma
Formvar film	Agar Scientific
Glucose oxidase	Sigma
Hydrogen peroxide	Sigma
Indomethacin	Sigma
Methanol	Analar
Melanex	ICI
Noradrenaline	Sigma
Normal Horse Serum	Gibco
Paraformaldehyde	Sigma
Picric acid	Sigma
Pyridoxalphosphate-t-azophenyl-2',4'-disulphonic acid	Sigma
Tetrodotoxin	Sigma

CHAPTER 3

SMOOTH MUSCLE, AND PURINERGIC CONTRACTION OF THE HUMAN, RABBIT, RAT AND MOUSE TESTICULAR CAPSULE

3.1 Abstract

The smooth muscle cells of the testicular capsule (tunica albuginea) of man, rat and mouse were examined by electron microscopy. They were characteristically flattened, elongated, branching cells and diffusely incorporated into the collagenous matrix and did not form a compact muscle layer. Contractile and synthetic smooth muscle cell phenotypes were identified. Nerve varicosities in close apposition to smooth muscle were seen in human tissue.

Contractile responses induced by ATP, α,β-meATP, NA, ACh and EFS of autonomic nerves were investigated. Nerve-mediated responses of the rabbit and human were recorded. The EFS-induced responses of human tunica albuginea smooth muscle were completely abolished by prazosin. In the rabbit, EFS-induced contractile responses were reduced by pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) by 36 % and by prazosin by 77%. Both antagonists together, almost completely abolished all EFS-induced contractions. The human tunica albuginea was contracted by NA, ATP and α,β-meATP, but not by ACh. The rabbit and rat tunica albuginea were contracted by NA, ATP, α,β-meATP and ACh. The mouse tunica albuginea was contracted by ACh, ATP and α,β-meATP, but relaxed to NA.

Immunohistochemical studies demonstrated that $P2X_1$ and $P2X_2$ receptors were expressed on the smooth muscle of the rodent and human testicular capsule.

The testicular capsule of all the species examined contained contractile smooth muscle. ATP, released as a co-transmitter from sympathetic nerves can stimulate the contraction of rabbit smooth muscle. Human, rat and mouse testicular smooth muscle demonstrated purinergic responsiveness, probably mediated through the $P2X_1$ and/or $P2X_2$ receptors.

3.2 Introduction

There is increasing evidence for falling sperm counts in man (Carlsen et al., 1992; Joffe, 1996). Despite the WHO standardization of semen analysis parameters (Rowe et al., 2000), it is difficult to define 'normal' in the multiple parameters of semen analysis, and there is widespread variation in laboratory measurement of semen analysis (Keel, 2004). Semen analysis is the end-product of sperm production, transport and ejaculation through the genito-urinary system. A reduction in sperm numbers may reflect reduced transportation through the genital tract, rather than reduced production. For instance, in young healthy volunteers, who gave a minimum of 5 samples in the course of a year, sperm concentration varied by an average of 4.8 fold with the maximum difference being 17.2 fold (Oshio, 2004). There is no clinical or laboratory measure of sperm transport failure, and exceedingly limited objective evidence for the mechanisms of sperm transport, with much evidence implied from relatively dated anatomical studies. It may therefore be that a significant number of patients labelled as idiopathic oligospermia have a degree of failure of the transport process rather than spermatogenesis. This subset of infertile patients is potentially amenable to stimulation of genital smooth muscle with either adrenergic or purinergic stimuli, should such an agent become available.

Our knowledge of the basic contractile mechanisms, and the frequency of such contractions in propelling sperm through the genital tract is surprisingly deficient. It is postulated that contraction of the tunica albuginea is one mechanism propelling sperm from the seminiferous tubules into the head of the epididymis (Hargrove et al., 1977). Since the demonstration of contractile smooth muscle in the tunica albuginea in the late 1960s, our understanding of autonomic signalling has advanced with the acceptance of purinergic co-transmission. The biphasic nature of vas deferens

contraction was instrumental in this acceptance (Sneddon and Westfall, 1983), and the importance of purinergic signalling in the contraction of genital smooth muscle has been confirmed by $P2X_1$ receptor deficient mice being infertile due to oligospermia, secondary to reduced vas deferens contraction (Mulyran et al., 2000). Contraction of the human vas deferens has recently been shown to have a significant purinergic component (Banks et al., 2006). The testicular capsule can be considered to be the most proximal part of the vas deferens, and in conjunction with the seminiferous tubules, constitutes the origin of the genital tract. Contraction of the tunica albuginea may be essential in moving sperm out of the testis and it is important to our understanding of sperm transport, that the contractile mechanisms governing the smooth muscle contraction are characterised.

This was a comparative study which examined the testicular capsule smooth muscle elements from man, rabbit, rat and mouse. The role of purinergic co-transmission in the contraction of the tunica albuginea smooth muscle was examined by electrical field stimulation of autonomic nerves, and the exogenous application of purinergic and autonomic agonists. The distribution of P2X receptors was examined by immunohistochemistry.

3.3 Methods

See Chapter 2

Human testes were obtained from patients undergoing orchidectomy for gender reassignment (n=5, ages 23-47) or metastatic prostate cancer (n=5, ages 67-88). Ethical approval was obtained, reference number RREC 2662. All patients were given verbal and written information prior to giving informed consent. A copy of the consent form was given to the patient and a further copy filed in the medical notes. Tissue was stored anonymously. All gender reassignment patients had ceased supplementary oestrogen therapy at least 6 weeks prior to operation.

Animal testes were obtained from adult male Sprague-Dawley rats (300-360 g, n=5), mice (35-42g, n=5) and rabbits (3kg, n=3).

Organ-bath studies were carried out at 35°C, to approximate to scrotal temperature.

3.4 Results

3.4.1 Electron microscopy

In the three species examined (man, rat, mouse) two layers of testicular capsule were identified. The main substance of the capsule was the tunica albuginea consisting of dense connective tissue and smooth muscle cells (Fig 3.1a). An inner layer, the tunica vasculosa, was identified which contained loose connective tissue incorporating the vasculature and nerve bundles. Previous investigators identified an outer mesothelial layer, one cell in thickness, termed the tunica vaginalis visceralis. This was not visualised in our preparations, though it has been reported that this is often lost in tissue preparation (Davis et al., 1970).

The three species examined demonstrated a similar distribution of smooth muscle cells within the tunica albuginea. Flattened branching cells were loosely arranged in single cell thick sheets with intervening collagenous connective tissue. These sheets were closer together on the inner aspect of the tunica albuginea, having less intervening collagen between them, but did not form an anatomically distinct muscle layer within the tunica albuginea (see Fig 3.1b). The smooth muscle cells were mostly arranged in a longitudinal pattern, though those of the human tunica albuginea were the least organised of the three species examined (Fig 3.1c). The smooth muscle cell appearance varied pending its position within the tunica albuginea. The cells of the outer aspect resembled the synthetic smooth muscle phenotype, having a greater cytoplasm to nuclear ratio, greater amounts of Golgi apparatus, fewer dense bodies and fewer myofilaments. In contrast, the cells of the inner aspect had a more classical appearance of the contractile smooth muscle phenotype (Fig 3.1d,e). These cells were flatter, had central cylindrical nuclei, numerous dense bodies among myofilaments, dense plaques adherent to the cell membrane, micropinocytotic vesicles and distinct

basal laminae except at regions of cell-cell contact. In between smooth muscle cells, large amounts of collagen and to a lesser extent elastin were present.

Specialized junctions between smooth muscle cells were seen in all three species examined. These consisted of a close apposition between cell membrane of smooth muscle cell processes (Fig 3.1d). However, these junctions were not examined at sufficiently high resolution to identify them as classical gap junctions.

In the human tunica albuginea proper, nerves, not associated with blood vessels. Retracted Schwann cells, in association with vesicle containing axon varicosities, were seen in immediate apposition to smooth muscle cells, which were strongly suggestive of functional innervation of smooth muscle (Fig 1e,f). In contrast, in the mouse and the rat no nerve fibres were identified within the tunica albuginea. Nerves were predominately associated with blood vessels in the tunica vasculosa (Fig 3.2).

3.4.2 P2X receptor immunohistochemistry

Only minimal $P2X_1$ receptor immunoreactivity was observed in the capsule of the human testis. Greater immunoreactivity was observed in the capsule of the rat (Fig 3.3a) and the mouse. $P2X_2$ receptor immunoreactivity was observed in all three species (Fig 3.3b).

$P2X_3$ receptors were expressed in a few variable punctate places in the human tunica albuginea but minimally so in the other species examined. No smooth muscle expression of $P2X_{4-7}$ receptors was demonstrated in any of the species examined. Germ cell expression was observed which confirmed that the antibodies where active.

3.4.3 Pharmacology

Strips of rabbit tunica albuginea were contractile to EFS of autonomic nerves in a frequency-dependent manner with peak contraction occurring at 16 Hz. Contractions were biphasic with an initial rapid phase followed by a longer plateau phase that took up to 30 seconds to reach a maximal contraction. Subsequent EFS in the presence of purinergic antagonist PPADS (30 μM) reduced the maximal contraction by 36%. EFS in the presence of the adrenergic antagonist prazosin (1 μM) reduced the maximal contraction by 77 %. When both antagonists were used together, EFS–induced contraction was almost completely abolished (Figure 3.4a). Some strips of human tunica albuginea were contractile to EFS of autonomic nerves in a frequency-dependent manner, but contractions were less repeatable and the maximal contraction occurred at 32 Hz. Contractions were completely abolished by prazosin (Figure 3.4b). In contrast, the rat and mouse tunica albuginea preparations were not reproducibly contractile to EFS of autonomic nerves.

Strips of human tunica albuginea contracted to exogenously applied NA, ATP and α,β-meATP in a concentration-dependent manner, although contractions were recorded only to the higher doses of ATP tested (Fig 3.5a,b). The orientation of the strip did not influence the contractions. The strongest contractions were to NA, with the maximal NA contraction approximately 6 times stronger than that of the maximal α,β-meATP contraction. Respective EC_{50} values were NA 2.6 μM (95% CI = 1.5-2.9 μM) and α,β-me-ATP 5.6 μM (95% CI = 2.9-11.1 μM). Contractions to ATP did not reach a maximum and so an EC_{50} could not be calculated. No contraction or relaxation was recorded to ACh.

Strips of rabbit tunica albuginea contracted to exogenously applied NA, ACh, ATP and α,β-meATP in a concentration-dependent manner. The strongest observed

contractions were to NA then to ACh with ATP and α,β-meATP being similar. Contractions to NA, ACh, ATP and α,β-meATP did not reach a maximum and so EC_{50} values could not be calculated (Fig 3.6a).

Rat tunica albuginea preparations contracted to ACh, ATP and NA in a concentration-dependent manner. The contractions to NA and ACh were very similar. Respective EC_{50} values were NA .68 μM (95% CI = .3-1.4 μM) and ACh 1.22 μM (95% CI = .09-15 μM). No maximum was reached with ATP so the EC_{50} could not be calculated (Figure 3.6b).

The mouse tunica albuginea contracted in response to ACh in a concentration-dependant manner, giving an EC_{50} value of .58 μM (95% CI = .3-1.1 μM). The capsule was also contractile in response to exogenous ATP, however, no maximum was reached (Fig 3.7a). The whole capsule preparation, when pre-contracted with ACh at its EC_{50} concentration, was found to relax in response to the addition of NA in a concentration-dependent manner giving an EC_{50} value of 8.0 μM (95% CI = 1.8-36 μM) (Fig 3.7b).

Contractions of mouse and rat tunica albuginea were recorded in response to α,β-meATP, however rapid desensitization occurred and so formal concentration-response curves were not recorded.

The strips of rabbit testicular capsule demonstrated marked spontaneous activity with small contractions occurring at a rate of 140 per hour. Human testis strips also demonstrated some spontaneous activity, but only following stimulation with NA or ATP. Contractions were less consistent (5/9 preparations), and at a mean rate of 75 contractions per hour.

3.4 Results

Figure 3.1

(a)   Electron-micrographs demonstrating the longitudinally orientated smooth muscle (↑) arrangement in the tunica albuginea of the rat testis. Smooth muscle cells are distributed singularly, embedded in connective tissue. They become closer to each other from the outer (O) to the inner (I) aspect of the sheet. The tunica vasculosa (TV) can be seen containing a blood vessel (BV). Arrows indicate smooth muscle cell bodies. Scale bar =10 μM.

(b)   Electron-micrograph of the mouse tunica albuginea demonstrating the increasing density of the smooth muscle cells on the inner aspect of the tunica albuginea. Arrows indicate smooth muscle cell bodies. Scale bar =2 μM.

(c)   Electron-micrograph of human tunica albuginea demonstrating the less organised arrangement of smooth muscle cells. Arrows indicate smooth muscle cell bodies. Scale bar =10 μM.

(d)   Electron-micrograph demonstrating a human smooth muscle cell (M) and the process (P) of another smooth muscle cell in area of cell-cell contact (thick arrow). Scale bar =1 μM.

(e)   Electron-micrograph demonstrating a smooth muscle cell (M) in immediate apposition to a vesicle-containing axon profile (*), which is free of Schwann cell (S) process. Scale bar =1 μM.

(f)   Electron-micrograph demonstrating a smooth muscle cell process (P) close to an axon profile, free of Schwann cell process. Scale bar =1 μM.
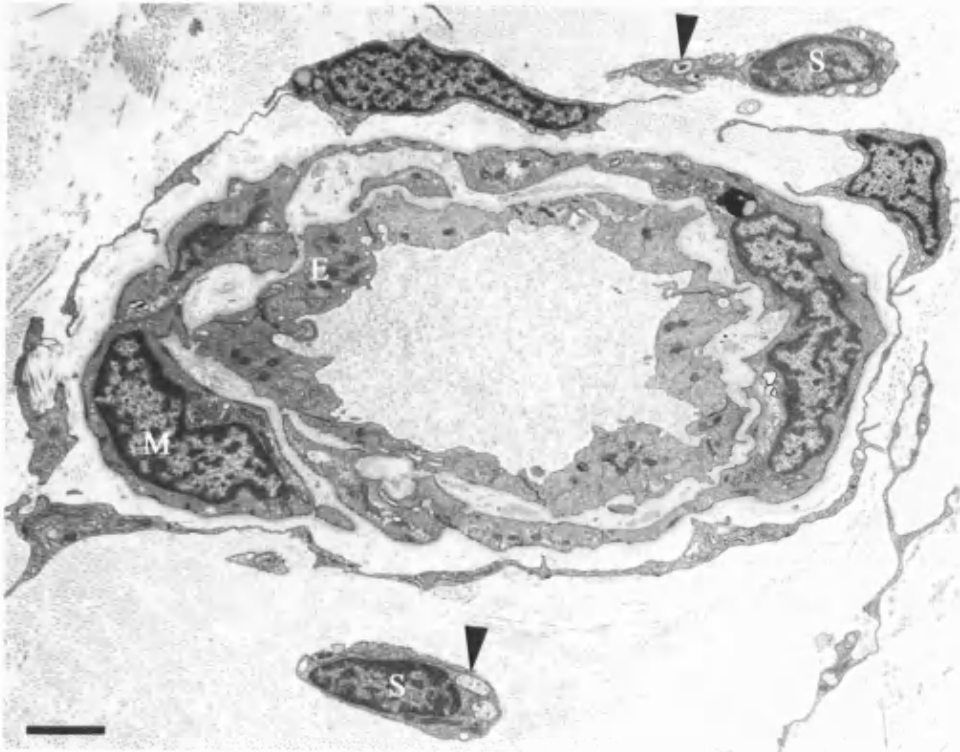
73
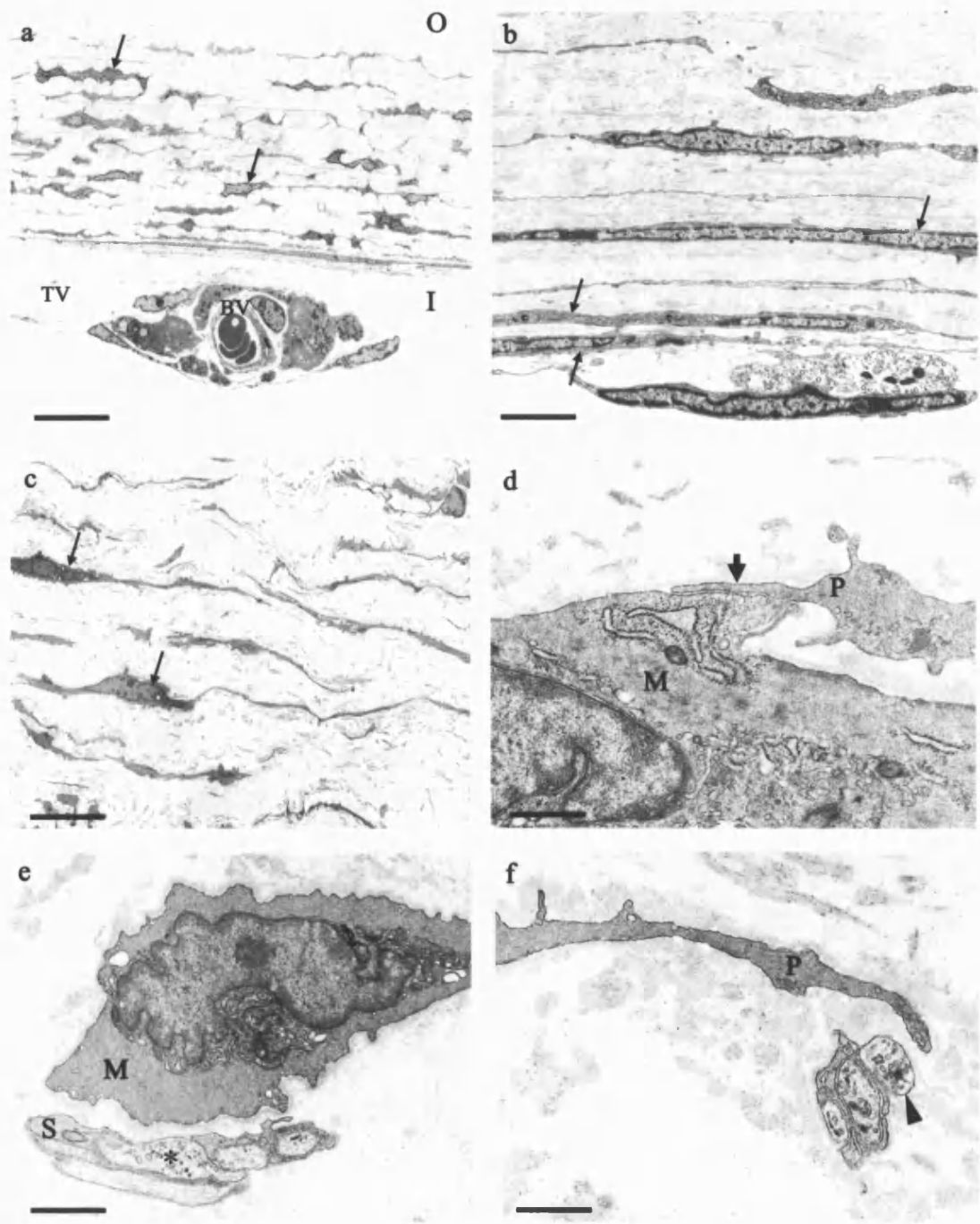
Figure 3.1

Figure 3.2

Electron-micrograph showing a typical blood vessel and associated nerve bundles (▲) together with Schwann cells (S) running together towards the deeper region of the human tunica albuginea. (E = endothelial cell, M = smooth muscle cell, S = Schwann cell). Scale bar =2 μM.

Figure 3.2

Figure 3.3

Immunohistochemistry of tunica albuginea

(a)   This transverse section shows some immunoreactivity to the $P2X_1$ receptor

subtype in the tunica albuginea of the rat. Arrows indicate smooth muscle

cell bodies. Scale bar = 25 μM.

(b)   This longitudinal section shows $P2X_2$ receptor immunoreactivity on human

tunica albuginea smooth muscle. Arrows indicate smooth muscle cell bodies.

Scale bar = 100 μM.

Figure 3.3

a
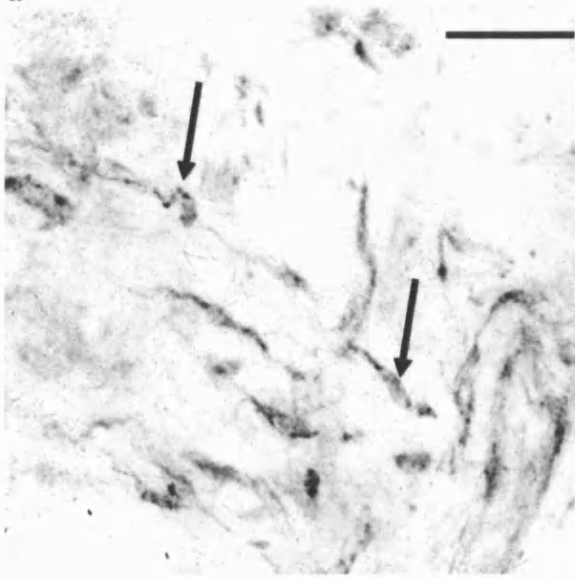
b

Figure 3.4

Frequency-response curves showing frequency-dependent contraction of the rabbit

and human tunica albuginea to EFS.

All symbols show mean % of contraction to KCl (120 mM) ± s.e. mean (n).

(a)   Contraction of the rabbit tunica albuginea to EFS (100 V, 0.3 ms, 2-64 Hz,

        15 s) (■).In the presence of PPADS (30 μM) (▲). In the presence of

        prazosin (1 μM) (▼), and in presence of both PPADS (30 μM) and prazosin
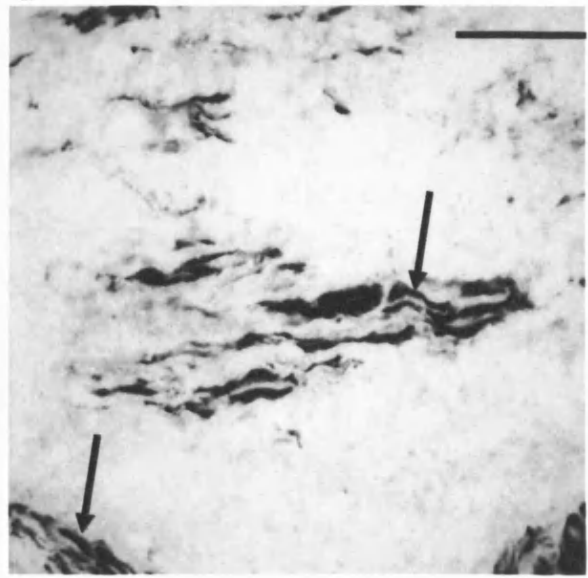
        (1 μM) (◆) (n=3).

(b)   Contraction of the human tunica albuginea to EFS (100 V, 0.3 ms, 2-32 Hz,

        30 s) (■) (n=6). Contractions were completely antagonised by prazosin (1

        μM) (▼).

Figure 3.4

a

% KCl contraction

Hz

b

% KCl contraction

Frequency [Hz]
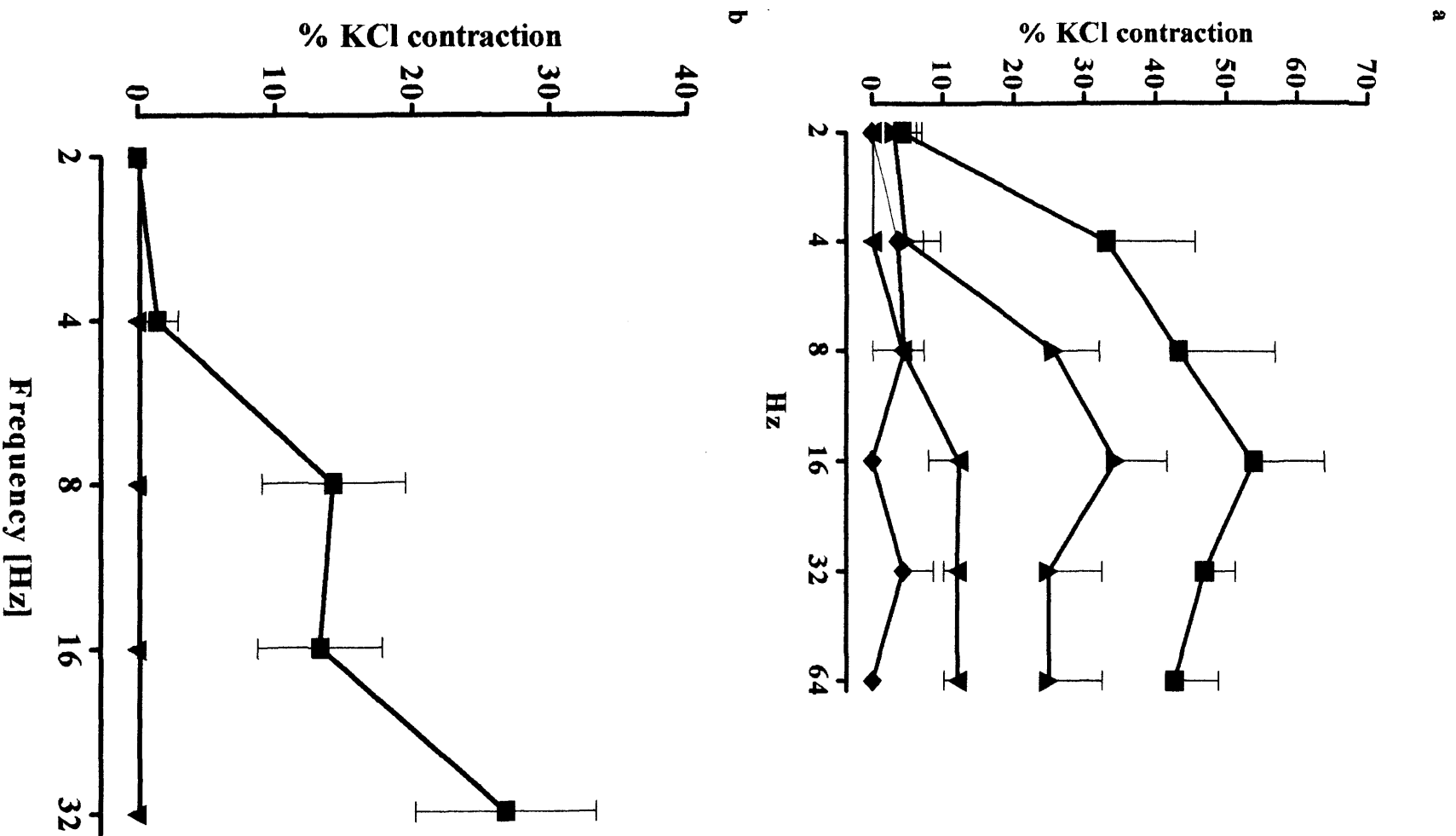
Figure 3.5

Concentration-response curves for NA, ATP and α,β-meATP on isolated strips of

human tunica albuginea. All symbols show mean % of KCl contraction (120 mM) ±

s.e. mean (n) (unless masked by symbol) in conjunction with non-linear regression

curve. Dotted line represents 95 % confidence interval (CI) of individual values.

(a)   Concentration-response curve for NA (■, n=7, strips=11).

(b)   Concentration-response curve for α,β-meATP (▲), (n=7 strips = 11) and

        ATP (◆), (n=12, strips =16). 95 % CI not shown for ATP.

Figure 3.5
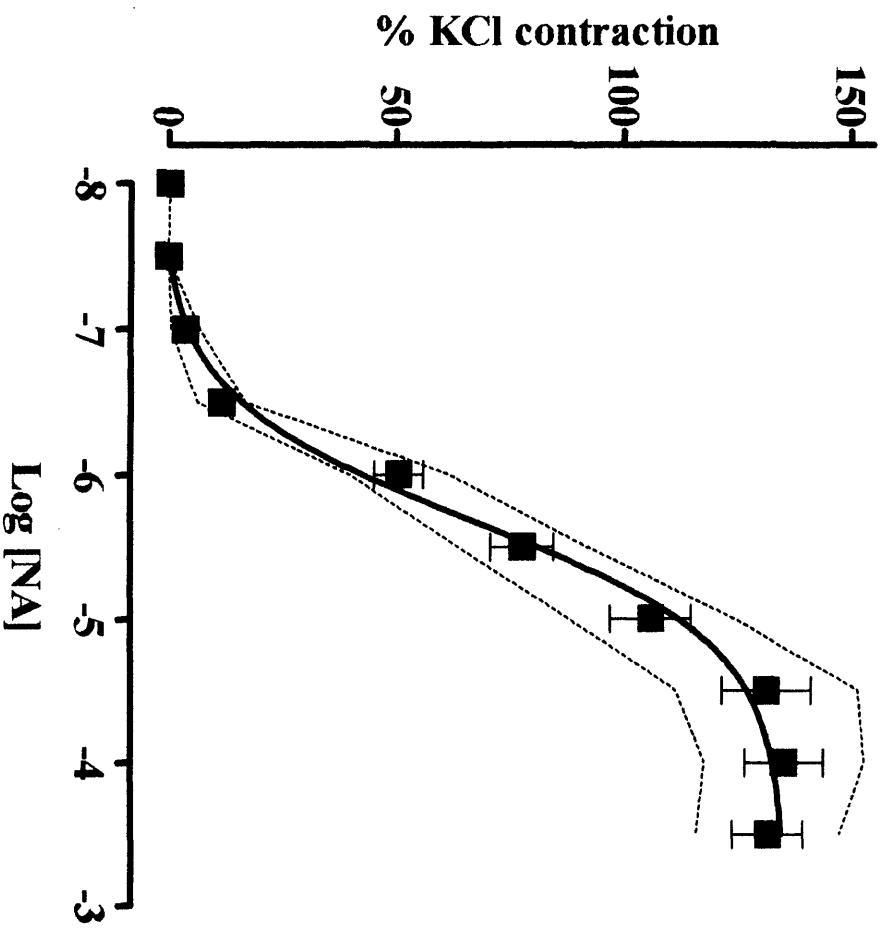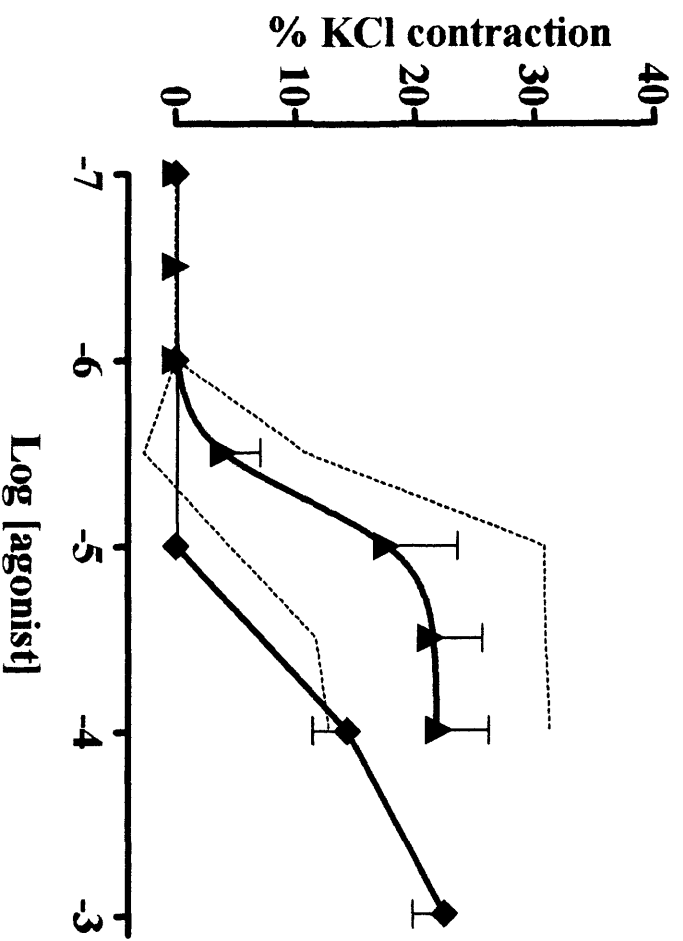
Figure 3.6

Concentration-response curves for NA, ACh, ATP and α,β-meATP on isolated

strips of rabbit tunica albuginea and whole tunica albuginea preparations of the rat.

All symbols show mean % of maximum KCl contraction ± s.e. mean (n), (unless

masked by symbol), together with non-linear regression curve where appropriate.

(a)   Rabbit tunica albuginea. Concentration-response curve for NA (■), ACh

(●), ATP (◆)(n=3, strips=3), and α,β-meATP (▲), (n=2, strips=2).

(b) Rat whole preparations of tunica albuginea. Concentration-response curve

for NA (■), ACh (●), ATP (◆), (n=6, preparations=12). Red dotted line

(---) represents 95 % CI for NA values, and green dotted line (---) represents

95 % CI for ACh values.

Figure 3.6

% KCl contraction

Log [agonist]

% KCl contraction

Log [agonist]

a

b

84
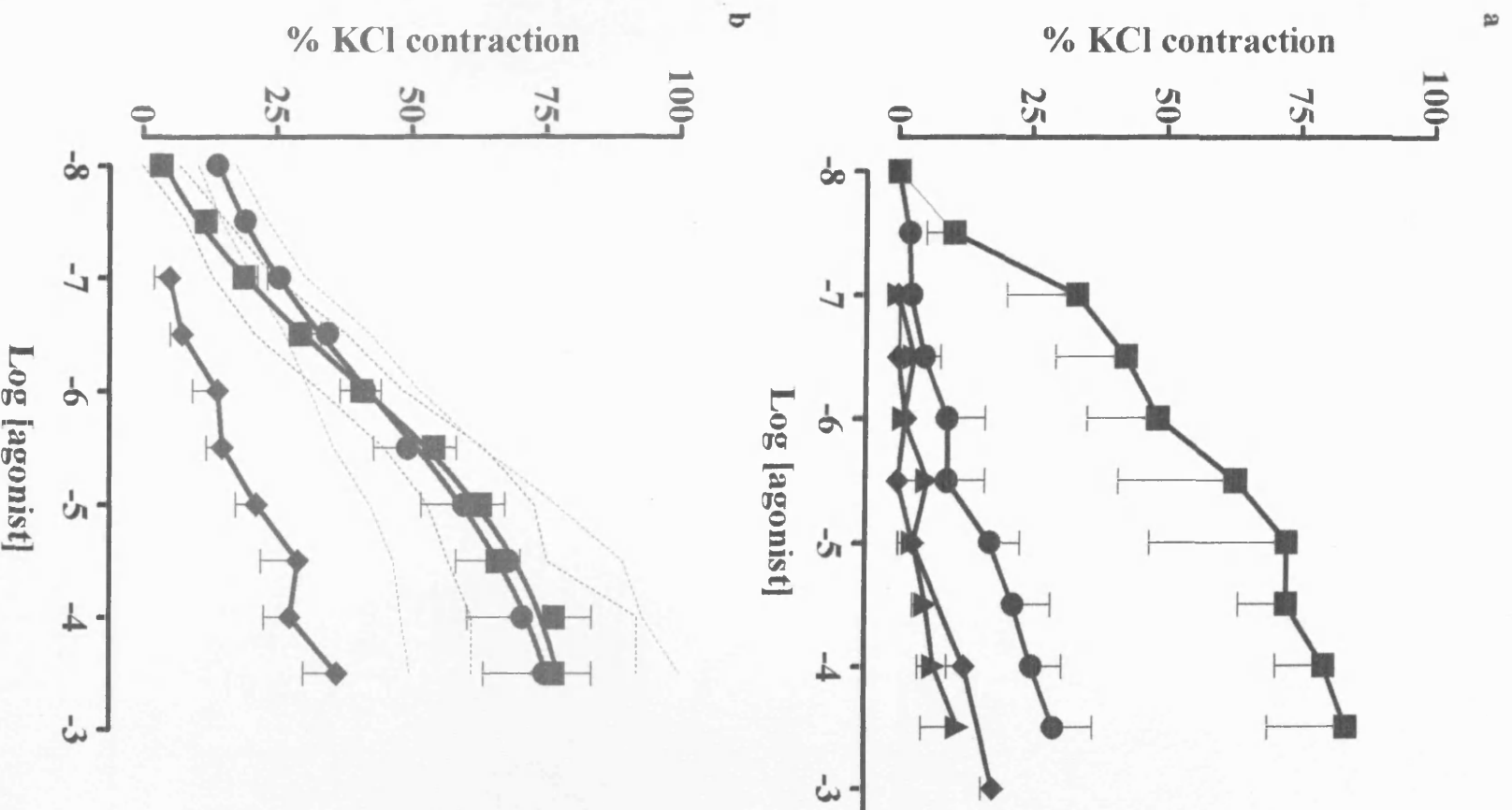
Figure 3.7

(a)   Concentration-response curves for ACh (●) and ATP (◆) on whole tunica

albuginea preparations of the mouse. All symbols show mean % of KCl

contraction ± s.e. mean (n), (unless masked by symbol) (n=6, strips=8),

together with non-linear regression curve. Dotted line represents 95 % CI for

ACh values.

(b) Relaxation concentration-response curves of whole preparations of mouse

tunica albuginea to NA (■), following pre-contraction with ACh at the EC_{50}

concentration All symbols show mean remaining contraction expressed as %

of contraction to ACh (EC_{50}) ± s.e. mean, (n=4, strips=4), together with non-

linear regression curve. Dotted lines represent 95 % CI for NA values.

Figure 3.7

86
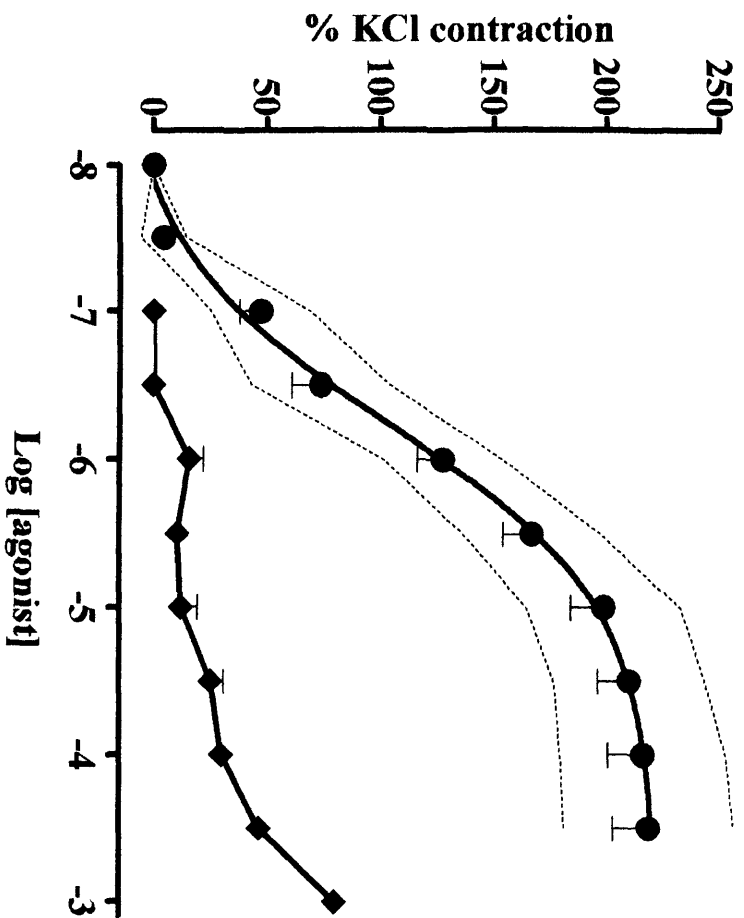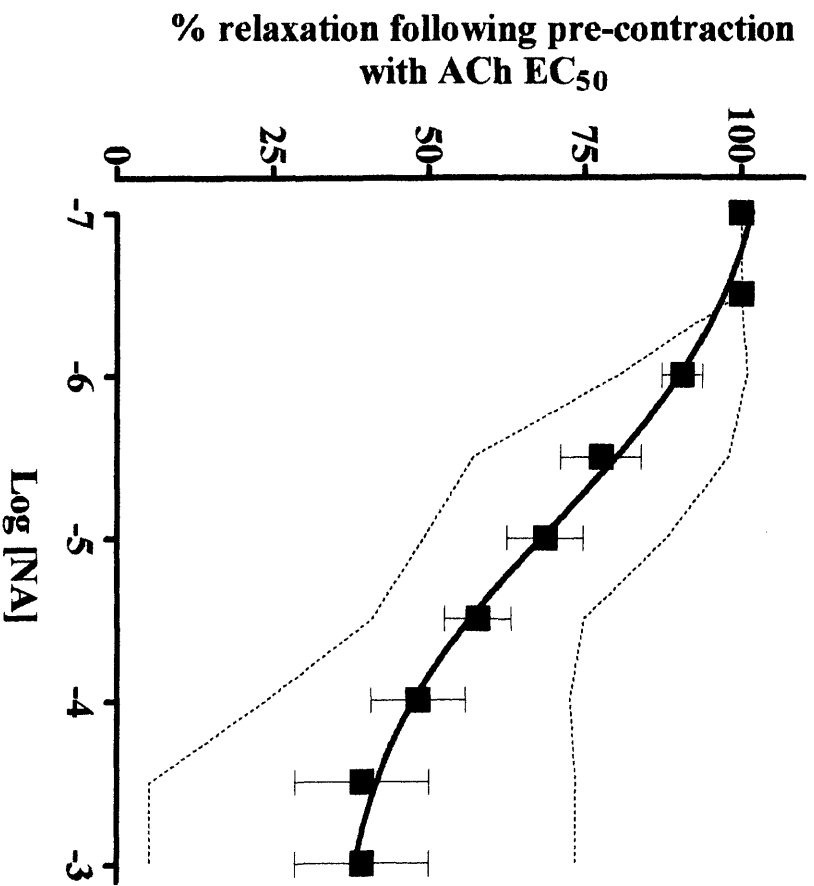
3.5 Discussion

Smooth muscle cells were first demonstrated in the tunica albuginea of rabbit testes in 1967 (Holstein, 1967). Subsequently, smooth muscle cells have been demonstrated in the tunica albuginea of several species including man, rat, guinea pig, cat, dog, pig, cow, sheep and horse (Davis et al., 1970; Langford and Heller, 1973; Leeson and Cookson, 1974; Ohanian et al., 1979; Chacon-Arellano and Woolley, 1980; Middendorf et al., 2002). The extent and conformity of the smooth muscle arrangement between species is not consistent, and only in the rabbit and man has a true layer been demonstrated, and indeed two layers perpendicular to each other have been described on the outer aspect of the rabbit tunica albuginea (Holstein and Weiss, 1967; Middendorf et al., 2002). The density of smooth muscle cells in different areas of the tunica albuginea has been shown to be variable (Holstein, 1967). Inter species variations exist and it has been suggested that more smooth muscle cells are located at the poles and the posterior border of the testis in the rete testicular area (Davis et al., 1970). In our own studies we found no conclusive pattern or density of smooth muscle cells in different areas of the testicular capsule. We found that the smooth muscle cell phenotype progressively changed from a secretory phenotype on the outer aspect to a more contractile phenotype on the inner aspect of the tunica albuginea. This arrangement is largely in agreement with the findings of Middendorff, who confirmed the phenotypic change by immunohistochemistry (Middendorff et al., 2002). However, we did not observe the inner smooth muscle cells to be a distinct layer. This transition and arrangement was seen in all three species examined, but was best demonstrated in the thin tunica albuginea of the mouse or rat, in which the entire testicular capsule could be observed in the same section. This observation may explain the mixed reports regarding the presence of true smooth muscle cells as

opposed to contractile myofibroblasts, and is in keeping with the observation that a smooth muscle cell is not a single entity but represents a heterogenous cell that is both contractile and synthetic at opposite ends of the spectrum (Campbell and Campbell, 1997).

The neuronal supply to the testis appears to vary markedly between species, with some immunohistochemical reports of dense networks covering the outer aspect of the tunica albuginea with nerves penetrating the substance of the testis and even directly innervating Leydig cells (Prince, 1992; Santamaria et al., 1997; Suburo et al., 2002). Other reports suggest the innervation is sparse and predominantly involved in vasomotor control and consequently thermoregulation (Bell et and McLean, 1973; Wrobel and Bradl, 1998). In the human testis, myelinated nerves penetrated the tunica albuginea, and nerve varicosities containing vesicles were seen in apposition to smooth muscle cells, consistent with neurogenic innervation of smooth muscle. The strips of human tunica albuginea that did respond to EFS had all been cut either parallel or adjacent to a blood vessel that was subsequently removed. This would imply that the neurogenic innervation of the smooth muscle is related to nerves associated with the blood vessels. In keeping with other investigators we found specialised junctions between smooth muscle cells, these were fused areas between smooth muscle cells, consistent with myogenic propagation of contraction, although no classical gap junctions were identified.

In 1969, Davis and Langford (Davis and Langford, 1969) first demonstrated that the rat testicular capsule contracted in response to NA and ACh. The present study demonstrated that the rabbit tunica albuginea was contractile to EFS of autonomic nerves. The P2 receptor antagonist PPADS reduced the contractile force by 36% and prazosin by 77 %. In combination virtually no contraction was detected. The

response of the rabbit tunica albuginea to exogenous α,β-meATP was uncharacteristically lower than that to ATP, which is suggestive that some desensitisation of P2X$_1$ receptors may have occurred, as seen in the rat and the mouse, or that P2X$_2$ receptors were involved. In the rabbit, the maximal contractile force induced by NA was approximately 4 times that of ATP or α,β-meATP. In contrast the human tunica albuginea contraction was completely antagonised by prazosin and the maximal contractions induced by NA were approximately 7 times that of α,β-meATP. The purinergic neural component of human tunica albuginea contraction may be smaller and masked by the adrenergic component. The pattern of a relatively smaller purinergic component would be in keeping with other studies on purinergic smooth muscle contraction of healthy human genito-urinary smooth muscle (Bayliss et al., 1999; Burnstock 2004). In keeping with previous findings (Davis and Langford, 1969) the rat tunica albuginea was contractile to both NA and ACh, although in our study we found marginally greater contractility with NA as opposed to ACh. In all the species examined, we have shown for the first time that the tunica albuginea is also contracted by purinergic agonists. The mouse tunica albuginea demonstrated a different pharmacological contractile profile to that of the rat and rabbit, where the tunica albuginea contracted to ACh and ATP, but relaxed to NA.

In other tissues, where purinergic co-transmission is demonstrated, such as the vas deferens or bladder, the contraction is classically biphasic with an initial rapid phase being attributable to ATP and a second slower phase attributable to either NA or ACh respectively (Fedan et al., 1981; Meldrum and Burnstock, 1983; Sneddon et al., 1984). The contraction of rabbit tunica albuginea to EFS was demonstrated to be biphasic. In the human tunica albuginea, where adrenergic transmission dominates, contraction to EFS was monophasic and contraction to exogenous agonists was slow,

taking up to 1 minute to reach maximum. This slow pattern was similar in the rat and the mouse.

ATP-induced smooth muscle contractions have been studied in other tissues and it is established that contraction is mediated through the $P2X_1$ receptor. α,β-meATP is selective for $P2X_1$ and $P2X_3$ receptors. $P2X_3$ receptors are largely expressed on sensory nerves involved in nociception (Burnstock, 2000; 2003), which suggests that smooth muscle contraction is largely mediated through the $P2X_1$ receptor subtype, with the exception of the rabbit where contractions to α,β-meATP were substantially less than ATP and $P2X_2$ receptors may be involved. Immunohistochemical studies using the seven P2X receptor subtype antibodies weakly demonstrated the presence of $P2X_1$ receptors on the smooth muscle membrane. There was also expression of $P2X_2$ receptors on smooth muscle cells, although often located intracellularly. Staining of both $P2X_1$ and $P2X_2$ receptors was generally low when compared to other tissues, such as the vas deferens, bladder and arteries. This is partly a reflection of the small smooth muscle content within testes and the relatively small purinergic component, at least in healthy tissues.

Sperm contained within the testis lack forward progressive motility and this is subsequently gained in the distal epididymis in man and rats (Hoskins et al., 1978; Yeung et al., 1993; Jeulin et al., 1996). A pressure gradient would move sperm out of the testis and it is postulated that contraction of the testicular capsule and myoid cells of the seminiferous tubules may generate this (Hargrove et al., 1977; Ellis et al., 1982; Maekawa et al., 1996). Fluid production within the testis would also create pressure and movement of the fluid would have the additional benefit of effectively carrying sperm out of the testis on an effluent tide. Movement of the ciliated cells of the ducti efferenti at the junction of the rete testis and caput epididymis were previously

thought to move sperm into the epididymis but this has been disproved due to the demonstration of normal numbers of sperm in the epididymis in patients with Kartagener's syndrome, in which cilia are immobile (Afzelius, 1976). Studies using radio-opaque sperm injected into the epididymis of rabbits have shown significant antegrade and retrograde movement of sperm from the epididymis (Prins and Zaneveld, 1980). As sperm are present in the ejaculate despite an absence of sexual activity it must be assumed that tunica albuginea contraction occurs subconsciously and without the need for physical sexual stimulation. In this study the rabbit tunica albuginea demonstrated marked spontaneous activity at a rate of approximately 140 per hour and the human tunica albuginea also demonstrated spontaneous contraction at a lower rate of 75 contractions per hour. Spontaneous contractions of the human and rabbit testis have been previously reported (Davis et al., 1970; Middendorff et al., 2002).

Concern has been raised over progressively falling sperm counts (Carlsen et al., 1992; Joffe, 1996). It is possible that this is, in part, due to a failure of sperm transport rather than spermatogenesis. Developmental studies have demonstrated that the development of testicular smooth muscle is coincidental with sexual maturity (Leeson, 1977; Leeson and Forman, 1981; Holt et al., 2004). This study has demonstrated an ultrastructural and pharmacological basis for testicular capsule contraction, which may play an essential role in moving sperm from the testis to the epididymis. This process is common to all species examined. Purinergic co-transmission was evident in the rabbit, and purinergic responsiveness was demonstrated in man, rats and mice. We suggest that continuous flux of spermatids from the seminiferous tubules within the testis into the rete testis and epididymis may be caused in part by low pressure, spontaneous contractions of the smooth muscle of

the tunica albuginea. Larger volume flux may be caused by slow, sustained contractions induced by sympathetic, parasympathetic and purinergic stimuli, as may be induced by sexual arousal. In man, this process is dominated by the adrenergic system in association with a smaller purinergic component, that maybe consistent with purinergic co-transmission, but is without a parasympathetic component. Sympathetic stimulation may have a role in improving sperm counts.

CHAPTER 4

THE PURINERGIC COMPONENT OF HUMAN

VAS DEFERENS CONTRACTION

4.1 Abstract

The aim of this study was to examine purinergic signalling in the human vas deferens. Strips of human vas deferens from the epididymal end were suspended in an organ bath and subjected to electrical field stimulation to establish frequency-response curves. These were repeated in the presence of the P2 antagonist pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS, 30 μM), the adrenergic α_1 antagonist, prazosin (1 μM) and tetrodotoxin (1 μM). Concentration-response curves were obtained by the exogenous application of NA and the P2X agonists ATP and the non-hydrolysable analogue α,β-meATP. The P2X receptor subtype distribution was assessed by immunohistochemistry using specific antibodies.

The response at 32 Hz in the presence of PPADS was reduced by 40% and in the presence of prazosin by 80%. NA caused concentration-dependent contractions (the EC_{50} was 11.8 μM). ATP also caused concentration-dependent contractions. Contractions to α,β-meATP (EC_{50} value = 6.27 μM) suggested that the functional receptor was $P2X_1$ and/or $P2X_3$. However, immunohistochemistry demonstrated $P2X_1$, but not $P2X_3$ receptor immunoreactivity on the smooth muscle cells. In summary, this study has demonstrated that ATP is a co-transmitter with NA in the contraction of the human vas deferens predominantly acting through the $P2X_1$ receptor.

4.2 Introduction

ATP, co-released with NA from sympathetic nerves and ACh from parasympathetic nerves, acting through P2X receptors, has been shown to cause contraction of genito-urinary smooth muscle of mammals (Burnstock, 1995; 2001).

This purinergic component of smooth muscle contraction is variable between tissues and species. The functional importance of co-released ATP demonstrated in laboratory animals is not always mirrored in human tissue. For instance, in the rabbit bladder the purinergic component of the parasympathetic mediated contraction is accountable for up to 40% of the smooth muscle contraction (Calvert et al., 2001), and in the guinea-pig up to 70% (Meldrum and Burnstock, 1983). In the normal human bladder it is very small, although in the pathological states such as detrusor instability, and interstitial cystitis, the purinergic component becomes significant, accounting for up to 40% of the contraction (Palea et al., 1993; Bayliss et al., 1999; O'Reilly et al., 2002).

NA and ATP have been shown to mediate contraction of the rat and guinea pig vas deferens smooth muscle. The contraction, in response to EFS of sympathetic nerves has been shown to be biphasic (Fedan et al., 1981; Meldrum and Burnstock, 1983; Sneddon et al., 1984). ATP, released from sympathetic nerves, acts through ion-gated P2X receptors to stimulate the initial fast phase of the contraction, with co-released NA causing the more sustained, but slower contraction, acting through G protein-coupled α adrenoceptors.

$P2X_1$ receptors have been demonstrated on the smooth muscle membrane of the vas deferens, where it mediates contraction (Vulchanova et al., 1996; Hansen et al., 1999; Lee et al., 2000a; b; Mulryan et al., 2000). The functional relevance has been confirmed by the demonstration that $P2X_1$ receptor null mice were shown to have a 90% reduction in fertility due to reduced vas deferens contraction, although their sperm were fertile. It was subsequently suggested that $P2X_1$ antagonists could be developed for male contraception (Mulyran et al., 2000).

The role of ATP as a co-transmitter and the distribution of the $P2X_1$ receptor in the human vas deferens have yet to be fully examined. This study examined the purinergic component of human vas deferens smooth muscle contraction in response to EFS of autonomic nerves, and also, the contractile effects of purinergic and adrenergic agonists. The tissue distribution of the P2X receptor subtypes was also examined by immunohistochemistry.

4.4 Methods

See Chapter 2

The hospital ethical committee granted approval for this study. Human tissue was taken from patients either undergoing a vasectomy for contraception or orchidectomy for metastatic prostate disease. Patients were fully informed and gave prior consent.

Each vas deferens was divided longitudinally into specimens measuring approximately 2 x 20 mm. An initial load of 1 g was applied at the start of each experiment.

4.3.1 Frequency-response curves

Strips of vas deferens were subjected to EFS (100 V, 0.5 ms, 1-64 Hz, 20 s) every 5 min. Three frequency-response curves were carried out to establish consistent contractile responses. Frequency-response curves were subsequently constructed after 20 min in the presence of prazosin (1 μM), or PPADS (30 μM) to establish the relative adrenergic and purinergic components of the contraction. Further frequency-response curves were then constructed in the presence of both antagonists, and then

finally in the presence of TTX (1 μM), to determine the part of the response due to direct electrical stimulation of smooth muscle. The Krebs solution in the organ bath was changed between each set of frequency-response curves.

4.3.2 Concentration-response curves

Non-cumulative concentration-response curves were constructed for NA (0.1-300 μM), ATP (0.1 μM-1 mM) and α,β-meATP (0.1-300 μM). Boluses of α,β-meATP were applied at least 20 minutes apart to avoid desensitisation of the $P2X_1$ receptor.

4.3.4 Immunohistochemistry

See general methods, chapter 2. All seven P2X receptor antibodies were diluted to 2.5-5 μg/ml.

4.4 Results

4.4.1 Pharmacology

EFS of sympathetic nerves of the vas deferens induced frequency-dependent contractions. These contractions were predominantly monophasic (Fig 4.1a, 1d), but became rhythmic at the highest frequencies. It was found that the maximum contraction was achieved at 32 Hz. The neurogenic contraction was significantly reduced by the α_1- adrenoceptor antagonist prazosin by over 80% at peak frequency (Fig 4.1b, Fig 4.2) (P=0.0001). EFS-induced contractions in the presence of the P2X receptor antagonist PPADS (30 μM) were also significantly reduced (P<0.0001, Fig 4.1e, Fig 4.2) from the initial contraction (Fig 4.1d, Fig 4.2). This was more evident at the higher frequencies. At the peak contraction frequency of 32 Hz this amounted to a 40% reduction from the initial contraction. Virtually all the neurogenic contraction was abolished when the EFS was carried out in the presence of both PPADS and prazosin (Fig 4.1f). At the lower frequencies tested prazosin almost completely abolished the smooth muscle contraction and PPADS caused minimal antagonism. At the higher frequencies tested, PPADS caused relatively greater antagonism and prazosin relatively less (Fig 4.2). EFS carried out in the presence of TTX resulted in some residual contraction, but only at the highest frequencies tested (Fig 4.1c). This confirmed that most of the contraction was nerve-mediated.

NA produced a concentration-dependent contraction of the vas deferens, EC_{50} value was 11.8 μM (95 % CI = 9.5-14.6μM) (Figure 4.3a). The contractile responses were typically slow rising, with a stimulus to maximal response time of approximately 12 s (Fig 4.1g). Supra-threshold rhythmic contractions developed when concentrations above 10 μM were used.

α,β-meATP caused contractions similar to those induced by ATP, but these were larger and more consistent (Fig 4.1h). Concentration-response curves obtained by using the non-hydrolysable ATP analogue α,β-meATP did reach a maximum (Fig 4.3b). Although some desensitisation was noted at the highest concentrations used, the EC_{50} value was calculated to be 9.2 μM (95 % CI = 5.6-15.1 μM). ATP, when applied exogenously produced a small, monophasic response (Fig 4.1i). Responses were less consistent and no maximum contraction was obtained despite using concentrations up to 1 mM (Fig 4.3b).

Spikes of spontaneous rhythmic contractile activity were observed in 9/14 vas deferens preparations. Spontaneous contraction frequency averaged 100 contractions per hour, with a mean contractile force of 5.8 mg/mg tissue. ATP, when applied at concentrations too low to induce individual contractions, rapidly increased the frequency and amplitude of these spontaneous contractions for a few seconds (Fig 4.1j). Spontaneous contractions were usually only observed following stimulation by exogenous agonists or EFS and were not completely abolished by TTX.

4.4.2 Immunohistochemistry

$P2X_1$ receptor expression was observed on the smooth muscle membranes in the outer longitudinal and middle circular muscle layers. Minimal expression was observed on the inner longitudinal layer (Fig 4.4a). At higher magnification, it appeared that the $P2X_1$ receptor expression was non-homogenous both in terms of the number of smooth muscle cells on which it was expressed and on single smooth muscle cells with expression being greater on some areas of the membrane (see Fig 4.4b). Expression of $P2X_2$ receptors was also noted in a similar pattern, but more diffusely distributed and in an intra-cellular position (Fig 4.4c,d). No expression of

other P2X receptor subtypes was observed on the smooth muscle. In control studies, no expression was observed in the absence of the primary antibody or following pre-adsorption of the primary antibody with its cognate peptide.

4.4 Results

Figure 4.1

Typical traces of responses to EFS, in the presence and absence of antagonists and individual responses to agonists. Start of time scale indicates the start of nerve stimulation or application of agonist to organ bath.

(a)   Response to EFS at 100v, 0.5ms, 32 Hz for 20s in absence of antagonist.

(b)   Stimulation in the presence of prazosin (1 μM).

(c)   Stimulation in the presence of TTX (1 μM).

(d)   Response to EFS in absence of antagonist.

(e)   EFS in the presence of PPADS (30 μM).

(f)   EFS in the presence of both PPADS (30 μM) and prazosin (1 μM).

(g)   Response to NA (100 μM).

(h)   Response to α,β-meATP (300 μM).

(i)   Response to ATP (1 mM).

(j)   Spontaneous contraction and subsequent reaction after ATP (10 μM).
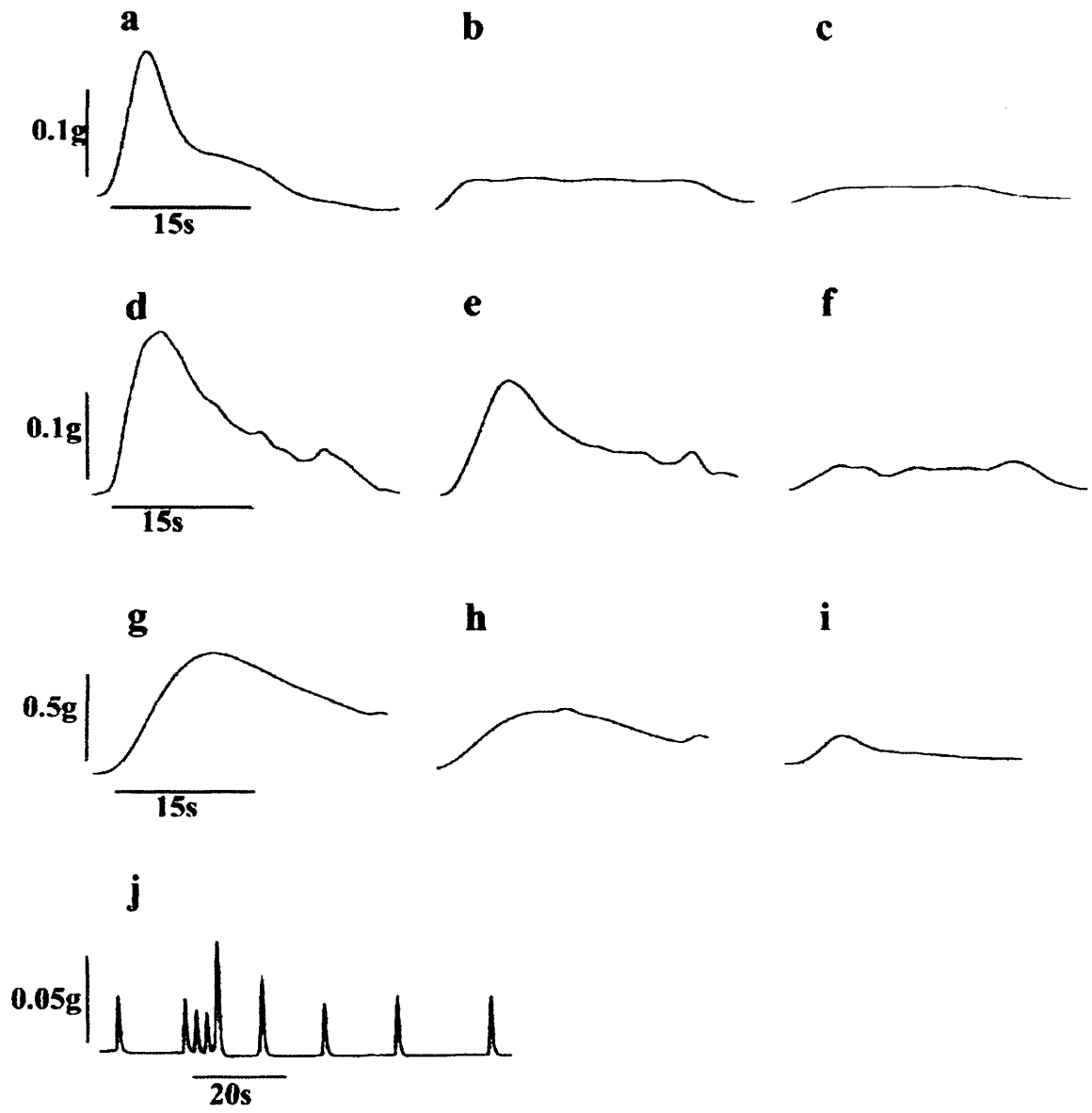
Figure 4.1

Figure 4.2

Frequency-response curve showing control contraction (■), then in the presence of PPADS (30 μM) (□), or prazosin (1 μM) (○), and then both PPADS (30 μM) and prazosin (1 μM) (∇). The contractions have been corrected for the TTX insensitive component, (n=6, all symbols show % max contraction ± s.e.m).

Figure 4.2
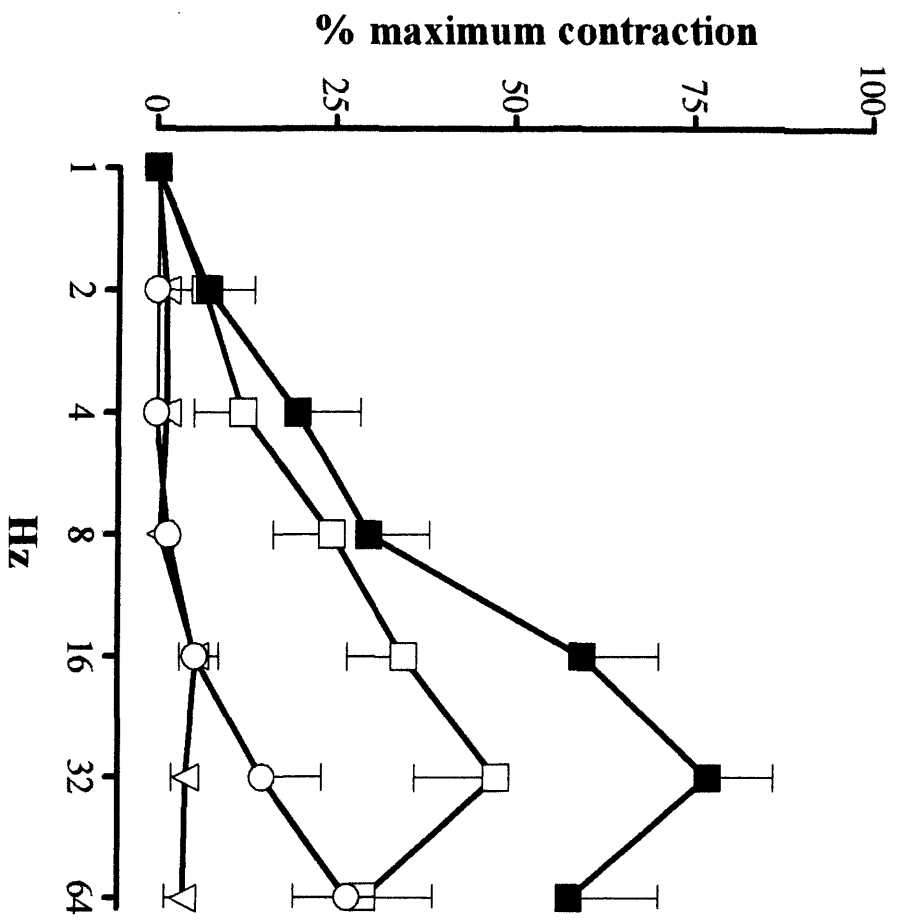
Figure 4.3

(a) Concentration-response curve to NA ($\bullet$),(0.05 μM-300 μM) (n=14).EC_{50} value =11.8 μM, (95 % CI = 9.5-14.6μM).

(b) Concentration-response curve to α,β-meATP (0.1 μM-300 μM)(∇), and ATP (0.1 μM-1 mM) ($\square$) (n=10). α,β-meATP EC_{50} value = 9.2 μM, (95 % CI =5.6-15.1 μM). EC_{50} not calculated for ATP as no maximum reached and range not shown.

All symbols show mean % max KCl contraction $\pm$ s.e.m., dotted line (---) indicates 95 % CI of individual values. The bold line indicates non-linear regression curve where a maximum contraction was obtained.

Figure 4.3
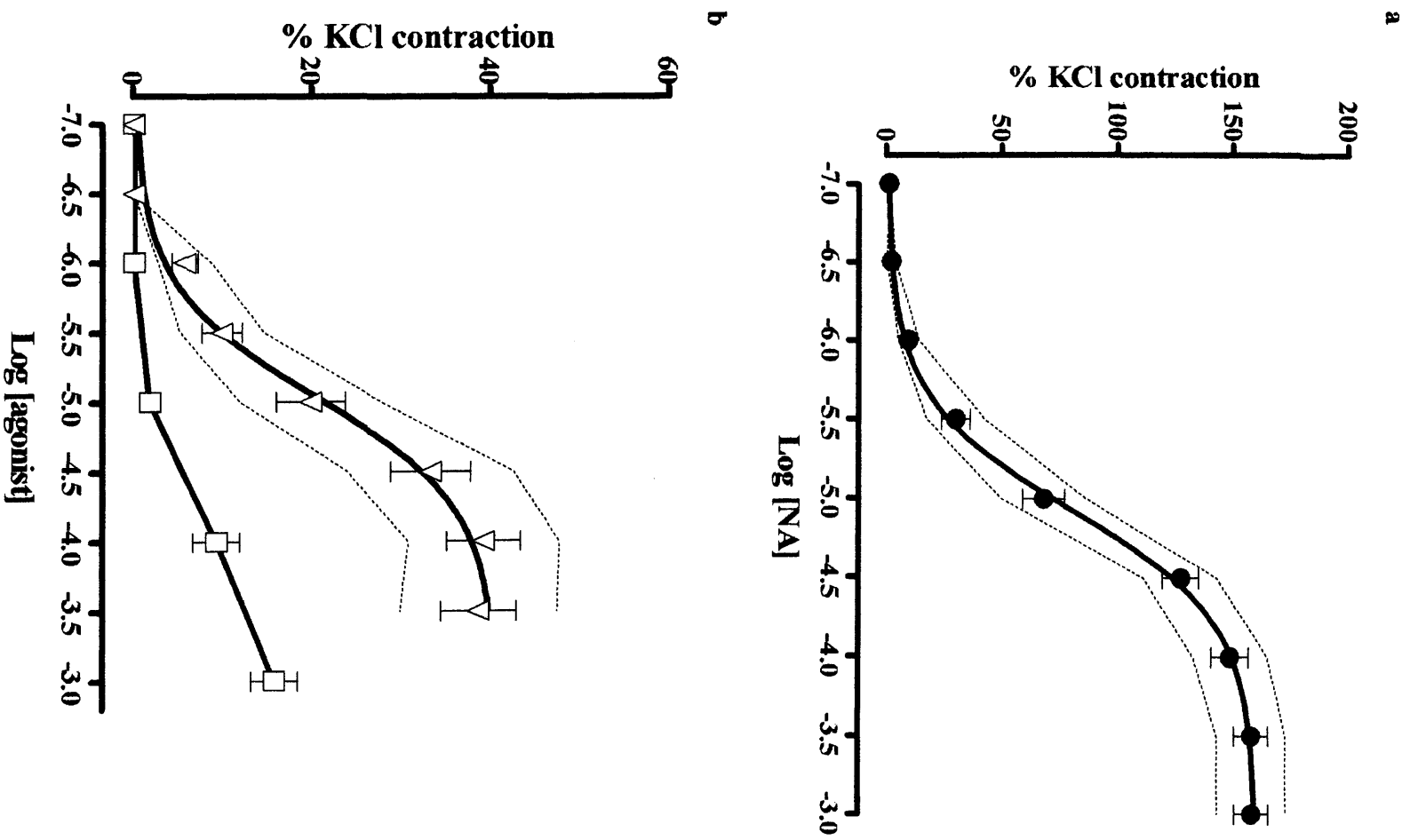
Figure 4.4

Photomicrographs demonstrating P2X$_1$ and P2X$_2$ receptor immunolocalisation on smooth muscle of human vas deferens.

(a) Photomicrograph demonstrating P2X$_1$ receptor localisation on outer longitudinal (OL) and middle circular (MC) smooth muscle layers. P2X$_1$ receptor expression was not observed on the inner longitudinal (IL) layer. The lumen is denoted by L. Scale bar 250 μm.

(b) Photomicrograph demonstrating P2X$_1$ receptor localisation on smooth muscle membranes. Scale bar 50 μm

(c) Photomicrograph demonstrating diffuse P2X$_2$ receptor expression on smooth muscle of the outer longitudinal and middle circular smooth muscle layers. Scale bar 125 μm.

(d) Photomicrograph demonstrating P2X$_2$ receptor expression in the cytoplasm of smooth muscle. Adapted from immunofluorescence, scale bar 25 μm.

Figure 4.4
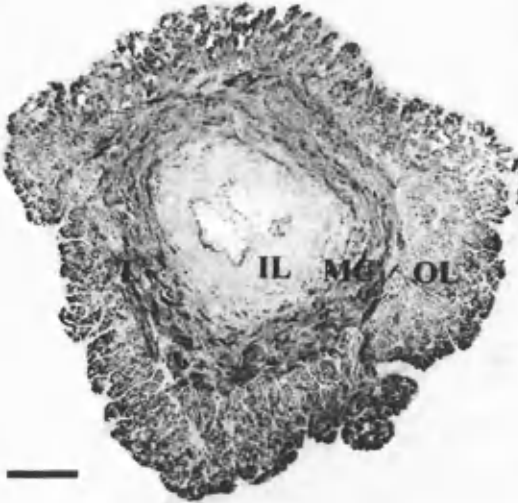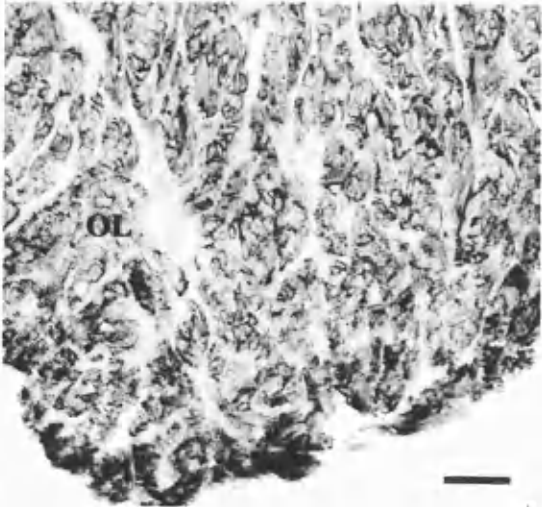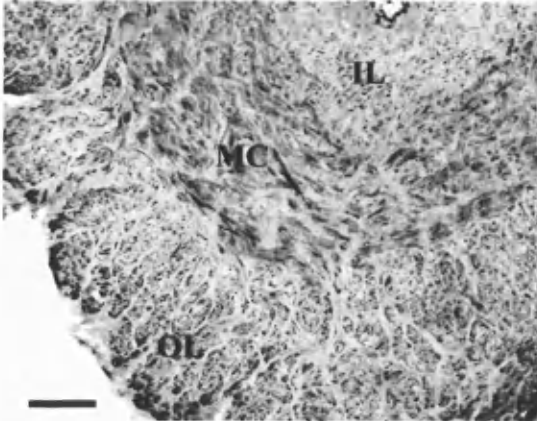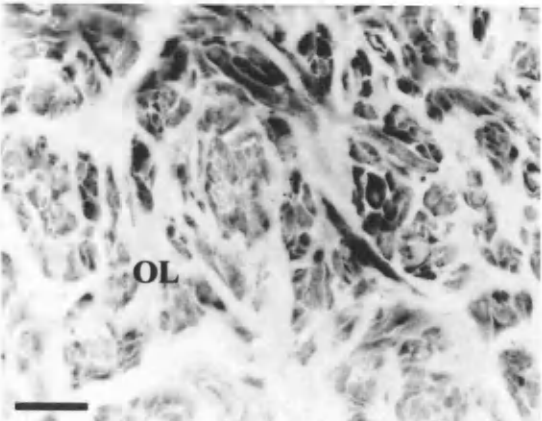
4.5 Discussion

The contraction of vas deferens from mammals such as the rat, rabbit and guinea pig in response to sympathetic nerve stimulation has been shown to be bi-phasic (Sneddon and Westfall, 1983; Meldrum and Burnstock, 1983; Sneddon et al., 1984; Fedan et al., 1991). In contrast, the human vas deferens did not contract to sympathetic nerve stimulation in a biphasic manner, but rather in a slower monophasic manner. At higher frequencies, supra-threshold rhythmic contractions were super-imposed. This pattern of contraction was also observed when the vas deferens contracted to higher concentrations of NA.

Maximal, nerve-mediated contractions, in the presence of PPADS were reduced by 40%. This suggested that the purinergic component of neurogenically induced vas deferens smooth muscle contraction at most amounts to approximately 40%. The purinergic antagonism was not uniform and was much greater at the higher frequencies. There was a corresponding reduction in adrenergic antagonism at the higher frequencies. This would suggest a differential in the amount of each neurotransmitter co-released from sympathetic nerves depending on the extent of stimulation. The purinergic component was most evident at maximal stimulation. We found that prazosin inhibited the contraction by over 80%, which confirmed that the adrenergic component is proportionally dominant, and is mediated through α_1-adrenoreceptors. The relative adrenergic and purinergic components, as assessed by antagonism, amounted to greater than 100%. This implied that synergism between the adrenergic and purinergic components existed. Synergism between ATP and NA in the vas deferens of other animals has previously been shown (Huidbro-Toro and Parada, 1988; Kishi et al., 1990; Witt et al., 1991).

The $P2X_1$ receptor has been shown to be present on the vas deferens smooth muscle membrane in the mouse and rat by immunohistochemistry (Lee et al., 2000a; Mulyran et al., 2000). α,β-meATP is specific for the $P2X_1$ and $P2X_3$ receptor subtypes (Khakh et al., 2001). The $P2X_3$ receptor has been principally demonstrated on sensory nerves, and is thought to be involved in nociception rather than smooth muscle contraction (Burnstock, 2000). The human vas deferens contracted to α,β-meATP in a concentration-dependent manner; which would suggest that the functional receptor on smooth muscle in human vas deferens is also the $P2X_1$ subtype.

The pharmacological evidence was further supported by the immunohistochemical demonstration of $P2X_1$, but not $P2X_3$ receptor expression on the outer longitudinal, and middle circular layers of smooth muscle. Expression was greatest on the outermost smooth muscle. $P2X_2$ receptor expression was also demonstrated in the outer longitudinal, and middle circular layers of smooth muscle cells of the human vas deferens. This distribution was similar to that of the $P2X_1$ receptors. Previous investigators have demonstrated a similar pattern of expression in the rat vas deferens (Burton et al., 2000) although one study reports $P2X_1$ receptor expression of the inner longitudinal muscle of the rat vas deferens (Lee et al., 2000a). $P2X_3$ receptor expression was not observed on the smooth muscle cells of the vas deferens of mice and rats. In the human vas deferens, sympathetic nerves run in the adventia and then spread into the muscularis forming an intramural network. A sub-mucosal plexus staining for acetylcholinesterase has also been demonstrated (Dixon et al., 1998). It has been shown that in the human vas deferens, the ratio of nerve varicosities to smooth muscle is of the order of 1:30, whereas in the rat the ratio is as much as 1:1 (McConnell et al., 1982). A previous study demonstrated that large clusters of $P2X_1$ receptors are present on smooth muscle opposite varicosities (Hansen

et al., 1998) whereas small clusters are not localised near varicosities. On this basis, in human vas deferens one would expect fewer clusters of P2X$_1$ receptors than in the rat, which may explain the relative paucity of staining in the human vas deferens compared to the rat vas deferens. It would also explain the relatively greater P2X$_1$ receptor expression on the outer smooth muscle layer, as the nerve density is greater in the outer smooth muscle layer. The purinergic component of vas deferens contraction from the rat, rabbit and guinea pig is greater at the prostatic end of the vas deferens compared to the epididymal end (Sneddon and Machaly, 1992). If the human vas deferens is comparable, then greater expression would be expected in more proximal sections of vas deferens. In this study it was not possible to collect proximal vas deferens specimens to examine the possibility.

At present, it is unclear exactly when the vas deferens of either laboratory animals or man contracts in physiological conditions. It is certainly thought to contract during ejaculation, but objective evidence for the timing and frequency of contraction is lacking. The tubular structure and three-layered smooth muscle arrangement of the vas deferens suggest that it contracts in a peristaltic fashion, however, objective evidence for this is lacking. Kimura demonstrated in dogs, that following hypogastric nerve stimulation, the epididymal section of vas deferens contracted, followed by the prostatic section 5-10 s later (Kimura et al., 1975). Radiographic studies in rabbits have demonstrated that dye injected at the vas-epididymal junction is moved both towards the urethra, and back into the epididymis during sexual rest. Sexual stimulation rapidly moved dye towards the urethra for ejaculation, but residual dye was retrogradely transported back to the caudal epididymis (Prins and Zaneveld, 1980). In our study, both spontaneous and neurogenic-induced contractions of the human vas deferens were demonstrated.

Autonomic-induced contractions were far more forceful and are probably responsible for the main propulsion of spermatozoa through the vas deferens, presumably following sexual arousal. Spontaneous contractions may be responsible for the slow antegrade and retrograde movement of sperm seen in sexual rest, assuming that sperm movement in man mirrors that of the rabbit. In the majority of preparations, spontaneous contractile activity was observed at a mean frequency of 100 per hour. In keeping with previous observations spontaneous activity was only observed following initial stimulation (Smith and Bray, 1990). Consistent with other investigators, we found a small part of the evoked response of the human vas deferens to be TTX-insensitive (Smith and Bray, 199; Hedlund et al ., 1985). TTX reduced the frequency and amplitude of these spontaneous contractions, but did not abolish them completely. These observations would suggest that inherent myogenic contractions occur in the vas deferens, particularly following stimulation. Exogenously applied ATP potentiated both the frequency, and the amplitude of spontaneous contractions. Studies have claimed dense expression of $P2X_2$ receptors on the lamina propria of the mouse, rat and guinea pig vas deferens (Burton et al., 2000). However, the role of the $P2X_2$ receptor has yet to be elucidated. Evidence is emerging for a possible action on the pacemaker cells of the myenteric plexus of the intestine where expression is on the interstitial cells of Cajal (Burnstock and Lavin, 2002). Whilst $P2X_2$ receptor expression on the lamina propria was not observed in our preparations, some diffuse expression on smooth muscle was noted. If the $P2X_2$ receptor does have a pacemaker effect, then it may be that the enhancement observed with ATP is due to stimulation of the $P2X_2$ receptor. The observation of spontaneous contractions in the human vas deferens would suggest 'background' antegrade or retrograde spermatozoan

movement, supplemented by relatively large volume movement due to forceful vas deferens contractions, induced by autonomic stimulation secondary to sexual arousal.

This study has demonstrated that the human vas deferens smooth muscle is contractile not only to adrenergic agonists, but also purinergic agonists. The adrenergic system has been shown to be functionally dominant, however purinergic co-transmission has also been shown to be functionally important in the contraction of human vas deferens in the in vitro-setting. $P2X_1$ and $P2X_2$ receptor expression has been demonstrated on human vas deferens smooth muscle. These physiological and immunohistochemical studies have suggested that the functional receptor in smooth muscle contraction is the $P2X_1$ subtype. These findings are in keeping with other species examined. ATP has been shown to modify spontaneous contractions, which suggests the regulation of background contractions by purinergic mechanisms. It is a possibility that by analogy with the human bladder that the ATP component of autonomic contraction is altered in pathological conditions (Palea et al., 1983; O'Reilly et al., 2002). The possibility of $P2X_1$ antagonists having a contraceptive role remains an exciting future development. Alternatively adrenergic and purinergic stimulation may be indicated in thetreatment of idiopathic oligospermia as suggested by 2 small case series, in which ephedrine or pseudoephedrine was used to stimulate aperistaltic vas deferens (Tiffany and Goldstein, 1985; Tillem and Mellinger, 1999).

CHAPTER 5

CHANGING P2X RECEPTOR EXPRESSION ON

MATURING SPERM IN THE EPIDIDYMIDES OF

MICE, HAMSTERS, RATS AND MAN

5.1 Abstract

Sperm passing from the testis into the caput epididymidis are unable to penetrate an oocyte and are immotile. These features develop as spermatozoa pass along the length of the epididymis. ATP is increasingly being shown to have trophic effects through the activation of P2 receptors. A number of P2X receptor subtypes have been demonstrated on developing spermatids in the rat testis, with changing expression pending on the stage of development. In the present study, $P2X_{1,2 \text{ and } 3}$ receptor expression was immunohistochemically demonstrated on the head of immature sperm in the human, mouse, hamster and rat caput epididymidis. $P2X_4$ receptor expression was also observed on the head of sperm in the caput epididymidis of mice, hamsters and man, but not rat. There was a subsequent loss of receptor expression on sperm in the cauda epididymidis, except in man where expression of $P2X_4$ receptors persisted. Comparision with PNA binding studies suggested the P2X receptors were located on the acrosome membrane. The change in expression of receptor subtypes is coincidental with the functionally essential morphological and maturational changes seen in sperm as they travel through the epididymis, and is suggestive of a role for purinergic signalling in sperm maturation.

5.2 Introduction

The daily sperm production from the human testes is estimated to be between 45 and 207 million sperm per day (Freund, 1962; Amann and Howard, 1980). Sperm leaving the testis are immature, and lack both forward motility and zona pellucida binding capability (Consentino and Cockett, 1986). These functions develop during the passage through the epididymis, following the interaction of sperm with the

epididymal microenviroment and epithelium (Jones, 1998; 1999). The epididymal epithelium progressively changes along the length of the epididymis, and the epididymal microenviroment is extremely complex, containing more than one hundred proteins and ions (Syntin et al., 1996; Dacheux et al., 2003). The epididymis is common to most animals, and is macroscopically divided into 3 regions, the caput, corpus and cauda, with each section being microscopically distinct (Chandler et al., 1981; Jones, 1999). The epididymis length is variable being approximately 4 metres in man (Turner, 1978), and in excess of 40 metres in bovines (Hoskins et al., 1978). The epididymal transit time mirrors this length variation, being between 2 and 16 days depending on species (Amann et al., 1976; 1980).

P2X receptors are increasingly being found to have novel trophic and apoptotic actions within tissues, as well their established role in purinergic co-transmission and pain initiation (Burnstock and Knight, 2004). Sperm synthesise their own ATP, and although seminal fluid and sperm have a high ATP content, there is no correlation with fertility (Jeulin and Soufir, 1992; WHO, 1992; Minelli et al., 1999). Anatomically-dependent P2 receptor expression has been noted on the mouse epididymal epithelium. Immunohistochemistochemistry demonstrated P2X $_{1,2,4,and\ 7}$ receptors on clear cells in the corpus and proximal cauda epididymal epithelium, with only $P2X_2$ receptor expression on the clear cells of the initial segment. RT-PCR also demonstrated $P2X_4$ and $P2Y_1$ receptors in all epididymal segements as well as $P2Y_2$ receptor expression in the caput and corpus epididymal epithelium. Dynamic function of these receptors was confirmed by the demonstration of calcium flux on stimulation with ATP, and it was suggested that these receptors are important for the ionic maintenance of epididymal fluid (Shariatmadari et al., 2003). Epididymal cell culture studies also support ATP-induced changes in the anion and fluid secretion by the

epididymis (Wong, 1988). The complex membranous changes termed the acrosome reaction, which sperm undergo prior to fertilization can be induced by ATP, and such stimulated sperm have higher fertilization rates (Rossato et al., 1999). Previously, $P2X_{2 \text{ and } 3}$ receptor expression has been demonstrated on the acrosome of testicular rat spermatids in developmental stages I to VIII, and $P2X_5$ receptor expression in stages X to XIII (Glass et al., 2001). To date there are no reports of P2X receptor expression on epididymal sperm despite the demonstration of P2X receptors on more immature sperm within the testis, evidence for purinergic signalling within the epididymis, and improved fertility rates with ATP-activated sperm. This study investigated the expression of P2X receptors on maturing sperm within the epididymis of mice, hamsters, rats and man.

5.5 Methods

See Chapter 2

Human epididymides were obtained from patients undergoing orchidectomy for prostate cancer (n=5, age range 67-88). Mouse, rat and hamster epididymides were obtained following sacrifice under Schedule 1 conditions. Epididymides were divided into respective caput and cauda ends. Sections from each end were placed on the same slide to ensure identical conditions for immunohistochemistry. Antibodies to all seven subtypes of P2X receptors were used at concentrations from 0.25-5 µg/ml. Peanut agglutinin antibodies were used for identification of the spermatid acrosome diluted to 10µg/ml.

5.4 Results

P2X$_1$ receptors were expressed on the sperm head from sperm contained in the caput epididymides of mice (Figure 5.1a), hamsters, rats and man. This expression was most pronounced in the most proximal sections of the caput epididymides, and in the rat, expression was only present on sperm contained in the ducti efferenti. In the rodent species, expression reduced progressively through the epididymis and was absent on sperm in the cauda epididymidis. Some persistence of expression was noted in sperm in the human cauda epididymis. The smooth muscle surrounding the distal tubules of the cauda epididymidis in the mouse strongly expressed P2X$_1$ receptors (Figure 5.1b).

P2X$_2$ receptors were demonstrated in a similar pattern to P2X$_1$ receptors, with expression on the sperm head of sperm in the caput epididymidis, but expression was absent on sperm in the cauda epididymidis. Expression was only seen on sperm in the most proximal sections of cauda epididymidis in man and rats (Figure 5.1c,d).

P2X$_3$ receptor expression was demonstrated on sperm heads on the sperm of the caput epididymidis from all species, with subsequent loss of expression on sperm in the cauda epididymidis.

P2X$_4$ receptor expression was observed on sperm from the caput epididymidis in all species, except the rat. In the hamster and mouse, expression was strong in the caput epididymis (Figure 5.1e) and declined throughout the epididymis. Some expression persisted in the proximal cauda epididymis (Figure 5.1f) but was absent on sperm in the most distal cauda epididymis (Figure 5.1g). In man, some P2X$_4$ receptor expression seen on caput epididymidis sperm (Figure 5.2a) persisted on sperm contained in the most distal cauda epididymidis. (Figure 5.2b).

No expression of P2X$_{5,6 \text{ and } 7}$ receptors was observed on the sperm in either the caput or cauda epididymides in any species examined.

The density of human sperm within the epididymal lumen was markedly less than the other species examined and many tubule cross sections did not demonstrate sperm. Significant debris was seen in sections of distal cauda epididymidis of rats and man, consistent with apoptotic sperm. It was therefore important to ensure the integrity of the acrosome. PNA lectin is known to bind to the intact acrosome (Mortimer, 1987), and in this study it confirmed the integrity of the acrosome on sperm in both caput and cauda epididymis (Figure 5.2c). In the symmetrical human and mouse sperm, PNA lectin predominantly bound to the equatorial segment of the acrosome. P2X receptor expression, when used with fluorescence, was found to be identical to that of PNA lectin and studies using consecutive slides confirmed this (Figure 5.2d). In the sickle shaped asymmetrical rat sperm the PNA expression bound to the acrosome in a classical sickle pattern (Figure 5.2e).

5.4 Results

Figure 5.1

Positive expression indicated by black precipitate. Sperm indicated by thin arrows. S=sperm, E=epithelium, SM=smooth muscle. Scale bars = 100 μm

(a) Photomicrograph showing sperm in mouse caput epididymidis expressing $P2X_1$ receptors.

(b) Photomicrograph showing sperm in the mouse cauda epididymidis no longer expressing $P2X_1$ receptors. There is strong expression of $P2X_1$ receptors on the smooth muscle surrounding the distal tubule of the cauda epididymidis.

(c) Photomicrograph showing sperm in the rat caput epididymidis expressing $P2X_2$ receptors.

(d) Photomicrograph showing sperm in the rat cauda epididymidis no longer expressing $P2X_2$ receptors.

(e) Photomicrograph showing sperm in the hamster caput epididymidis expressing $P2X_3$ receptors.

(f) Photomicrograph showing a reduction in expression of $P2X_3$ receptors on sperm in hamster cauda epididymidis.

(g) Photomicrograph showing a loss of expression of $P2X_3$ receptors on sperm in the most distal tubule of the hamster cauda epididymidis.

Figure 5.1
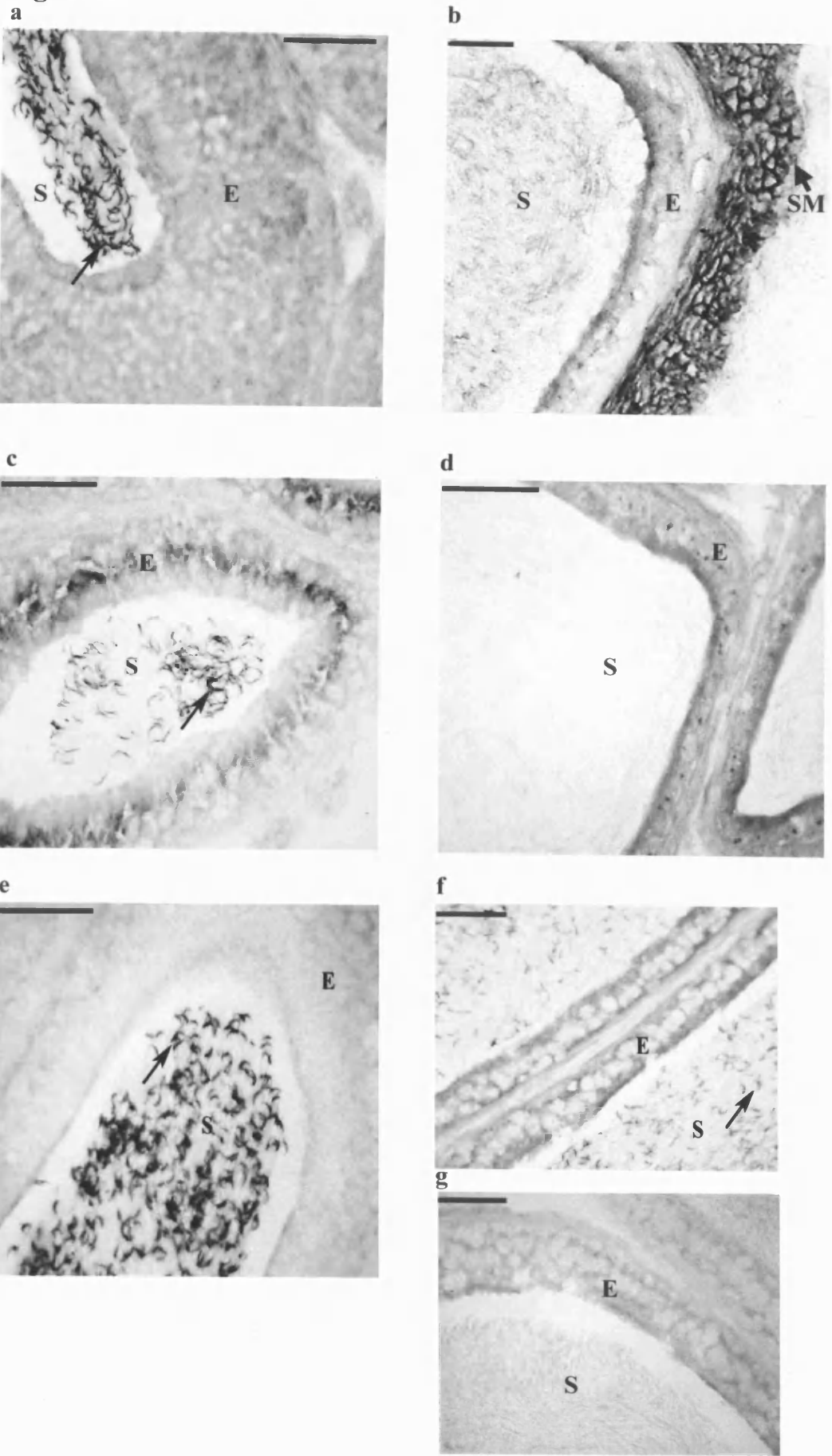
Figure 5.2

Positive expression indicated by black precipitate or bright fluorescent yellow/green.

Sperm indicated by thin arrows. S=sperm, E=epithelium. Scale bars = 50 μm.

(a) Photomicrograph showing sperm in human caput epididymidis expressing P2X$_4$ receptors.

(b) Photomicrograph showing persistent expression of P2X$_4$ receptors on sperm in the human cauda epididymidis.

(c) Photomicrograph showing fluorescent PNA binding to the symmetrical acrosome of human sperm in caput epididymidis.

(d) Photomicrograph showing fluorescent P2X$_1$ receptor expression on the symmetrical acrosome of human sperm in caput epididymidis.

(e) Photomicrograph showing fluorescent PNA binding to the asymmetrical acrosome of rat sperm in caput epididymidis.

Figure 5.2
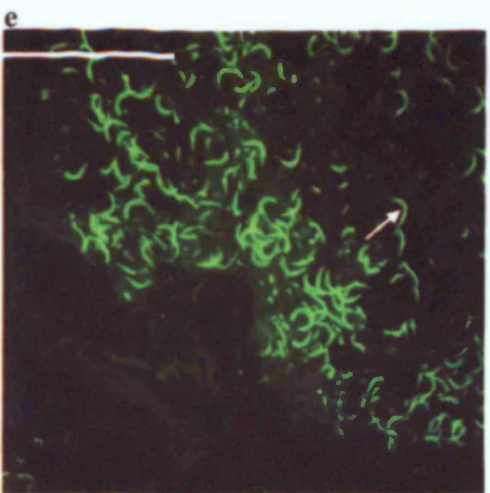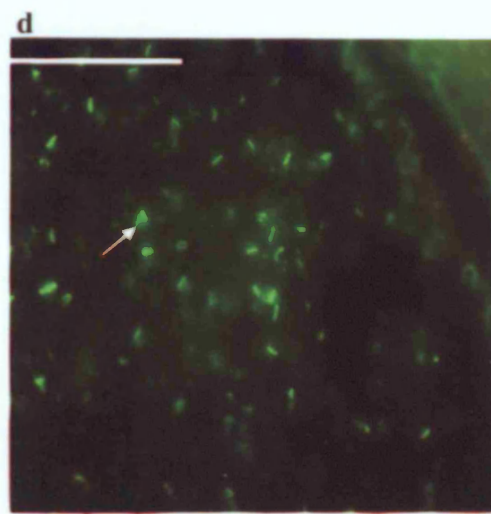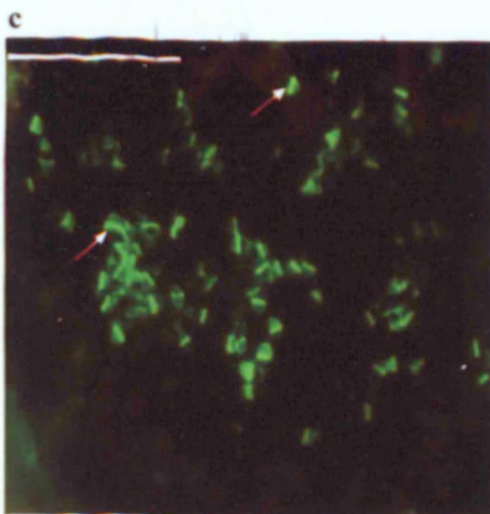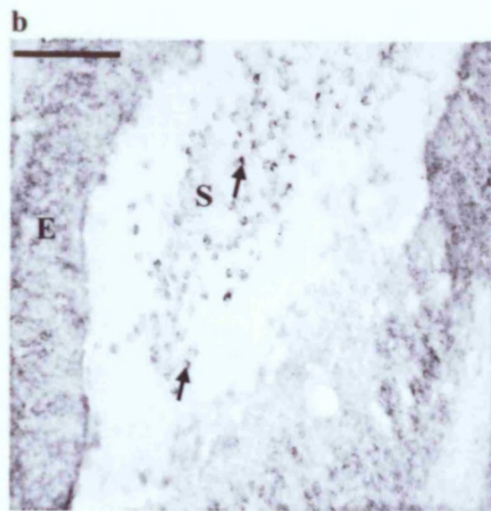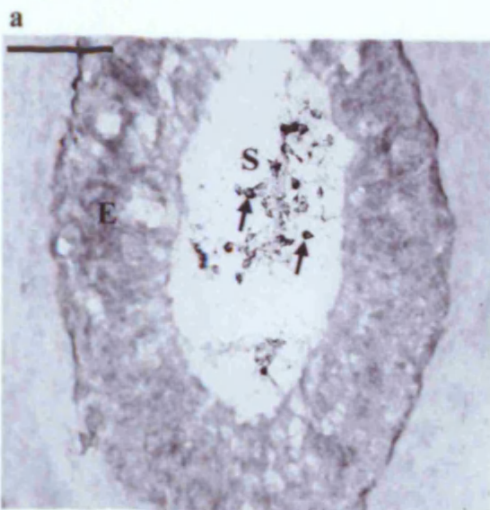
5.5 Discussion

This study has demonstrated, for the first time, that $P2X_{1,2 \text{ and } 3}$ receptors are expressed on the head of immature sperm contained within the caput epididymidis of mice, rats, hamsters and man. There is a subsequent loss or reduction of $P2X_{1,2 \text{ and } 3}$ receptor expression on mature sperm in the cauda epididymidis. There was persistence of $P2X_4$ receptor expression on sperm in the cauda epididymidis of man and mice. The expression is probably on the acrosome, as the immunohistochemical pattern was similar to that of PNA which is known to bind to the intact acrosome. The developing rat spermatid, within the testis, has been shown to differentially express P2X receptors. $P2X_2$ and $P2X_3$ receptors were demonstrated together on developing spermatids in stages I to VIII and were thought to be on the developing acrosome. $P2X_5$ receptors were demonstrated in stages X to XIII in rat testes (Glass et al., 2001). The observed P2X receptor expression and subsequent loss, is coincidental to the key maturational stage of the epididymis. Anatomically, the proximal epididymidis has a distinct epithelium with a high epithelial cell height supportive of a nutrient or absorptive function, which contrasts with the low epithelium seen in the cauda epididymidis, which appears structurally more appropriate for storage of mature sperm (Chandler et al., 1981). Estimates of epididymal sperm storage suggest that 52% of epididymal sperm are stored in the cauda, 23% in the corpus and 25% in the caput, which appears consistent between species (Jones, 1999).

In the normal human epididymidis, it was found that only 22.9% of sperm from the distal caput were motile in comparison to 68.3% from the mid to distal corpus with a slight reduction in the cauda (Yeung et al., 1993). Human micro-canulation studies suggest that the development of motility appears suddenly at the junction of the distal caput and proximal corpus epididymidis (Dacheux et al., 1992).

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Similarly it has been demonstrated that the ability of human epididymal sperm to bind to zona-free hamster oocytes increased with successive epididymal segments, and that only sperm from the cauda epididymidis were able to penetrate the oocyte (Hinrichsen et al., 1980). Human studies involving fertility rates following epididymo-vasostomy, suggest that only passage through the caput epididymidis is required for fertility, although fertility rates increased with more distal anastomoses (Schoysman, 1986).

During epididymal sperm maturation, the plasma membrane undergoes remodelling by the uptake of secreted glycoproteins, removal and utilization of phospholipids from the inner leaflet of the bilayer, and processing of existent glycoproteins (Jones, 1998). Over 200 proteins have been identified in epididymal fluid, many of which are specific to different regions of the epididymis (Dacheux et al., 1998). The epithelial-sperm signalling and ionic changes that initiate and control these complex events are far from understood. The presence of P2X receptors on immature sperm, but not mature sperm may indicate a role for purinergic signalling in sperm maturation. Ionic changes induced by stimulation of P2 receptors may be important in either maintaining epididymal fluid composition, or facilitating sperm membrane changes. This is supported by human studies on ejaculated sperm that have shown ATP rapidly induces the acrosome reaction, and that the mode of action is through the action of Na^+ channels and is Ca^{2+} independent. Functional in vitro fertilisation studies using sperm from patients with male factor infertility, demonstrated a significantly higher fertilisation rate, when the acrosome reaction was induced by exogenous ATP (Foresta et al., 1992; 1996; Rossato et al., 1999).

Seminal fluid has a relatively high ATP content, which has generally been viewed as an energy substrate (Singer et al., 1983) and although reduced seminal ATP levels are implicated in infertility (Irvine et al., 1985), investigative studies have failed to

find a correlation between seminal ATP and infertility (Mieusset et al., 1989; Vigue et al., 1992; WHO, 1992). Sperm are known to have high mitochondrial content based around the midpiece of the spermatozoa and synthesise their own ATP with the result that seminal fluid ATP levels remain very constant in the presence of substrates for ATP synthesis (Jeulin and Soufir, 1992; Minelli et al., 1999). The ATP content of epididymal fluid has not yet been confirmed, but possible sources of ATP include ATP released from viable sperm, or ATP released from apoptotic sperm. Further possibilities include release from the epididymal epithelium, as ATP and adenosine have been shown to be released from Sertoli cells as well as germinative and myoid peritubular cells of the seminiferous tubules (Gelain et al., 2003). Neurogenic release may be a further source of ATP as it is known to be co-released with NA and ACh in the stimulation of smooth muscle contraction of the genital tract (Burnstock, 2001; Banks et al., 2005). Previous studies have demonstrated the importance of neurogenic innervation to the epididymal fluid composition (Ricker, 1996).

The loss of receptor expression in the distal epididymidis was clearly evident, and may imply that the receptor is altered, possibly following stimulation, resulting in loss of expression, which,may be part of a negative feedback loop. Alternatively the expression may be masked by a proteinacous substance present in distal epididymal fluid. Several studies have demonstrated the presence of proteins specific to regions of the epididymis (Young et al., 1985; Jones 1987; Gatti et al., 2004), which may have an inhibitory role (Turner and Giles, 1982). The persistence of $P2X_4$ in the distal cauda epididymidis would suggest that masking is not universal or receptor alteration is more likely.

This study has demonstrated the expression of several P2X receptors on the heads of immature epididymal sperm in 4 different species, which shows a high degree of

species similarity. This expression appears to be lost on more mature sperm in the distal epididymidis although this is incomplete for P2X$_4$ receptors in man. This may suggest that ATP has an extracellular signalling role in the complex process of sperm maturation.

CHAPTER 6

ALTERATIONS IN PURINERGIC AND CHOLINERGIC COMPONENTS OF CONTRACTILE RESPONSES OF ISOLATED DETRUSOR CONTRACTION IN A RAT MODEL OF PARTIAL BLADDER OUTFLOW OBSTRUCTION

6.1 Abstract

Following 3 weeks of partial bladder outlet obstruction, the rat bladders became significantly hypertrophied and doubled in weight. Spontaneous activity was markedly increased, but the contractile response to a single bolus of potassium chloride (120 mM) was unaltered. The neurogenic-induced contractile responses of strips of detrusor from obstructed bladders were significantly greater than those of sham-operated bladders, with the responses of strips of detrusor from obstructed bladders to electrical field stimulation showing a significantly greater atropine-sensitive component compared to sham-operated detrusor. However, the response of detrusor strips to EFS that was susceptible to desensitisation by α,β-meATP, was not significantly changed in obstructed bladders. The sensitivity of the strips of detrusor from obstructed bladders to carbachol, ATP and β,γ-ATP was reduced with respect to sham-operated detrusor. Immunohistochemical studies did not show a difference in the P2X receptor subtypes expressed on detrusor smooth muscle from obstructed and sham-operated rats.

In the rat, following moderate bladder hypertrophy, the atropine sensitive component was significantly up-regulated, but the ATP sensitive component was marginally reduced, although not significantly. In conclusion, these results suggest that up-regulation of the P2X component of bladder contraction seen in humans with symptoms of bladder overactivity, and in other species models of bladder obstruction, is not mirrored in the rat, or occurs later in the pathological process of bladder hypertrophy.

6.2 Introduction

Bladder overactivity represents a massive health and economic problem with symptoms affecting up to 9 million people over 40 years old in the UK (McGrowther, 2004). The condition of bladder overactivity is characterised by involuntary bladder contractions causing pressure rises during bladder filling, which result in a strong and uncontrollable urge to pass urine, which often leads to incontinence (Abrams et al., 1990). The mechanisms underlying bladder overactivity are poorly understood. It is clear that there are marked species differences in the proportions of the cotransmitters ACh, ATP in the parasympathetic nerves supplying the bladder of different species including man (see Burnstock, 2001). In the healthy human bladder, the purinergic component of parasympathetic cotransmission is minor, although P2 receptors are present on the smooth muscle. However, in pathologically overactive bladders, the purinergic component has been shown to account for up to 50% of the contraction (Palea et al., 1993; Bayliss et al., 1999; O'Reilly et al., 2002). A significant increase in the purinergic component of detrusor contraction was also seen in a rabbit model of partial bladder obstruction (Calvert et al., 2001). In a rat model, however, sensitivity to purinergic receptor agonists was shown to be reduced following 10 days of obstruction (Sjuve et al., 1995) and in a long-term rat obstruction model, only the cholinergic element of EFS-induced detrusor contractions was increased (Lluel et al., 2002). Animal models of bladder outlet obstruction must be interpreted with caution as they may represent the response to acute retention and subsequent detrusor failure, as indicated by bladder weight increases of several hundred percent and reduced contractile responses to potassium chloride (KCl). In the present study, the effects of partial bladder outlet obstruction on the cholinergic and purinergic components of contraction of the rat detrusor were investigated.

6.3 Methods

6.3.2 Induction of partial bladder outlet obstruction.

Female Sprague-Dawley rats weighing 173-220g were anaesthetised using a mixture of halothane and oxygen. Under sterile conditions, a lower midline incision was made, the peritoneum was mobilised cranially and the pre-vesical fat was retracted to expose the bladder neck and urethra running on the ventral surface of the vagina. A 1 mm section of proximal urethra was mobilised approximately 2-3 mm from the bladder neck. Care was taken to avoid mobilising the bladder neck to prevent damage to bladder neck nerves. Similarly, care was taken to avoid puncturing the large peri-urethral veins. Following successful mobilisation of the urethra, a jeweller's silver 'jump' ring with an internal diameter of 1 mm was passed around the urethra and closed exactly. The ring remained lose on the urethra and did not appear to cause immediate obstruction. The bladder was then gently squeezed to ensure it was not fully obstructed and that the urethra had not been punctured. The bladder and wound were irrigated with saline to prevent drying and the formation of adhesions. In most situations it was possible to perform the operation without entering the peritoneal cavity. The wound was closed with absorbable suture, local anaesthetic (0.3ml 0.25 % marcaine), and a single dose of analgesic (.28 ml temgesic) was administered. The rats were observed overnight in a designated recovery room, and then returned to normal livery conditions with food and water *ad libitum* the following day. Sham-operated female rats underwent an identical procedure without placement of the ring. Rats were sacrificed under Schedule 1 guidelines at 3 weeks ± 2 days.

6.3.2 In Vitro Pharmacology

See general methods, Chapter 2

Bladders were weighed intact following division at the urethra-bladder neck junction. The bladder was divided immediately above the level of the ureters to exclude the bladder neck. The remainder of the bladder was then pinned and divided into strips measuring approximately 3 x 20 mm. 3 μM indomethacin was added to the all Krebs solutions in order to reduce the spontaneous detrusor activity. An initial load of 1 g was applied to the preparations. Despite allowing the preparations at least 1 hr to stabilise, pilot studies found that initial responses to EFS from partially obstructed bladder preparations were inconsistent, and contractions often increased following the first frequency-response curve, subsequent frequency-responses were more consistent. Therefore control contractions were repeated 3 times to ensure reproducibility. The contractions from the last two frequency-response curves were averaged to give a control contraction.

Non-cumulative concentration response curves were constructed using CCh (0.01 μM-0.3 mM), ATP (0.1 μM-1 mM) and β,γ-meATP (0.1 μM-0.3 mM). A single concentration of KCl (120 mM) was applied at the end of the experiment. The bladder strips were weighed following completion of the experiment.

6.3.3 Immunohistochemistry

See general methods Chapter 2. All P2X receptor antibodies diluted to 2.5-5μg/ml.

6.4 Results

6.4.1 Procedure tolerability / rat growth. Partial bladder obstruction did not adversely affect rat growth as assessed by the mean weight increase over 3 weeks (sham-operated 22.5 ± 3.7, partial bladder obstruction 27.6 ± 3.9 g, P = 0.4). Two rats died approximately 3 days post procedure from urinary retention. In both cases the ring was closed so that it overlapped very slightly. In all further cases the ring was closed exactly. It was important to do this, as even the tiniest gap resulted in failure of the procedure to induce bladder hypertrophy. No other post-operative complication was observed and the procedure appeared to be very well tolerated. It was not necessary to massage bladders in the immediate post-operative period to facilitate voiding as in other studies (Lluel et al., 2002).

6.4.2 Bladder weight. The mean weight of sham operated rat bladders was 70.3 ± 1.3 mg. The partially obstructed rat bladders were significantly heavier at almost exactly double the weight of sham-operated rats at 141.8 ± 14.5 mg (un-paired Student t- test P = 0.0016), (Figure 6.1a). Detrusor strip weight was 11.1 ± 0.7 mg from sham-operated rats and 16.6 ± 0.8 mg from partial bladder obstruction rats.

6.4.3 Response to KCl. There was no significant difference in the response of detrusor strips from sham-operated or obstructed rats to a standard concentration of KCl (120 mM). Sham detrusor 0.182 ± 0.019 g per g of tissue and obstructed bladder 0.179 ± 0.011 g per g of tissue (Figure 6.1b).

6.4.4 Response to Electrical Field Stimulation. EFS induced TTX-sensitive frequency- dependent contractions. Small TTX-insensitive contractions were

observed at the higher frequencies consistent with direct muscle stimulation. All results are adjusted for this. The contractile responses of obstructed detrusor to EFS were significantly greater with respect to sham-operated detrusor. The mean peak contraction at 16 Hz was 80% greater in obstructed rat detrusor, (111.3 +/- 12.8 % and 62.5 +/- 11.7 % contraction of KCl response, obstructed and sham detrusor respectively) (Two way ANOVA P = 0.0015), (Figure 6.2).

6.4.5 Atropine sensitive component of EFS induced contraction. The percentage of control contraction inhibited by atropine (1 µM) was greater for obstructed rat detrusor at all frequencies tested and increased with increasing frequency. Maximal inhibition occurred at 16 Hz and amounted to an 81 % reduction in contraction (range 42-81 %). The percentage of initial contraction inhibited by atropine (1 µM) in sham-operated rats also increased with increasing frequency, but was less, and at 16 Hz amounted to 47 % (range 24-56 %). (Two way ANOVA, P = 0.0013), (Figure 6.2).

6.4.6 Purinergic component of EFS induced contraction (α,β-meATP desensitised component). The percentage of control detrusor contraction inhibited following desensitisation with α,β-meATP (2 x100 µM) was greater in sham-operated rats with respect to obstructed rats. This component was constant in sham-operated rats at a mean inhibition of 73 % (Figure 6.3a). In obstructed rats, the α,β-meATP (2 x100 µM) induced inhibition was more variable and inconsistently increased with frequency. At 0.5 Hz inhibition was 50 % and at 32 Hz was 70 %, mean inhibition was 62 % (Figure 6.3b). The difference in the purinergic components was not significant. (Two way ANOVA, P = 0.16).

The combination of atropine (1 μM) and α,β-meATP (2 x100 μM) caused significantly greater inhibition in partially obstructed rats. In partially obstructed rats virtually no contraction was observed under these circumstances (Figure 6.3b), however in the sham-operated rats some residual contraction was observed at the higher frequencies tested (Figure 6.3a).

6.4.7 Sensitivity to carbachol. Detrusor strips contracted in a concentration-dependent manner to CCh (0.01 μM-0.3 mM). The sensitivity of detrusor strips from obstructed rats to CCh was significantly reduced with respect to sham-operated rats. The EC_{50} was 0.96 μM (95 % CI = .9-1.0 μM) for sham-operated rats and 3.0 μM (95 % CI = 2.5-3.5 μM)for obstructed rats, (Two way ANOVA, P = 0.001), (Figure 6.4a).

6.4.8 Sensitivity to purinergic agonists. Detrusor strips contracted in a concentration dependent manner to both ATP (0.1 μM-1 mM) and β,γ-meATP (0.1 μM-0.3 mM). The sensitivity to the most stable purinergic agonist β,γ-meATP was significantly less in the obstructed rats (Two way ANOVA P = 0.0078), (Figure 6.4b). The responses of detrusor strips to ATP were smaller than those for β,γ-meATP. The sensitivity to ATP was also significantly reduced in the obstructed rats (Two way ANOVA P = 0.0031) (Figure 6.5).

6.4.9 Immunohistochemistry. $P2X_1$ receptor expression was observed on the detrusor smooth muscle membrane from both sham-operated and control rats. There was no discernable difference in the expression (Figure 6.6). Minimal expression of $P2X_2$ receptor was observed within smooth muscle cells from either group. $P2X_3$ receptor expression was observed in a subepithelial position in both groups. No

smooth muscle expression was observed. Some expression of $P2X_4$ receptors was observed on the transitional epithelial cells from both groups. Expression of $P2X_5$ receptors was not observed. Some expression of $P2X_6$ receptors was observed on the basal membrane from both groups. No expression of $P2X_7$ receptors was observed.

6.4 Results

Figure 6.1

(a)   Comparison of bladder weights (mg) from sham-operated controls to partial bladder obstruction rats. Bars represent mean $\pm$ s.e. (sham-operated, n=5, partial bladder obstruction, n=6).

(b)   Comparison of tension generated by KCl (120 mM) on detrusor strips from sham-operated controls (strips=24, n=5) and partial bladder obstruction rats (strips =31, n=6).

Figure 6.1

bladder weight mg

contraction to KCl
g/mg tissue

b

138
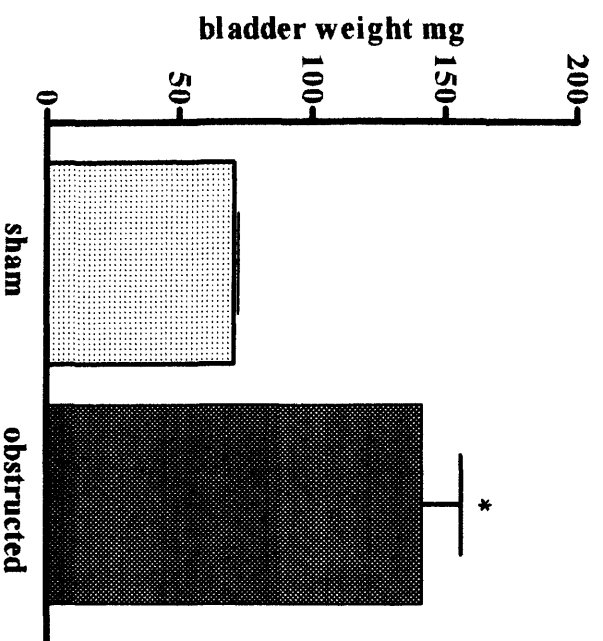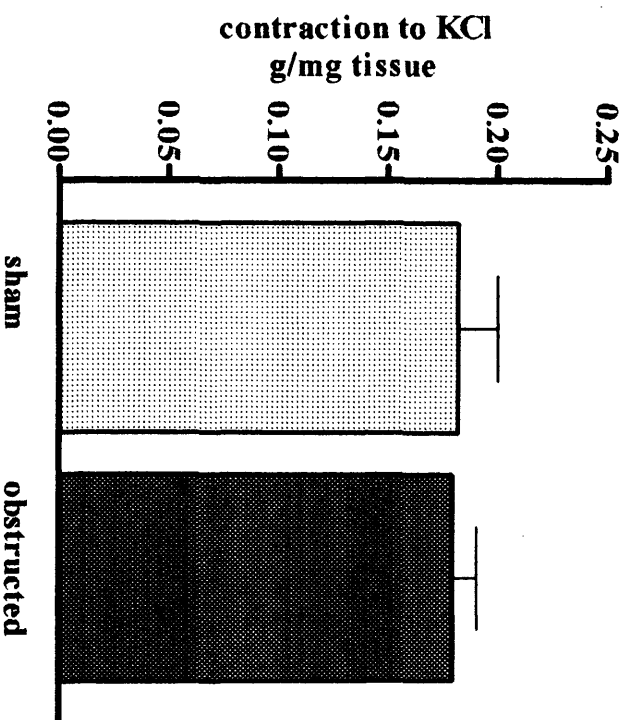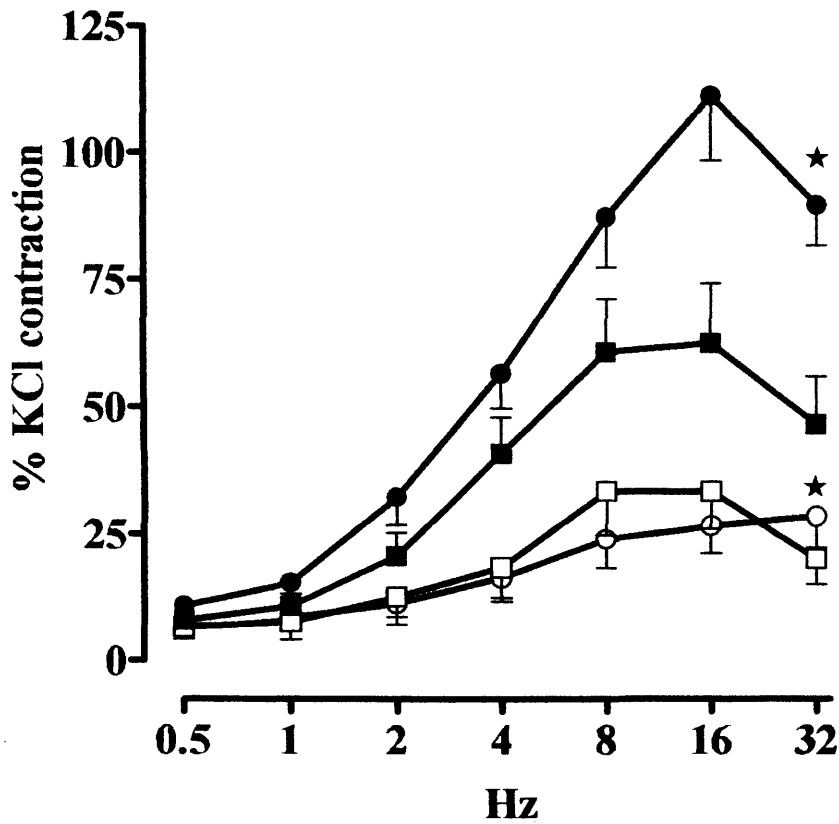
Figure 6.2

Figure 6.2

Frequency-response curve showing contraction to EFS, (100 V, 0.3 ms, 0.5-32 Hz, 20 s) of sham-operated control (■)(n=5) and partial bladder obstruction (●)(n=6) strips of detrusor. Subsequently, in the presence of atropine (1μM), sham-operated (□), partial bladder obstruction (o). All symbols show mean % contraction of the KCl response (120 mM) ± s.e. (unless masked by symbol). All data shown has been adjusted to subtract the TTX insensitive component. ★ indicates significance, (P=<.05).

Figure 6.3

(a)   Sham-operated

(b)   Partial bladder obstruction

Frequency-response curves showing initial contraction to EFS, (100 V, 0.3 ms, 0.5-32 Hz, 20 s) of either sham-operated (a) or partial bladder obstruction (b) strips of detrusor (●). Subsequently following desensitisation with α,β-meATP (■) (sham, n=5), (partial bladder obstruction, n=6), in the presence of atropine ($1\mu M$)(○) or in the presence of both atropine ($1\mu M$) and following desensitisation with α,β-meATP (▲). All symbols show mean % maximum contraction ± s.e. (unless masked by symbol). All data shown has been adjusted to subtract the TTX insensitive component.

Figure 6.3

a. Sham-operated
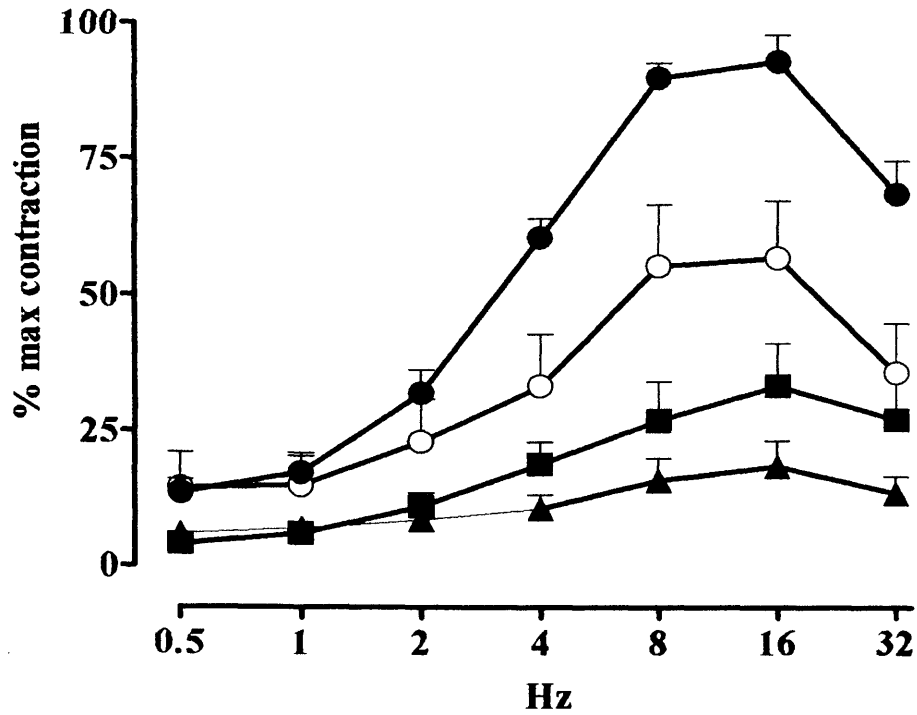
b. Partial bladder obstruction
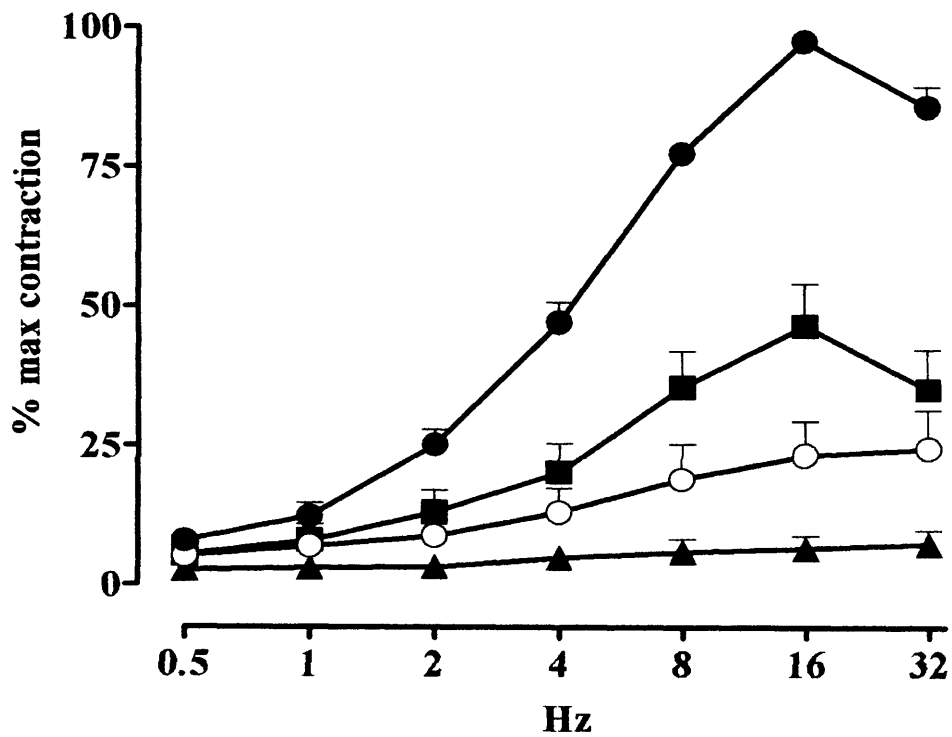
Figure 6.4

(a)   Concentration-response curve for CCh, Sham-operated controls (■, n=5), red

dotted line (---) indicates 95 % CI for Sham-operated control values. Partial

bladder obstruction (●, n=6), green dotted line (---) indicates 95 % CI for

individual values.

(b)   Concentration-response curve for β,γ-meATP. Sham-operated controls

(■, n=5) and partial bladder obstruction (●, n=6).

All symbols show the mean % of the contraction to KCl (120 mmol) ± s.e. (unless

masked by symbol) and non-linear regression curve where concentration-response

curve reached maximal contraction. ★ indicates significance, (P=<.05).

Figure 6.4

a

b

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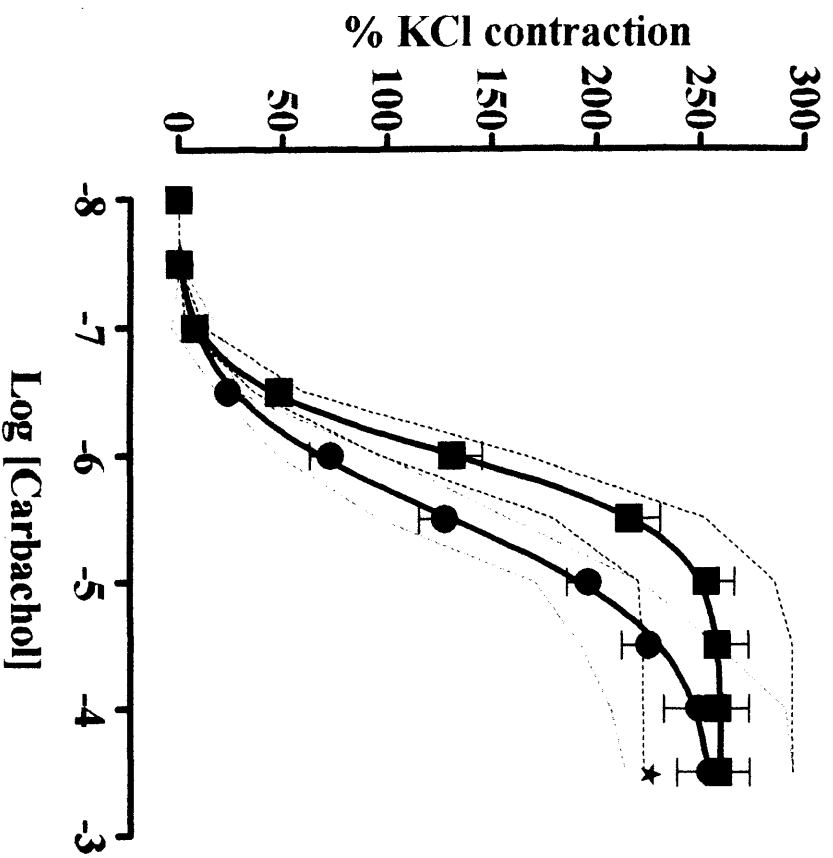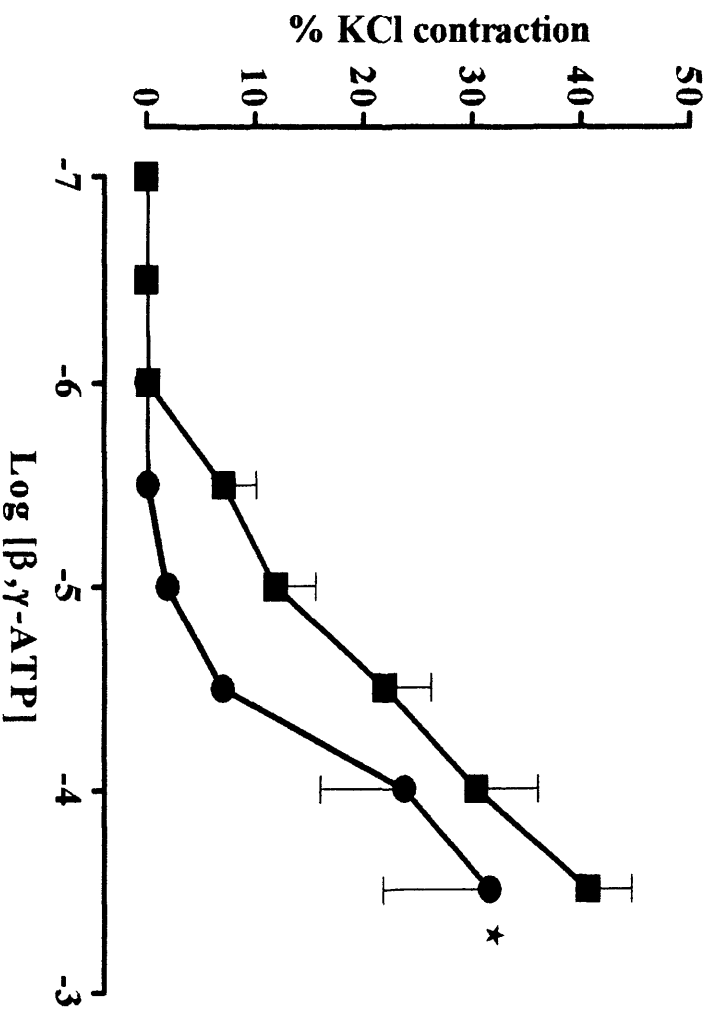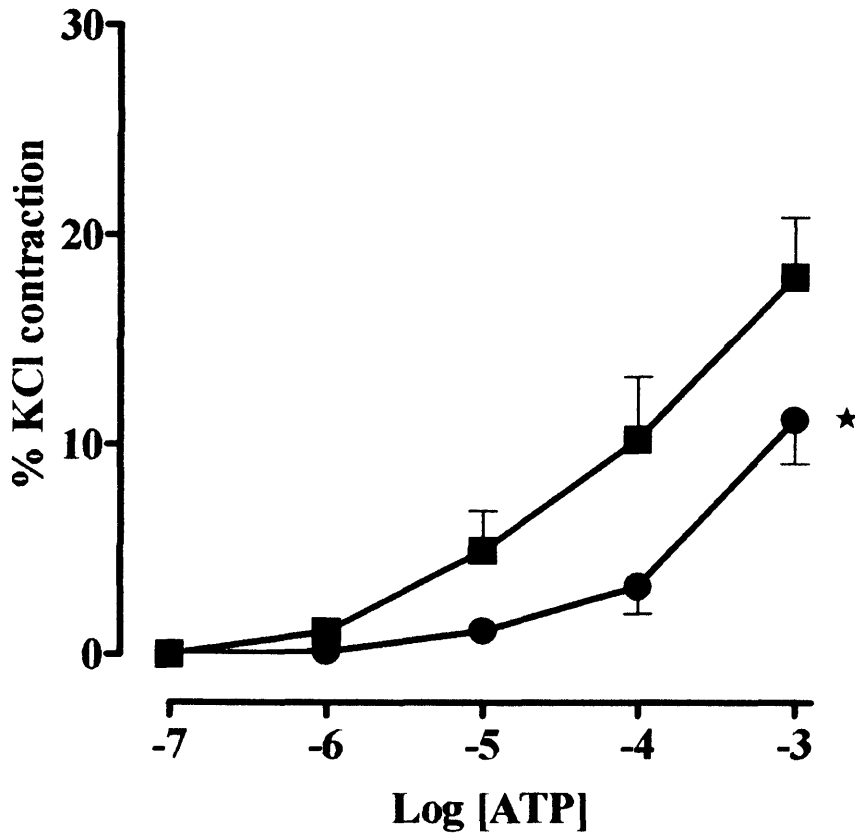
Figure 6.5

Figure 6.5

Concentration-response curve for ATP.

Sham-operated controls (■, n=5) partial bladder obstruction (●, n=6).

All symbols show the mean % of the contraction to KCl (120 mmol) ± s.e. (unless masked by symbol). ★ indicates significance, (P=<.05).

Figure 6.6

All scale bars=50 μM

a.   High power photomicrograph demonstrating $P2X_1$ receptor expression on

membrane of detrusor smooth muscle of sham operated control rats. No

epithelial expression was observed.

b.   High power photomicrograph demonstrating $P2X_1$ receptor expression on

membrane of detrusor smooth muscle of partial bladder obstruction rats.

No epithelial expression was observed.

Figure 6.6
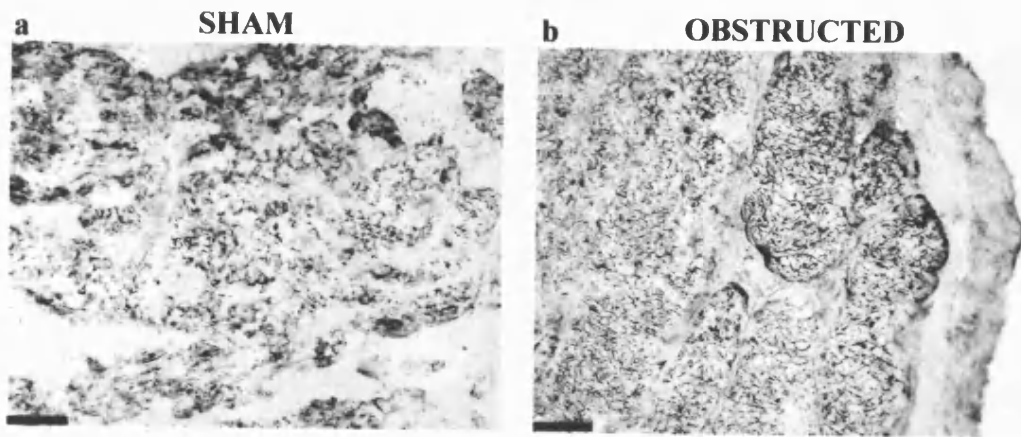
6.5 Discussion

The rat model of partial bladder outflow obstruction has been shown to cause detrusor overactivity (Malmgren et al., 1987; O'Connor et al., 1997). In some studies, very large bladder mass increases and reduced KCl responses have been reported (Lluel et al., 2002). These studies must be interpreted with caution, as they may be more representative of the recovery response of the bladder to acute obstruction. The partial bladder obstruction model in the current study was well tolerated and resulted in a relatively mild increase in bladder mass with evidence of genuine detrusor hypertrophy, increased nerve-mediated contraction and marked spontaneous activity. As a consequence of the hypertrophy the obstructed strip size was slightly heavier but small differences in detrusor strip size have not been shown have a significant effect on tension responses (Longhurst et al., 1995). Macroscopically the bladder detrusor smooth muscle and epithelium were thickened. Hypertrophy was confirmed histochemically. There was no deterioration in smooth muscle contractility as assessed by the contractile response to KCl. In our study the cholinergic component of the nerve-induced contraction accounted for up to 80 % in obstructed bladders, although only 50 % in sham-operated bladders. The purinergic component was reduced, although not significantly. In a longer, 12 week bladder outlet obstruction model in the rat, a significant increase in the cholinergic component and a non-significant increase in the purinergic component were observed, but the contraction to KCl was significantly reduced indicating reduced bladder contractility (Lluel et al., 2002).

Purinergic co-transmission in the contraction of the bladder smooth muscle of many animals is well documented (Burnstock, 2001). In the normal human bladder it is thought to have a minimal contribution to normal physiological contraction (Bayliss

et al., 1999). However, P2X receptors are expressed on normal human detrusor smooth muscle, and contractile function was observed to exogenous ATP (O'Reilly et al., 2002). Human studies are challenging due to difficulty in confirming bladder overactivity, and ensuring that controls represent normal tissue. A number of studies have suggested that the purinergic component of human bladder contraction is substantially up-regulated in the pathological state. The purinergic co-transmission component is increased to account for 40 % of the contraction in interstitial cystitis (Palea et al., 1993), up to 50 % in idiopathic female detrusor overactivity (O'Reilly et al., 2002), and in cases of proven bladder overactivity, significant up-regulation has been demonstrated (Bayliss et al., 1999). It has been shown that the purinergic component of human bladder contraction is increased and the cholinergic component reduced with age (Yoshida et al., 2001). Another study contradicts this, suggesting that the purinergic component, as assessed by antagonism, is 5% in overactive detrusors and 3% in controls, with a significant up-regulation of the myogenic or TTX resistant component in overactive bladder tissue (Mills et al., 2000). The up-regulation of the purinergic component cannot be explained by altered sensitivity, as detrusor myocytes from stable and unstable bladders were equally sensitive to cholinergic and purinergic agonists (Wu et al., 1999). A reduction in ecto-ATPase enzyme activity has been demonstrated from unstable or obstructed human bladders and this may account for the increased potency of ATP in these pathological states (Harvey et al., 2002). Increased expression of the $P2X_2$ receptor has been demonstrated in detrusor overactivity (O'Reilly et al., 2002).

The increase in the cholinergic component of detrusor contractility seen in the present study cannot be explained by denervation supersensitivity, since although the nerve-stimulated response was raised, the response to exogenous CCh was decreased.

Brading (1997) has suggested that a feature of bladder overactivity is denervation supersensitivity, in which there is a reduction in the number of excitatory nerves and a reduction in acetylcholinesterase (AChE), resulting in agonist supersensitivity. In contrast it has been suggested that there is an increase in cholinergic nerve proliferation in bladder outlet obstruction (Steers et al., 1990; 1991). It has been proposed the relative ratio of parasympathetic cotransmitters can change in pathological conditions (Burnstock, 2001). A study into the neuroanatomical changes of hypertrophied rat detrusor demonstrated a modest increase in PGP staining nerves around smooth muscle bundles, but minimal change within the smooth muscle bundles. In contrast nerves expressing AChE within the bundles were reduced, though between bundles were unchanged (Sutherland et al., 1998). In another rat study of mild obstruction, no change in choline acetyltransferase expression was observed, and no dysfunction in responses to EFS (Levin et al., 1993). In the present study, the neurogenic atropine-sensitive component was increased, which would be consistent with a reduction in AChE. However, it does not explain the reduced sensitivity to CCh, although the CCh sensitivity would be independent of AChE due to being non-hydrolysable. The sensitivity to all agonists tested was reduced, which would suggest a common change in contractile mechanism. In previous rat models, the detrusor has been shown to rapidly adapt to partial outflow obstruction, with bladder weight increasing up to 12 fold in 6 weeks, with the fastest growth rate being at 3 days following obstruction (Uvelius et al., 1984a; Sutherland et al., 1998). The growth is mainly accounted for by smooth muscle hypertrophy, with the smooth muscle cross section increasing significantly. The collagen content also increased, but the relative concentration was reduced to 1/3 of control levels. The smooth muscle bundles were preserved, with the collagen increase being mostly between bundles with minimal

collagen infiltration within bundles (Uvelius et al., 1984b; 1991). It therefore seems unlikely that the reduced sensitivity to all exogenous agonists seen in the present study could be explained by tissue fibrosis obstructing agonist permeability. The response to KCl was similar which would suggest that there was no fundamental change in the smooth muscle contractility. Our observations, therefore suggest a change in the expression of receptors or transduction mechanisms.

The rat bladder is known to express several P2X receptor subtypes, with the strong expression of $P2X_1$ receptors on detrusor smooth muscle (Lee et al., 2000). In the present study no difference was observed between normal and obstructed bladder in the expression of P2X receptor subtypes. Subtype expression concurred with previous reports (Lee et al., 2000). In the awake rat, bladder contraction was induced by ATP, and then reduced following pre-treatment with α,β-meATP (Igawa et al., 1994). Similarly in the $P2X_1$ receptor-deficient mouse, despite expression of other subtypes of P2X receptors, no detrusor contractions were recorded to the P2X agonists ATP, α,β-meATP and β,γ-meATP. This was further supported by patch-clamp studies on detrusor smooth muscle demonstrating no effect on the holding current in $P2X_1$ receptor-deficient mice. The bladders of $P2X_1$ receptor-deficient mice were functionally and morphologically normal. It was thought that the cholinergic element of the bladder contraction was sufficient to maintain function (Vial and Evans, 2000). This is in contrast to contraction of the vas deferens from $P2X_1$ receptor-deficient mice, which was found to be markedly reduced, resulting in infertility (Mulryan et al., 2000). The evidence would strongly suggest that the functional P2X receptor involved in smooth muscle contraction in rat, mouse and probably human bladder is the $P2X_1$ subtype. In the rabbit testis where α,β-meATP is less potent than ATP, the dominant P2X receptor subtype may be the $P2X_2$ subtype

(Banks et al., 2006). The function of other P2X subtypes expressed in smooth muscle is less clear, as is the functional role of the suggested heteromultimers $P2X_{1/5}$ and $P2X_{1/2}$. The $P2X_3$ receptor has been shown to have a mechano-sensory role in micturition in mice, and $P2X_3$ receptor null mice demonstrated bladder hyporeflexia (Cockayne et al., 2000; Rong et al., 2002). There was no discernable reduction in the expression of P2X receptors. It is therefore possible that the marginally reduced neurogenic purinergic component may be due to either reduced ATP release or increased ecto-ATPase activity.

This study has shown that the cholinergic component of rat detrusor contraction is enhanced in mild obstruction and the purinergic component is marginally reduced. This is in contrast to the rabbit and human in which the reverse occurs. This study would suggest that the rat detrusor adapts to obstruction in a different manner to that of man, or that purinergic up-regulation is not a feature of early adaptation.

CHAPTER 7

GENERAL DISCUSSION

In this chapter, I have attempted to put the results into a broader context and I have addressed certain points raised in the course of this study. I have discussed the extent of testing of my hypothesis, and have discussed the limitations of the techniques used. I have addressed some of the inevitable questions that arise from this study, and have suggested some further lines of investigation.

The main aspects that I will discuss are, the species conformity in the presence of smooth muscle in the tunica albuginea of the testis, the significance of the direct innervation seen in human tissue and the presence of purinergic signalling in the contraction of this smooth muscle. I will address the significance of the presence of P2X receptors in the contraction of the human tunica albuginea and vas deferens and suggest the possibility of a 'testis-epididymis-vas deferens sperm pump' propelling sperm through the genital tract. I will comment on the possible role of purinergic signalling in sperm maturation, and on the potential of purinergic signalling in male contraception. Finally, I will address the use of a rat model of partial bladder outlet obstruction to replicate the upregulation of purinergic signalling seen in human detrusor overactivity.

In order to examine the role of purinergic signalling in the testicular capsule, it was important to clarify the extent of smooth muscle in the tunica albuginea as there has been little published on this topic since the initial interst in the early 1970s. Electron microscopy confirmed a high level of species similarity in both the appearance of the smooth muscle cells, and their arrangement in sheets. Secretory phenotype smooth muscle cells were observed in the outer part of the tunica albuginea which presumptuously secrete the collagen that give the tunica albuginea its tough

capsule, with contractile phenotype smooth muscle cells on the inner aspect presumably being responsible for functional contraction. This arrangement was present in man and was similar to the other species examined, and may be common to the tunica albuginea of all mammals. The smooth muscle arrangement appeared to be a continuum and did not support the suggestion of a defined inner contractile smooth muscle layer (Middendorf et al., 2002). It was significant to demonstrate the neuronal innervation of human smooth muscle, which has not been shown previously, and was not seen in the other species studied. The demonstration of specialised close junctions between the long processes of the smooth muscle cells would suggest myogenic propagation of neuronal stimuli and the possibility of the tunica albuginea contracting as a syncytium. Central to my hypothesis was the role of purinergic signalling in the genito-urinary tract, and this study demonstrated that contraction of the tunica albuginea to purinergic agonists was common to all species examined. However, other autonomic stimuli were less consistent between species. In man, it is likely that ATP is co-released with NA, and that human tunica albuginea contraction is under sympathetic nervous system control alone. This is in contrast to the mouse in which the contraction is under parasympathetic cholinergic control, with adrenergic stimulation resulting in smooth muscle relaxation. In the rat and rabbit cholinergic, adrenergic and purinergic agonists contracted the tunica albuginea. Purinergic signalling is thought to represent a primitive signalling system (Burnstock, 2001), and the fact that it was common to all the the species examined may support that it was a primitive common signalling pathway that has evolved to be coupled with other transmitter systems depending on reproductive behaviour. Whilst I have demonstrated purinergic signalling in the tunica albuginea it was unfortunately beyond the remit of this study to examine the importance of tunica albuginea contraction in the movement

of sperm out of the testis. Despite the demonstration that testicular capsulotomy causes infertility and the subsequent degeneration of seminiferous tubules (Qin and Lung, 2000a; b), it is still uncertain whether testicular contractions are essential to the movement of sperm out of the testis, and consequently whether such contractions are significantly implicated in infertility.

In contrast, the significance of purinergic signalling to the movement of sperm in the mouse vas deferens has been clearly demonstrated by oligospermic infertility in $P2X_1$ knockout mice, however, the role of purinergic signalling to the contraction of the human vas deferens was essentially unknown. This study has clearly demonstrated the presence of purinergic cotransmission in the contraction of the human vas deferens. The expression of $P2X_1$ and $P2X_2$ receptors on the outer longitudinal and middle circular layers conforms to the arrangement seen in other species; however, it is not clear why there is no expression on the inner longitudinal muscle layer. Previous studies in labaroratory animals have shown differing contractile response profiles to nerve stimulation (Furness and Burnstock, 1969). Comparison of the relative adrenergic and purinergic components of human vas deferens contraction would suggest that the adrenergic component dominates. This may be the reason the human vas deferens contracts to nerve stimulation in a relatively slow monophasic manner, without an initial fast peak due to significant ATP release, as seen in some laboratory animals. This significant difference between man and mouse would suggest that $P2X_1$ antagonism alone would not result in infertility, and that comments suggesting such antagonism would be contraceptive in man may have been premature (Lincoln, 2000; Mulyran et al., 2000).

In the course of background reading for this thesis it was surprising not to find an objective study examining when the smooth muscle of the vas deferens or tunica

albuginea contracts. The three layered tubular structure of the vas deferens implies peristalsis, but this has not been clearly demonstrated, and one study in dogs suggested that sections of vas deferens contract as a whole, rather than in a peristaltic wave (Kimura et al., 1975). In the epididymis there is increasing tubule musculature from proximal to distal which is supportive of peristaltic propulsion of sperm, but objective evidence is lacking. Radiographic studies have demonstrated antegrade and retrograde movement of radio-opaque dye within the vas deferens with storage in the cauda epididymis (Prins and Zaneveld, 1980; Kihara et al., 1995). It is also unclear whether contractions are continuous or occur in response to specific stimuli. It was an interesting observation that rabbit tunica albuginea demonstrated spontaneous contractions, but in man, spontaneous contractions of the tunica albuginea and vas deferens only occurred following prior stimulation. The fact that ejaculates contain sperm, despite sexual abstinence suggests a continuous flux of sperm through the genito-tubular system. This has led me to question whether there is a 'tunica albuginea-epididymis-vas deferens sperm pump', continually contracting to move sperm from the testis, through the epididymis and finally along the vas deferens into the ejaculatory ducts in preparation for ejaculation. It is likely there is antegrade and retrograde movement to prevent senescence of sperm. I would suggest that larger volumes of sperm are moved in response to adrenergic and purinergic induced contraction of smooth muscle, secondary to sympathetic discharge in states of arousal. The demonstration of direct neurogenic innervation in man adds weight to the requirement for sympathetic stimulation for human tunica albuginea contraction, as opposed to a preponderance of background myogenic contraction.

The role of purinergic signalling in smooth muscle spontaneous contraction frequency is an area of general investigation. $P2X_2$ receptors have been demonstrated

on interstitial cells of Cajal in the vas deferens and intestine and it has been suggested that they have a pacemaker function on smooth muscle contraction (Burton et al., 2000; Burnstock and Lavin, 2002). In the present study, ATP increased the rate of spontaneous vas deferens contractions, even when applied at an insufficient concentration to evoke a contraction, which in conjunction with the immunohistochemical demonstration of $P2X_2$ receptors is supportive of a pacemaker role for $P2X_2$ receptors. This role may also be important in the tunica albuginea where immunohistochemical expression of $P2X_2$ receptors appeared stronger than that of $P2X_1$ receptors. Futher evidence for neural control for sperm transport comes from sympathetic denervation studies that demonstrated significantly lower daily sperm counts in the presence of normal testis function (Consentino et al., 1984). Partial sympathetic denervation was also shown to alter rat cauda epididymal fluid protein content, independent of sperm accumulation (Ricker et al., 1996). It was also shown that sperm from sympathetically denervated rat cauda epididymides had normal potential for fertility, but embryo development was inhibited (Ricker, 1997). This would suggest that there may be an optimum maturation period within the epididymis and that prolonged epididymal transit time may be detrimental to sperm as demonstrated by poor sperm quality in spinal injury patients (Brackett et al., 2000; Utida et al., 2005)

An obvious limitation of this thesis is that whilst I have shown the presence of P2X receptors and contraction of genital smooth muscle to purinergic stimuli, I have not demonstrated that such contractions move sperm. A number of essential studies are needed to determine normal sperm transport from the testis to the epididymis and subsequently the vas deferens. The essence of these would be to investigate the presence of peristaltic waves within the genital tract. The simplest would be in vitro

whole testis pressure recordings, which our own pilot studies suggested were possible. Microcannulae and subcutaneously implanted pressure transducers have been successfully used during *in vivo* urodynamic studies. Such techniques could be used to record pressure waves from the different parts of the genital tract. This basic, but essential knowledge is critical to our understanding of sperm transit. Alternatively, radiolabelled studies have the potential to track the continuous movement of sperm. It is possible that such techniques might be useful in diagnosing genital tract smooth muscle dysfunction in man in a clinical setting. The role of purinergic signalling in genital tract dysfunction will be much harder to confirm, as it would be ethically impossible to examine vas deferens from patients with possible vas deferens dysfunction, as it is essential for the possibility of natural conception. It would also be extremely difficult to create an animal model of purinergic vas deferens dysfunction, without denervating the tract, although the $P2X_1$ knockout mouse can be considered such a model. The Mulyran paper sparked interest into the possibility of an, as yet, elusive selective $P2X_1$ receptor antagonist as the male contraceptive pill (Mulyran et al., 2000; Dunn, 2000). Whilst such a treatment may be successful in the veterinary field, the predominance of the adrenergic system in man is likely to limit its therapeutic usefulness, and it would need to be in combination with adrenergic antagonism to be effective. A $P2X_1$ or $P2X_2$ receptor stimulant preferably in combination with an adrenergic stimulant would potentially increase sperm flux through the genital tract and would be potentially useful in the treatment of oligospermia. Such a treatment might compensate for reduced spermatogenesis. Two case series suggest a successful outcome of treatment with sympathomimetic agents in vas deferens aperistalsis, although the accuracy of such a diagnosis must be questioned (Tiffany and Goldstein, 1985; Tillem and Mellinger, 1999). A simple

starting point would be to see if semen parameters, particularly total numbers and concentration, in normal subjects could be improved by freely available sympathomimetics such as pseudoephedrine (Sudafed®). Such a trial would lend itself to a placebo-controlled, double-blind crossover study.

The positive expression of P2X receptors on sperm contained within the proximal epididymis and the subsequent loss of expression in the distal epididymis is suggestive of a trophic role for ATP in sperm maturation. Although expression varied, it was demonstrated in four different species including man, in a very similar pattern, which may imply a common phenomenon. Human studies would benefit from examining epididymides from younger patients. The clinical importance of this expression remains to be elucidated. Functional studies would be extremely difficult to undertake due to the lack of understanding of the epididymal milieu and sperm interaction with it. Rossato, in her study demonstrated higher fertility rates with ejaculated sperm incubated with ATP, which was suggestive of a clinically important function for P2X receptors (Rossato et al., 1999). The development of several P2X receptor subtype null mice gives an insight into the functional significance of receptor subtypes. The $P2X_1$ receptor null mouse was infertile due vas deferens aperistalsis, but sperm were fertile in an *in vitro* setting (Mulyran et al., 2000). There are no reports of infertility associated with the other knockout mice studies, $P2X_2$, $P2X_{2/3}$, however, full reproductive details of these animals have not been reported (Vlaskovska et al., 2001; Coutinho-Silva et al., 2005). On the evidence available, it would seem that the expression of P2X receptors during spermiogenesis and maturation is not critical for fertilisation. Fertilisation depends on adequate communication between a mature and competent sperm and a similar female oocyte. The acrosome of sperm is a mosaic of glycoprotein domains and the fluid nature of

the membrane means that there is the potential for extremely rapid movement and incorporation of glycoproteins and molecules. Ion channels are instrumental in the dialogue between a sperm, its environment, and an egg. The ability of sperm to swim to, and fertilise an egg, is modulated by ion permeability changes induced by environmental stimuli in both male and female genital tracts, together with components of the outer layer of the egg. Calcium is probably the key messenger in this information exchange. Certain sperm ion channels are turning out to be unique (Ren et al., 2001) of which a P2 channel may yet be one. These channels make them attractive targets for contraception as antagonisation would hinder fertilisation rather that either spermiogenesis or the transport of sperm with their probable hormonal or ejaculatory side effects. New approaches to measure sperm currents, particularly with the advent of nanotechnology, will allow previously impossible patch clamp studies of sperm. The same technology could also be used to measure currents at epithelial microvilli and may elucidate epididymal epithelium-sperm interactions (Gorelik et al., 2002). The presence of ATP within the epididymal and seminal fluid suggests it may have a role in exocrine cell signalling in the complex membranous changes that occur firstly in spermiogenesis, and secondly in the processes resulting in fertilisation. The measurement of epididymal fluid ATP concentrations within the different epididymal sections would give valuable information in support of a role in sperm maturation. Isolating the role of purinergic signalling on the maturational membrane changes undergone by epididymal sperm would appear extremely difficult.

It was my hypothesis that purinergic signalling seen in the genito-urinary tract of laboratory animals may differ from that of man. I have demonstrated that purinergic signalling is important in the contraction of human, mouse, rat and rabbit

tunica albuiginea. I have also demonstrated human vas deferens contraction has a significant purinergic component, but that this is proportionally less than published reports in laboratory animals and is likely to be functionally less significant than the dominant adrenergic component. Purinergically mediated and/or modulated contraction of gental tract smooth muscle may be of significance in the propulsion of sperm from the testis to ejaculatory ducts. I have also demonstrated a P2X receptor basis for a possible trophic role for ATP in sperm maturation in the epididymis. The pattern and receptor subtype expression was loosely consistent between man and the labaratory animals investigated, which may be representative of differing sperm transit times through the epididymis.

Purinergic signalling has been implicated is human detrusor overactivity. Animal models of bladder outlet obstruction are well established in this field, and have shown plasticity of the purinergic component. Human studies in this field are difficult because of ethical considerations in obtaining sufficient amounts of detrusor tissue, difficulties in confirming pathological states, and ensuring normal tissue is indeed normal. Furthermore, problems of matching even basic patient characteristics, such as age,   . This is highlighted in a paper by Harvey, in which the subsets of unstable bladders and obstructed bladders were combined to generate greater numbers (Harvey et al., 2002). In Chapter 6 it was hoped to replicate an earlier bladder obstruction study in the rabbit which had demonstrated purinergic upregulation (Calvert et al., 2001). One possibility to explain purinergic upregulation is an increase in the receptor density, and it was intended to examine whether up-regulation of the purinergic component occurred as a result of increased P2X receptor expression. The bladder outlet obstruction model was successfully applied to the rat, and undoubtedly induced detrusor overactivity. Indeed some of the spontaneous

activity of obstructed tissue was greater than the evoked contractions of sham-operated controls. The study demonstrated a significant up-regulation of the cholinergic component rather than the purinergic component and no discernable difference in the P2X receptor expression. This model confirmed neurogenic plasticity in detrusor overactivity, however, the rat model of obstruction did not appear to be representative of human tissue. This highlights the difficulty of using animal models to replicate a human disease process. In man, detrusor overactivity usually develops insidiously and covers a spectrum of symptoms and signs, which do not necessarily correlate. This rat model induced rapid detrusor hypertrophy and instability over 3 weeks.

The field of purinergic signalling continues to be hampered by the paucity of receptor specific antagonists. P2X knockout mice help to circumnavigate this problem, but neurogenic plasticity may mean that the impact of absent receptors is masked by compensatory adaptation, as possibly demonstrated by normal bladder function in $P2X_1$ knockout mice. The $P2X_3$ knockout mouse has demonstrated hyporeflexia of the bladder. More recent developments, using antisense oligonucleotides (ASO) and double stranded, short interfering RNAs (siRNA) to the $P2X_3$ receptor gene, have demonstrated significantly reduced pain responses in rat chronic pain models, confirming the role of $P2X_3$ receptors in inflammatory pain and possibly suggesting a therapeutic vehicle (Barclay et al., 2002; Dorn et al., 2004). In detrusor overactivity it is possible that up-regulation of purinergic signalling may be of more therapeutic relevance to the sensory mechanisms than to the contractile mechanisms. The possibility of 'damping down' bladder afferents by antagonism of $P2X_3$ receptors is currently an exciting possibility receiving serious attention from pharmaceutical companies. The new field of gene array may greatly help to examine

up or down regulation of the genes encoding the purinergic receptors and help elucidate their function in pathological situations. Furthermore, the possibility of nanotechnology pipettes in conjunction with patch-clamping has the potential to unravel the ionic messages of P2X receptors and the cross talk with other signalling systems.

In this study I have confirmed the presence and function of P2X receptors on the smooth muscle of the human testis and vas deferens as well demonstrating a possible trophic role for purinergic signalling in sperm maturation. Although the general pattern of P2X receptor expression was similar between man and the laboratory animals examined or previously studied, the role of purinergic signalling in contraction of human genito-urinary muscle appeared less significant. Although receptor plasticity was demonstrated in the pathological bladder model used, it was not possible to confirm purinergic dysfunction in the genito-urinary tract.

Currently there are no P2X pharmaceutical agents on the formulary. The $P2Y_{12}$ receptor antagonist, clopidigrel, is now in widespread use as an anti-thrombotic agent and has had such a significant impact on stroke prevention and vascular graft or stent success that it is the third biggest blockbuster drug on the market (Economist, 2005). The fields of bladder overactivity and infertility represent billion pound markets. Purinergic modulation continues to offer therapeutic potential and I would suggest that it is only a matter of time before a P2X receptor agent joins its illustrious cousin on the formulary.

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