PHARMACOLOGICAL AGENTS THAT DISTINGUISH BETWEEN P2X RECEPTOR SUBTYPES

by

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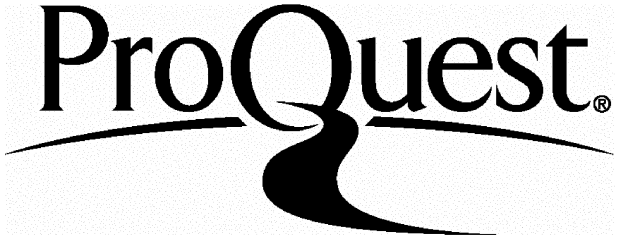

Pharmacological agents that distinguish between P2X receptor subtypes

The activity of novel pharmacological agents at recombinant P2X receptors was studied to find agents that distinguish between P2X receptor subtypes, particularly $P2X_1$ and $P2X_3$. Adenine nucleotide derivatives and diadenosine polyphosphates (Ap_nA, n = 2-6) were investigated as P2X receptor agonists. PAPET and HT-AMP were agonists, to varying degrees, at $P2X_{1-4}$ receptors. PAPET displayed higher affinity but lower efficacy than ATP at $P2X_1$ and $P2X_3$ receptors. HT-AMP showed higher affinity than ATP at $P2X_3$ receptors yet acted as a partial agonist at $P2X_{1-4}$ receptors. Diadenosine polyphosphates also showed selectivity in their actions at $P2X_{1-4}$ receptors. Ap_2A was inactive and $Ap_{3-6}A$ showed varying affinities and efficacies as agonists at $P2X_{1-4}$. Ap_3A was most effective at distinguishing between $P2X_1$ and $P2X_3$ receptors with over 100 fold difference between their respective EC_{50} values A series of PPADS derivatives, involving chemical manipulation of the phenylazo moiety and/or the pyridoxal phosphate moiety, showed nanomolar activity at $P2X_1$ and $P2X_3$ receptors with variable degrees of selectivity between these receptor subtypes. The most potent compounds were studied in detail and shown to be nonsurmountable antagonists. A comparison of like data for recombinant $P2X_1$ receptors and native $P2X_1$-like receptors in vas deferens revealed a number of pharmacological anomalies. Co-expression of $P2X_1$ and $P2X_2$ revealed a novel pH-sensitive phenotype although this heteromeric receptor is unlikely to account for the difference between $rP2X_1$ and the native P2X subtype(s) in this tissue. A step forward has been made in the search of pharmacological agents that distinguish between $P2X_1$ and $P2X_3$ receptors. Antagonist-resistant ATP responses in the vas deferens lend weight for other contraction-mediating P2 receptors in this preparation. Greater diversity of purinergic signalling was revealed through co-expression of P2X receptors and underlines the need for further novel pharmacological tools.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 PREFACE

For many years, research had focused on the intracellular role of Adenosine 5′-triphosphate (ATP). The accepted role of ATP as the ubiquitous "energy currency" of all living cells, and its rapid degradation in the extracellular medium, resulted in an initial resistance to accept that ATP could act as a neurotransmitter. However, considerable evidence has accumulated establishing ATP as a potent extracellular signalling molecule (Burnstock, 1997). The actions of ATP can be mediated through many subtypes of P2 receptor, both ionotropic (P2X receptors) and metabotropic (P2Y receptors) in nature, allowing for both direct and indirect control of the target tissue. Fine control and diversity of actions in the nervous system can be achieved, in part, through the complex synergism that occurs through release of multiple transmitters, modulatory substances and distinct receptor subtypes for ATP at synapses and neuroeffectors.

Pharmacological analysis, in the form of classical organ bath techniques, indicated that there were subtypes of nucleotide receptors (Burnstock *et al.*, 1978b; Burnstock and Kennedy, 1985). An early problem, that still exists today, is the lack of selective and potent agonists and antagonists at P2 subtypes therefore hampering the identification and characterisation of ATP receptors in tissue preparations. However, cloning of P2 receptors provided a great leap forward in receptor study allowing the identification of genetic sequences and molecular structures for distinct P2 receptor subtypes. Electrophysiological analysis goes hand in hand with certain molecular biology applications. Isolation of encoding cDNA/mRNA permits their introduction into expression systems, to allow the isolated study of distinct P2 receptor subtypes. In this way, six P2Y receptors and seven P2X receptors have been cloned and

characterised. This particular method of receptor study has proved invaluable not only in allowing characterisation of individual subunits but also combinations of subunits, specifically of the P2X subtype. The results from these recombinant investigations have, in the majority of cases, been related back to results from tissues in which subunit message expression was be found. Here, again, molecular biology has proved useful in mapping subunit distribution through *in situ* hybridisation and application of immunohistochemical techniques.

The defining principle that the human body functions under is that of homeostasis. It is one of control, controlling the variables in the body within a defined range, to maintain the body in a dynamic steady-state. One mechanism for maintaining fine control is through the release of endogenous mediators; for example, hormones in the case of the endocrine system, production of T-cells in the case of the immune system and release of neurotransmitters in the case of the nervous system. In the realm of homeostasis it is desirable that the receptor/endogenous ligand interaction is understood in detail on two levels. Firstly, under normal physiological conditions it is the interaction that controls homeostasis. Secondly, the majority of treatments for disorders of the nervous system are designed to affect this coupling, either by facilitation through mimicry of the endogenous ligand or antagonism of the endogenous ligand at its receptor target.

There is great awareness of the potential of ATP signalling as a therapeutic target, for example, in conditions such as urinary incontinence and pain (Burnstock, 1999a; Williams & Jarvis, 2000). However, the ability to manipulate the effect of ATP at P2 receptors is severely hindered by the lack of potent, selective and competitive antagonists even for simple pharmacological analysis.

As my work has focussed on P2X receptors, Chapter 1 is divided into three main sections that are concerned with the history of P2X receptors. Firstly, the role of ATP as a fast neurotransmitter, where I discuss the early evidence for purinergic transmission and establishment and nomenclature of purinoceptor subtypes. Secondly, I give a detailed account of the cloned P2X receptor subtypes, in particular the pharmacological, structural and kinetic properties of $P2X_{1-4}$, the subtypes that I concentrated on during my research. The third section describes the main antagonists of P2 receptors that have been developed since the inception of the purinergic concept. Following this section is an account of diadenosine polyphosphates, endogenous mediators that have been shown to have agonist activity at endogenous P2X receptors and are examined in detail in this thesis as agents to distinguish between recombinant P2X receptor subtypes. Chapter 2 describes the methodology employed throughout my investigations. Chapter 3 begins the experimental results chapters with a comparison of the pharmacology of recombinant $P2X_1$ and native $P2X_1$-like receptors. Thereafter, Chapters 4 – 7 deal with pharmacological agents that can distinguish between recombinant P2X receptor subtypes. In chapter 8, I provide further evidence demonstrating the complexity of purinergic signalling through co-expression of a novel combination of recombinant P2X receptors. Finally in chapter 9, I discuss my findings in relation to the possible pathophysiological roles of ATP and how they may be counteracted through novel drug intervention. Furthermore, I discuss the diversity of purinergic signalling that occurs through heteropolymerisation and the continuing quest to find agents that can distinguish between native P2X receptors.

1.2 ATP: A Fast Excitatory Transmitter

1.2.1 Purinergic Nerve Hypothesis

It is currently recognised that there are in excess of twenty substances that serve as neurotransmitters in the nervous system including acetylcholine, neuroactive peptides, monoamines, excitatory amino acids, gases (CO and NO) and purines. Until the early 1960s the autonomic nervous system was generally considered to consist of three divisions sympathetic (adrenergic), parasympathetic (cholinergic) and enteric. However, there was evidence stretching as far back as the end of the 19th century for a type of nerve-mediated response in the urinary bladder and intestine that was atropine-resistant (Langley and Anderson, 1895, Bayliss and Starling, 1900). Langley and Anderson (1895) reported that the contractile response of the urinary bladder to parasympathetic or transmural nerve stimulation was only partially blocked by atropine in smaller mammals. This was largely ignored until the 1970s when intensive research began to elucidate the mechanisms behind these atropine-resistant responses. Several theories were considered based mainly on the notion of atropine resistant acetylcholine receptors (Carpenter, 1977) or high local concentrations of acetylcholine in excess of that which could be effectively antagonised by exogenous atropine (Chesher and Thorpe, 1965). Finally, a non-adrenergic and non-cholinergic (NANC) innervation to the urinary bladder was considered (Ambache and Zar, 1970; Burnstock *et al.*, 1972; Dean and Downie, 1978). Electrophysiological evidence was presented for synaptic events that were neither adrenergic nor cholinergic in origin from guinea-pig taenia coli (Burnstock *et al* 1963, Bennett *et al* 1966) supporting previous pharmacological evidence showing NANC responses in this tissue (Burnstock *et al.,* 1963,

1964a). The first indication that ATP might be a neurotransmitter came from earlier work when it was shown that ATP was released from sensory nerves following antidromic stimulation (Holton and Holton, 1953; Hilton and Holton, 1954; Holton, 1959). After testing a wide range of compounds which might satisfy the criteria for establishment of a neurotransmitter as defined by Eccles, 1964, ATP was proposed to mediate NANC responses in the gut (Burnstock *et al.*, 1970). In 1972, Burnstock published an extensive review of the work on ATP and its possible role as a neurotransmitter. He proposed the existence of nerves that release ATP as their primary transmitter, "purinergic" nerves. There was initial resistance for such a theory, especially in the light of the lack of selective pharmacological agents to block the action of ATP. However, a weight of evidence has since been presented to support the purinergic hypothesis (Burnstock, 1990; Hoyle, 1992; Zimmerman, 1994; Ralevic and Burnstock, 1998).

1.2.2 ATP as a co-transmitter

Implicit in much of the work investigating purinergic signalling is that ATP is co-released with another transmitter. The original hypothesis of neurotransmission described a process where each neurone synthesises and releases one transmitter. However in 1976, Burnstock proposed that this original idea be reconsidered to take into account the increasing body of evidence that nerves could synthesise, store and release more than one transmitter with other "classical" neurotransmitters, for example, noradrenaline or acetylcholine.

It had been known for a number of years that ATP is stored and released together with catecholamines from the adrenal chromaffin cells. Langer and Pinto (1976) suggested that, following noradrenaline depletion with reserpine, the residual NANC response of the cat

nictitating membrane might be due sympathetic nerve-mediated ATP release. Subsequently, evidence was presented for neurogenic purine release from sympathetic nerves in the guinea-pig vas deferens. Hypertonic extracellular solution, preventing electrical excitation of the muscle, failed to prevent tetrodotoxin (TTX)-sensitive efflux of tritiated noradrenaline or adenosine (Westfall *et al.*, 1978). Equally, release was unaffected by prazosin or αβ-methylene ATP (αβ-meATP – a stable ATP analogue), thereby excluding postjunctional transmitter release (Lew and White, 1987; Kasakov *et al.*, 1988). Similar results regarding the overflow of transmitter from sympathetic nerves have been obtained in vascular smooth muscle preparations of the rabbit (Su, 1975, 1983) and cerebral and basilar arteries of the dog (Muramatsu *et al.*, 1981; Muramatsu and Kigoshi, 1987). Furthermore, in the appropriately treated pulmonary artery of the rabbit KCl, in place of nerve stimulation, increased ^{3}H-purine overflow which was Ca^{2+} dependent and attenuated by 6-hydroxydopamine (6-OHDA) and cold storage (Katsuragi and Su, 1980). Also in the saphenous artery, destruction of seemingly-adrenergic nerves with 6-OHDA abolished the neurogenic response that remained following noradrenaline (NA) depletion, suggesting a common source for the mediator of both components of the nerve response (Warland and Burnstock, 1987).

Sensitive techniques such as the firefly luciferase assay and high pressure liquid chromatography allow detection of overflow of endogenous ATP. Similar to the results seen with tritiated adenosine, concomitant release of ATP and NA was reported during nerve stimulation on guinea-pig vas deferens and vascular smooth muscle that is unaffected by prazosin or αβ-meATP yet abolished by TTX and 6-OHDA (Westfall *et al.*, 1987; Kasakov *et al.*, 1988)

Sympathetic nerve endings in the vas deferens are known to possess nicotinic receptors

(Carneiro and Markus, 1990). In guinea-pig vas deferens preincubated with tritiated noradrenaline, increasing concentrations of nicotine produced an increasing contraction and overflow of [^{3}H] NA and ATP (measured by luciferase technique). 6-OHDA blocked nicotine overflow of ATP (von Kugelgen and Starke, 1991). Moreover, EJPs and NA overflow was abolished by 6-OHDA treatment and sympathetic neurone blocking agents in rodent vas deferens and rat tail artery (Burnstock and Holman, 1961; Cheung, 1982; Allcorn, 1986).

Sympathetic nerve terminals contain small dense-cored and large dense cored vesicles that act as NA storing organelles. Both vesicle types also contain ATP (Fried *et al.*, 1984). Interestingly, there is evidence for frequency dependence upon the ratios of cotransmitter release from sympathetic nerves (Burnstock, 1988; von Kügelgen and Starke, 1994; Vizi *et al.*, 1997; Todorov *et al.*, 1999).

In the guinea-pig urinary bladder, ATP containing neurones can be detected using quinacrine staining (Burnstock *et al.*, 1978a). Furthermore, ATP overflow is detected upon nerve stimulation, using the luciferase technique, which is TTX sensitive and Ca^{2+} dependent. (Burnstock *et al.*, 1978a, 1978b).

There is now an increasing body of evidence suggesting that ATP can be coreleased along with other established neurotransmitters in other regions of the autonomic and central nervous systems (Sperlagh and Vizi, 1996; Bardoni, *et al.*, 1997; Pankratov *et al.*, 1998; Burnstock, 1999b, Jo and Schlichter, 1999).

1.2.3 ATP at the parasympathetic neuroeffector junction

Although a NANC theory of transmission had been established in the urinary bladder (see **section 1.2.1**), definitive evidence identifying the chemical mediating these responses had not been forthcoming.

Exogenous application of ATP was seen to cause contractions of the bladder in the rabbit, guinea-pig and rat, although only weakly so in the latter (Burnstock *et al.*, 1972, 1978a; Dean and Downie, 1978). Desensitisation using ATP produced inhibition of neurogenic contractions in the rabbit and guinea pig bladder (Dean and Downie, 1978, Lukacsko and Krell, 1981) and in rat when concomitant prostaglandin synthesis was prevented (Choo and Mitchelson, 1980). The introduction of the $ANAPP_3$ (see **section 1.4.1**) and stable ATP analogues provided further answers into the nature of the parasympathetic innervation in the urinary bladder. Degradation-resistant βγ-meATP, produced concentration-dependent contractions of the rat urinary bladder which mimicked the atropine-resistant response to nerve stimulation (Brown *et al.*, 1979). Responses to ATP and field stimulation were abolished after desensitization with αβ-meATP (Kasakov and Burnstock, 1983). $ANAPP_3$ antagonised responses to βγ-meATP, ATP and nerve stimulation in the cat and guinea-pig bladder (Westfall *et al.*, 1983; Theobald, 1982, 1986). Along with evidence concerning ATP-mediated contractions in the vas deferens, much of this information was used in the proposal of a P_{2X} receptor subtype in these tissues (Burnstock and Kennedy, 1985).

EJPs in smooth muscle cells of the urinary bladder were first recorded in 1983 (Creed *et al.*, 1983). Although the data was interpreted as an arguement against the involvement of ATP as the NANC transmitter, evidence soon emerged to contradict this proposal. EJPs were

resistant to atropine and could be desensitized by prolonged application of αβ-meATP (Hoyle and Burnstock, 1985; Fujii, 1988). Neuropeptides had been postulated to mediate the atropine resistant component. However, it was demonstrated that various neuropeptides elicited a slow sustained excitation, which contrasted with the fast transient response of ATP and to field simulation. The neuropeptide response was also unaffected following desensitisation of the P2 receptor with αβ-meATP (MacKenzie and Burnstock, 1984; Meldrum and Burnstock, 1985). Furthermore, ATP-induced currents in bladder smooth muscle cells appeared to be carried by Na^+ and Ca^{2+} (Inoue and Brading, 1991). Later, high affinity binding sites for αβ-me ATP were identified in the rat urinary bladder (Bo *et al.*, 1994).

Following the introduction of suramin and PPADS (see **section 1.4.2 and 1.4.5**), it was clear that P_{2X} receptor mediated responses could account for smooth muscle excitation in the urinary bladder (Hoyle *et al.*, 1990; Ziganshin *et al.*, 1993; Bailey and Hourani, 1994). Subsequently, transcripts for the P_{2X} receptor cloned from the vas deferens were detected in the urinary bladder (Valera *et al.*, 1994).

1.2.4 ATP at sympathetic neuroeffector junction

1.2.4.1 Vas deferens

The vas deferens has become an important tissue in the study of purinergic signalling and is often used to conduct research into the properties of novel P2 receptor antagonists. Burnstock and Holman (1961) conducted some of the earliest work measuring excitatory junction potentials, (EJPs), and depolarisations in the smooth muscle cells. Phentolamine, an α-

adrenoceptor blocker, did not completely abolish the smooth muscle cell response or spontaneous electrical potentials recorded therein. Further evidence pointed to another source, other than noradrenaline, for the production of EJPs. Reserpine pretreatment, to abolish adrenergic transmission, did not totally abolish EJPs in the guinea pig vas deferens (Burnstock and Holman, 1962; Burnstock *et al.*, 1964b). It had been noted that nerve-mediated contractions of the vas deferens consisted of two components, believed to be due to two different innervations (Ambache and Zar, 1971). In rats, the initial phase of contraction to single pulse stimulation peaked at approximately 300 ms, with the second slower component peaking after approximately 650 ms (Mallard *et al.*, 1992). The second slower component of the vas deferens to nerve stimulation was seen to be abolished by α-adrenoceptor antagonists, enhanced by drugs which inhibited neuronal uptake and absent from reserpinised tissues. In each case the initial component remained (McGrath, 1978). With the introduction of novel pharmacological agents new light was shed upon the nature of signalling in the vas deferens. The photoaffinity label $ANAPP_3$, selectively blocked responses to exogenous ATP (Hogaboom *et al.*, 1980) and the first phase of the contractile response to nerve stimulation (Fedan *et al.*, 1981) as well as EJPs in the vas deferens (Sneddon *et al.*, 1982). Burnstock (1978) reported that the slowly degradable analogue αβ-meATP acted selectively on P2 receptors. Repeated administration of αβ-meATP resulted in selective desensitisation to ATP and the first phase of contractile response to nerve stimulation (Meldrum and Burnstock, 1983). Similarly, EJPs were abolished by desensitisation by αβ-meATP (Sneddon and Burnstock, 1984a). These results made a compelling case for ATP mediated transmission at the sympathetic neuroeffector junction of the vas deferens and were used in part to argue a case for two separate classes of P2 receptor

(Burnstock and Kennedy, 1985). Subsequently it was reported that the depolarisation of the smooth muscle by ATP could be mediated by opening non-selective cation channels (Benham and Tsien, 1987). The introduction of new antagonists, particularly suramin and PPADS, gave added weight to the pharmacological argument for P_{2X} receptor mediated contractions of the vas deferens (Dunn and Blakeley, 1988; Lambrecht *et al.*, 1992; Mallard *et al.*, 1992; Sneddon, 1992; McLaren *et al*, 1994; Bailey and Hourani, 1995). The strong case for ATP transmission in the vas deferens made it a natural choice for receptor studies. Binding studies and solubilisation of αβ-meATP binding sites resulted in the isolation of a glycosylated membrane protein (Bo *et al.*, 1992). This line of research culminated in the cloning and sequencing of the first P_{2X} receptor in 1994 which functioned as a non selective cation channel, the $P2X_1$ receptor (Valera *et al.*, 1994, see **section 1.3.2.2**).

1.2.4.2 Excitatory ATP transmission in blood vessels

EJPs in some arteries (Holman & Surprenant, 1979; Surprenant, 1980) and arterioles (Hirst & Neild, 1980) are only partially blocked by α-adrenoceptor antagonists (Hirst & Neild, 1980; Holman & Surprenant, 1980). In the rabbit ear artery, fast EJPs are mimicked when ATP is applied by iontophoresis (Suzuki, 1985) and prazosin-resistant neurogenic contractions can be abolished by desensitisation with αβ-meATP (Kennedy *et al.*, 1986). Furthermore, ATP evoked a rapid inward current that was carried by Ca^{2+} and Na^{+} (Benham and Tsien, 1987). In the rat tail artery, a case for the involvement of ATP in vascular smooth muscle excitation can be made due to the fact that the initial phase of neurogenic contraction is inhibited by αβ-meATP and that ATP analogues could evoke concentration-dependent

inward currents (Bao *et al.*, 1989, 1990, Evans & Kennedy, 1994). Prazosin-resistant EJPs can also be blocked by αβ-meATP desensitisation (Sneddon and Burnstock, 1984b), although it is thought that ATP has only a minor role to play in sympathetic vasoconstriction in rat tail artery. In the saphenous artery of guinea pig and rabbits, αβ-meATP and $ANAPP_3$ were employed to demonstrate a role for ATP in smooth muscle excitation (Cheung & Fujioka, 1986; Warland and Burnstock, 1987). ATP appears to account for a significant part of sympathetic nerve mediated responses of the mesenteric arteries and small jejunal arteries from several species (von Kügelgen and Starke, 1985; Muramatsu *et al.*, 1984; Muramatsu, 1986). A minor role for ATP in the neurogenic contraction of vascular smooth muscle has been proposed in rat aorta (Kitajima, *et al.*, 1993). Following the finding that tritiated αβ-meATP could be used as a radioligand to label P_{2X} receptors (Bo and Burnstock, 1989), a study was then conducted to find direct evidence that P_{2X} receptors existed in vascular smooth muscle of several species. It was reported that there was a wide variation in density of $[H^3]$αβ-meATP binding sites in the vascular smooth muscle in different arteries although the density correlated with the strength of reported P_{2X} receptor mediated contractions in these tissues (Bo and Burnstock, 1993).

1.2.5 ATP as a fast excitatory neurotransmitter in sensory and central neurones

After the original work, by Holton, on the sensory nerves of the rabbit ear, further evidence was obtained highlighting the potential role of ATP in sensory transmission in other primary afferent fibres. In dissociated cell cultures of dorsal horn neurones and dorsal root ganglion

(DRG), ATP excites a subpopulation of around a quarter of the total sampled cell population, the mechanism for which was primarily through an increase in Na^+ conductance (Jahr and Jessell, 1983). Subsequently, further instances of specificity of ATP responses within populations of the same neurone type were described in neurones of the spinal cord receiving inputs from sensory afferent fibres (Fyffe and Perl, 1984; Salter and Henry, 1985). Similarly, excitation of neurones by ATP from vestibular, trigeminal and spinal ganglia was also thought to occur as a result of an inward current carried by Na^+ ions (Krishtal *et al.*, 1983; Li & Perl, 1995). Recently it has been shown conclusively that P2X receptors can mediate fast synaptic transmission in the rat dorsal horn (Bardoni *et al.*, 1997). Excitation of a small population of postsynaptic neurones in lamina II, a region known to receive input from nociceptive primary afferents, occurs upon application of ATP (Bardoni *et al.*, 1997). Further studies have been conducted demonstrating the ability of ATP to excite sensory neurones, including the DRG (Bean 1990; Bean *et al* 1990; Robertson, 1996, Rae *et al.*, 1998) and nodose ganglia (Khakh *et al.*, 1995a) resulting in increased intracellular calcium as well as increased Na^+ current.

As well as its possible role as a mediator of pain through excitation of sensory afferent fibres, ATP has been reported to mediate fast synaptic transmission at sympathetic and enteric ganglia. In 1992, results were published concerning the excitatory effects of ATP on sympathetic coeliac neurones from guinea-pigs using patch-clamp techniques. Fast excitatory postsynaptic potentials (EPSPs) were recorded from these neurones that were mimicked by ATP, blocked by suramin and desensitized by $\alpha\beta$-meATP and were unaffected by antagonists of other neurotransmitters (Evans *et al.*, 1992; Silinsky *et al.*, 1992). Intracellular recordings from myenteric neurones demonstrated that in addition to evoked fast EPSPs,

believed to be cholinergic in origin, there was a population of neurones where EPSPs were less sensitive to hexamethonium and could be inhibited by suramin. In these neurones ATP caused a fast depolarisation with properties indicating that it was mediated by a ligand-gated ion channel (Galligan and Bartrand, 1994). Fast excitatory roles for ATP have also been proposed in the peripheral auditory (Housley, 1998) and visual systems (Taschenberger *et al.*, 1999). Chromaffin cells have been used as neuronal models to study the effects of ATP. It was noted that ATP had excitatory effects on rat PC12 cells (Nakazawa *et al.*, 1990 a,b) and bovine chromaffin cells (Diverse-Pierlussi *et al.*, 1991) that resulted in raised intracellular calcium and catecholamine release. In PC12 cells, the currents that were recorded resembled those measured in sensory neurones (Krishtal 1998 a,b).

As evidence was forthcoming for the role of ATP as a transmitter between neurones in the peripheral nervous system, the first work was published providing evidence for a neurotransmitter role for ATP in the central nervous system. Patch-clamp techniques were employed to record spontaneous and evoked synaptic currents in the rat medial habenula and these events could be mimicked by exogenous applications of ATP (Edwards *et al.*, 1992). The time course and pharmacology of these currents were such that the researchers proposed that the current was mediated via a ligand-gated ion channel as opposed to a second messenger system. Alternative transmitters known to exist in this tissue were ruled out through the resistance of the currents to specific antagonists of these transmitters. Also, the currents could be abolished by suramin or desensitized with αβ-meATP, then considered a prototypic P2X agonist (Edwards *et al.*, 1992). Subsequently, ATP transmission has been reported in several areas of the brain (hippocampus, cerebral cortex, cerebellum and locus coeruleus) and is likely to be mediated through P2X receptors (Shen and North, 1993; Inoue,

1998; Mateo *et al.*, 1998; Pankratov *et al.*, 1998; Sansum *et al.*, 1998).

Evidence for ATP acting as a fast excitatory transmitter at parasympathetic ganglia is rarer. However, ATP has been reported to cause an inward current that is carried primarily by Na^+ ions in rabbit vesical parasympathetic ganglia (Nishimura and Tokimasa, 1996). Furthermore, αβ-meATP can evoke depolarisations of similar magnitude to ATP in a small population of guinea-pig intracardiac neurones (Allen and Burnstock, 1990). In a proportion of isolated rat major pelvic ganglion (MPG) neurones ATP can evoke a rapid inward current that slowly desensitises (Zhong *et al.*, 1998). However, MPG neurones from the guinea-pig have at least three distinct P2X receptor subtypes (Dunn *et al.*, 2001).

1.3 Receptor subtypes for Nucleosides and Nucleotides

1.3.1 P2 Receptor subtypes

In 1972, Burnstock noted that there appeared to be two types of receptors for ATP, one excitatory and one inhibitory. Subsequently, Burnstock (1978) proposed that two receptor types, P_1 and P_2, could be distinguished based on the rank order of potencies of ATP, ADP, AMP and adenosine as well as by sensitivity to antagonists. This section outlines the further subdivisions that took place in the P2 receptor field and highlights the important role that molecular cloning played in the advancement of purinoceptor research, specifically of the P2X subtype.

Prior to the advances that molecular cloning brought to the purinergic field, research was centred on the use of whole tissue preparations for pharmacological and ligand binding techniques. The greatest problem, which still persists, is the lack of selective and potent

antagonists. Further consideration had to be given to the possibility of ligand interactions with ectonucleotidases. Agonists may be broken down at different rates and antagonists may interfere with agonist breakdown through blockade of ectonucleotidase activity. Moreover, agonists and their metabolites, especially those of ATP, may have the ability to act on more than one subtype of receptor in a tissue. These limitations all have the potential to influence the apparent affinity and efficacy of ligands, that is the pharmacological criteria that the subtypes were distinguished upon.

In 1985, Burnstock and Kennedy proposed two distinct subdivisions of the P_2-purinoceptor class. The two subtypes were separated largely on the rank order of potency of structural analogues of ATP and potency of the antagonist $ANAPP_3$ (Table 1.1). Essentially, the P_{2X} subtype mediating contraction in the vas deferens and urinary bladder showed a high sensitivity to αβ-meATP and the P_{2Y} subtype mediating relaxation present in the taenia coli and longitudinal muscle layer of the rabbit portal vein showed a high affinity to 2-MeSATP. The development of a number of other ligands aided further research that supported this proposal. Suramin was found to antagonise both P_{2X} and P_{2Y} mediated responses. However, reactive blue 2, a histochemical dye, appeared to show selectivity for the P_{2Y} mediated responses (Kennedy, 1990). Moreover, the mechanisms by which these receptor subtypes transduced their signal appeared to differ. As previously mentioned in **section 1.2.4.1** the P_{2X} receptor on smooth muscle cells appeared function as a non-selective cation channel (Benham and Tsien, 1987). This contrasts with the P_{2Y} subtype which appeared to initiate a release of internal calcium via a second messenger system, which subsequently opened potassium channels and evoked a hyperpolarising current (Friel and Bean, 1988; Boyer *et al.*, 1989). The use of ATP analogues led to the identification of further P2 subtypes (table 1.1,

Table 1.1. P2 receptor subtype profiles prior to cloning of P2X receptor subtypes

P2 RECEPTOR SUBTYPE	AGONIST POTENCY ORDER	KNOWN ANTAGONISTS
P_{2x}	αβ-meATP, βγ-meATP > ATP = 2-MeSATP[1]	$ANAPP_3$[2], Suramin[3I, 3III], PPADS[4]
P_{2Y}	2-meSATP > ATP > αβ-meATP, βγ-meATP[1]	Suramin[3II], Reactive blue 2[5]
P_{2T}	2-MeSADP > ADP ≥ αβ-meADP[6]	ATP[7], Suramin[8], FPL66096[9]
P_{2Z}	BzATP >> ATP = 2-MeSATP = 2-chloroATP > ATPγS[10]	Oxidized ATP[10], FSBA[10]
P_{2S}	αβ-meADP = αβ-meATP > ADP = ATP[11]	PCMBS[11]
P_{2U}	UTP ≥ ATP = ATPγS > 2-MeSATP = αβ-meATP[12]	None
P_{2D}	Ap_4A > ADPβS > AMP-PNP > αβ-meATP[13]	None

[1] Burnstock & Kennedy, 1985.
[2] Hogaboom *et al.*, 1980; Sneddon *et al.*, 1982
[3] Dunn & Blakely, 1988[I]; Hoyle *et al.*, 1990[II]; Mallard *et al.*, 1992[III]
[4] Ziganshin *et al.*, 1993
[5] Burnstock & Warland, 1987, Kennedy, 1990
[6] Macfarlane *et al.*, 1983
[7] Cusack & Hourani, 1982
[8] Hourani *et al.*, 1992
[9] Humphries *et al.*, 1994
[10] Wiley *et al.*, 1994
[11] Wiklund & Gustafsson, 1988a,b
[12] Cusack, 1993
[13] Pintor *et al.*, 1993b

see also Dalziel and Westfall, 1994). ADP stimulates a G protein coupled receptor, P_{2T}, on thrombocytes which results in the calcium mobilisation via phospholipase C activation and their subsequent aggregation (Cusack and Hourani, 1982; Rink and Sage 1990). Degranulation of mast cells, lymphocytes and macrophages was seen to be achieved through the action of ATP (of which ATP^{4-} is thought to be the most active form) on a P_{2Z} subtype (Greenberg *et al.*, 1988; Wiley and Dubyak, 1989). A P_{2S} subtype was proposed to exist in the guinea-pig ileum that was sensitive to αβ-meATP, yet showed no cross desensitisation with ATP. Contractile responses were indomethacin sensitive but Reactive blue 2 insensitive (Wiklund and Gustafsson, 1988a,b). A pyrimidine-sensitive P_{2U} subtype was cloned from mouse neuroblastoma cell lines (Lustig *et al.*, 1993) and is present on PC12 cells and vascular smooth muscle (Cusack, 1993).

A presynaptic P3 purinoceptor was proposed that mediates an inhibition of the release of noradrenaline from nerves of the caudal artery and rat vas deferens (Shinozuka *et al.*, 1988; Forsyth *et al.*, 1991).

1.3.2 The P2X Receptor Subtype

1.3.2.1 Influence of Agonist Breakdown on Classification

The difference in the relative potency of nucleosides and nucleotides was the main basis for the original division of P_2 purinoceptors (Burnstock and Kennedy, 1985). It was readily acknowledged that the breakdown or uptake of agonists would be a factor that may distort these determinations. However, the full extent as to which the influence of ectonucleotidases had on the potency order of nucleotides at the P_{2X} receptor, through breakdown of susceptible

agonists, was not realised until experiments were conducted on dissociated smooth muscle cells. Evans and Kennedy (1994), showed that ATP and 2-MeSATP, agonists known to be readily degraded by ectonucleotidases, were in fact more potent than αβ-meATP at the P_{2X} receptor mediating contractions in the rat tail artery. These experiments revealed a potency order of ATP = 2-MeSATP ≥ αβ-meATP. The activity of the ectonucleotidase enzymes is dependent upon the presence of extracellular Ca^{2+} and Mg^{2+} (Ziganshin *et al.*, 1994a). In the rat isolated vagus nerve, the potency of ATP and 2-MeSATP was markedly increased in a Ca^{2+} and Mg^{2+} free solution such that the dose-response curves for both of these agonists lay to the left of that for αβ-meATP (Trezise *et al.*, 1994a). Similarly pharmacological intervention in the form of the ecto-ATPase inhibitor ARL67156 shifted the ATP dose-response curve to the left without affecting that of αβ-meATP (Crack *et al.*, 1994). Recently a novel mechanism has been proposed that involved the co-release of ATP and soluble ectonucleotidase enzymes (Todorov *et al.*, 1997; Westfall *et al.*, 2000). Clearly agonist breakdown plays a significant in the physiological role of ATP, limiting its potency as well as production of inhibitory active metabolites. However, the agonist activity of ATP could not be studied in isolated until the cloning of the first P2X receptor in 1994.

1.3.2.2 The molecular identification of P_{2X} receptor subtypes

Prior to the molecular characterisation of distinct P_{2X} receptor subtypes and despite the lack of pharmacological tools, there was evidence for multiple ATP-gated excitatory receptors. As discussed in **section 1**, αβ-meATP can activate an excitatory P_{2X} receptor that acts as a non-selective cation channel and shows marked desensitization. However, electrophysiological

studies conducted on PC12 cells and in the guinea pig submucosal plexus indicated the presence of a P_{2X} receptor that was αβ-meATP insensitive and slowly desensitized (Barajas-Lopez *et al.*, 1994, see also Humphrey *et al.*, 1995). A subsequent study showed similar results from cultured SCG cells as well as a further novel profile in cells from the nodose ganglion that were αβ-meATP sensitive and slowly desensitized (Khakh *et al.*, 1995a). Furthermore, βγ-meATP could distinguish between the P_{2X} receptors present on the vas deferens and vagal neurones (Trezise *et al.*, 1995).

It was clear that ATP could mediate excitatory responses through cation channels throughout the body. However, finding common properties or distinct differences between excitatory ATP responses between tissues such that they could be categorised proved difficult due to the lack of pharmacological agents available. Only with the intervention of molecular technology did it become possible to begin identify which receptor type may account for the ATP excitatory response in a particular tissue.

A cDNA encoding the P_{2X} receptor from the rat vas deferens ($P2X_1$) was cloned in 1994 (Valera *et al*, 1994). Through expression in *xenopus* oocytes and mammalian cell lines, it was shown to be a cation selective channel with a hydrophobicity plot suggesting two transmembrane spanning domains with a long extracellular domain, and intracellular N- and C- terminals. This topology was noted to be similar to a mechanosensitive channel and the amiloride-sensitive Na^+ channel (Valera *et al.*, 1994). The $P2X_2$ receptor was cloned from rat PC12 cells that was also shown to be a cation channel with a similar membrane topology to $P2X_1$. However, there were clear differences in the pharmacological and kinetic profiles, as well as the amino acid sequence, of these receptors (Brake *et al.*, 1994). Therefore, for the first time it was possible to clearly distinguish between, and name the receptor responsible

Table 1.2. Agonist EC_{50} values (μM) at recombinant $P2X_{1-4}$ receptors[a]

	$P2X_1$	$P2X_2$	$P2X_3$	$P2X_4$
ATP	0.5	4.6	1.2	10
ADP	11	>100	>100	ia*
UTP	>100	ia*	>100	ia*
CTP	nd*	23	>100, 17.9[e]	250
αβ-meATP	2	ia*	2.3[d], 0.5[d]	7[f],19[g]
2-MeSATP	0.4	7.1	0.2	74
ATPγS	3.1	7.4	7[d], 0.7[d]	23
L-βγ-meATP	2	ia*	>100	ia*
BzATP	0.7[b], 0.002[b]	23, 0.4[c]	10[d], 0.03[d]	25
References	1, 2, 3	1, 4, 5	2, 6, 7, 8	9, 10

[a] rat P2X orthologue unless stated
[b] Human $P2X_1$ expressed in *Xenopus* oocytes and 1321N1astrocytoma cells respectively
[c] Human $P2X_2$
[d] Rat $P2X_3$ expressed in *Xenopus* oocytes and 1321N1 astrocytoma cells respectively
[e] human $P2X_3$
[f] Mouse $P2X_4$
[g] Human $P2X_4$
* nd: Not determined; ia: inactive

[1] Evans *et al.*, 1995
[2] Bianchi *et al.*, 1999
[3] Valera *et al.*, 1994
[4] King *et al.*, 1997b
[5] Lynch *et al.*, 1999
[6] Chen *et al.*, 1995
[7] Evans *et al.*, 1998
[8] Garcia-Guzman *et al.*, 1997
[9] Bo *et al.*, 1995
[10] Jones *et al.*, 2000

for, types of excitatory ATP responses. Currently seven ionotropic P2X receptors ($P2X_{1-7}$, see table 1.2 for agonist data at $P2X_{1-4}$, that is the receptors of interest in this study) have been cloned and characterised in mammalian tissues. The cDNA sequences share 32 – 52% sequence homology, ranging from 379 – 595 amino acid residues in length (Humphreys *et al.*, 1998). Despite the difference in amino acid content there is remarkable conservation in the structural features of P2X receptors. For example, they all have the overall hydrophobicity conforms in the two transmembrane domains. These domains are connected by a hydrophilic extracellular loop in which six lysine, ten cysteine and thirteen glycine residues are conserved. The P2X receptors are all permeable to sodium, potassium and calcium ions (Valera *et al.*, 1994; Brake *et al.*, 1994; Bo *et al.*, 1995; Chen *et al.*, 1995; Collo *et al.*, 1996 Surprenant *et al.*, 1996). Interestingly, Ca^{2+} may have a dual role in limiting the depolarisation through certain P2X receptors as well as activation of intracellular Ca^{2+}-dependent processes. It has been demonstrated that currents carried through P2X receptors on certain neuronal cells can be limited by extracellular and intracellular Ca^{2+} (Bean, 1990; Nakazawa and Hess, 1993). Conversely, it has been demonstrated that removal of extracellular Ca^{2+} converts the rapidly-desensitising $P2X_3$ receptor into a non-desensitising phenotype (King *et al.*, 1997a). The exact permeability ratio of Ca^{2+}: Na^{+} of native P2X receptors varies from 0.3 in rat sensory neurones (Bean *et al.*, 1990) to 10 in rat medial habenula neurones (Edwards *et al.*, 1997).

1.3.2.3 Structural Features of P2X Receptors

The topology of P2X receptor subunits (Figure 1.1) is currently thought to consists of an

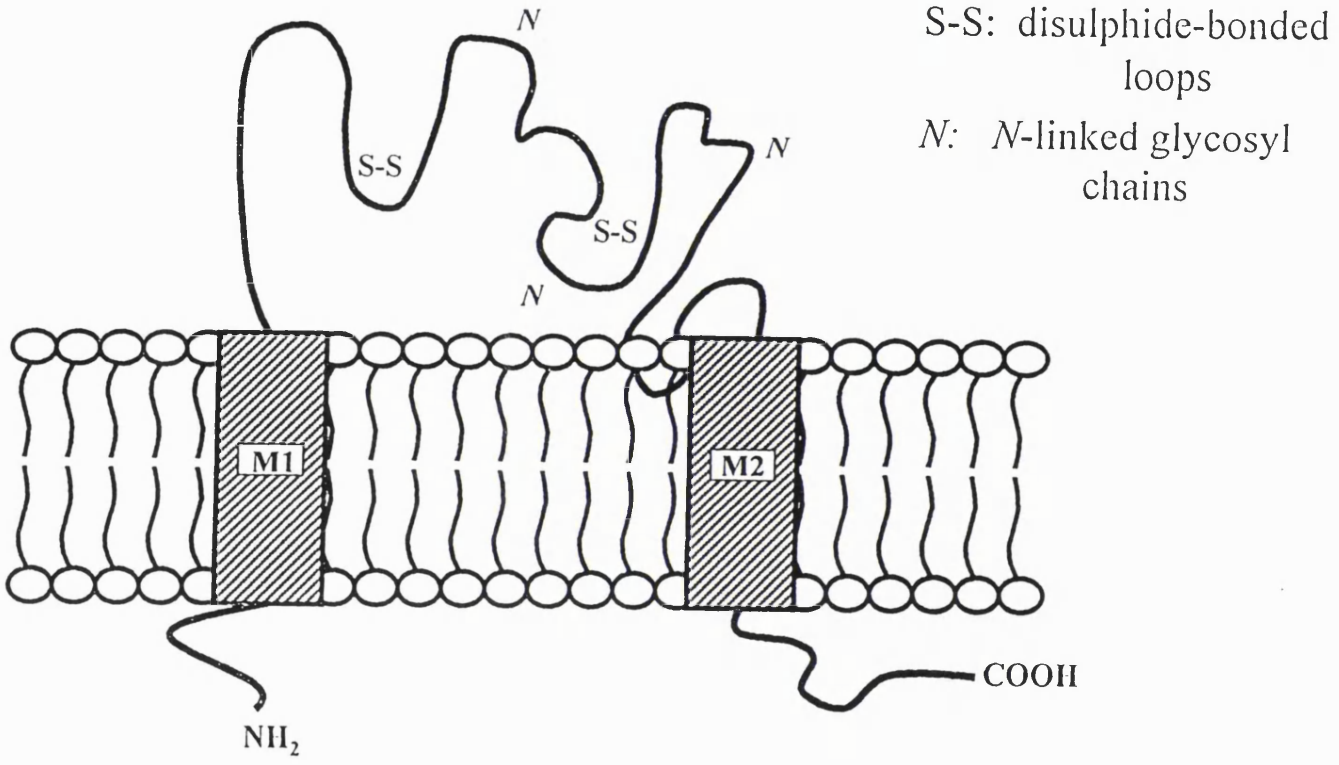


Figure 1.1 Predicted transmembrane topology of P2X receptors (Brake *et al.*, 1994).

intracellular N- and C-terminus, two transmembrane domains, a large extracellular loop that contains a H5 hydrophobic region which loops in and out of the membrane next to M2. This topology is distinct from the nicotinic superfamily and excitatory amino acid gated channels (Brake *et al.*, 1994; Valera *et al.*, 1994). The technique of substituted cysteine accessibility method (SCAM) has been used successfully to identify the transmembrane regions and residues within, which are important in channel formation and gating. This method involves introducing point mutations into the P2X receptor cDNA such that individual amino acids are replaced with cysteine and then testing the ability of sulphydryl-reactive agents to modify these residues. Comparison of channel kinetics is used to identify affected residues. $P2X_2$ is most commonly used in these experiments due to its efficient expression and non desensitising currents in expression systems. It was proposed that residues 30-44 in TM1 and 336-349 in TM2 may line the pore region of the channel due to their size and charge selectivity (Rassendren *et al.*, 1997; Jiang *et al.*, 2001). Similar results regarding TM2 were obtained by Egan and coworkers when they confirmed that the second transmembrane domain was involved in pore formation and that residues 342 and 349 play an important role in formation of the channel gate (Egan *et al.*, 1998). Interestingly, a natural deletion of one leucine residue in the apolar region (351 – 353) of TM2 of $P2X_1$ results in loss of function but not assembly or incorporation into the membrane (Oury *et al.*, 2000). Egan and colleagues (1998) also cast doubt on the tertiary structure of the transmembrane region that had been proposed to form either an α-helix or a β-sheet. Due to the polar nature of a number of amino acid residues in the M2 region, it was calculated that it might form an amphipathic α-helix (Brake *et al.*, 1994). Alternatively, a model based on a voltage K^+ channel was proposed that the M2 region contains a β-strand (Van Rhee *et al.*, 1998). Such a proposal

brings into consideration the stoichiometry of P2X subunits. Inward rectifying K^+ channels have a predicted stoichiometry of 4 subunits (Kubo *et al.*, 1993). Although a tetrameric assembly has been proposed (Burnstock and Wood, 1996; Kim *et al.*, 1997) there is also evidence suggesting that 3 subunits contribute to the formation of the P2X receptor channel pore (Nicke *et al.*, 1998; Ding and Sachs, 1999; Stoop *et al.*, 1999). The use of chimeric $P2X_2$ receptors is being increasingly used to study the regions of P2X receptor that are involved in the desensitization properties of the receptor. Introduction of residues 14-47 from the M1 region of $P2X_2$ into the corresponding region of $P2X_1$ conferred a non desensitising phenotype. Equally, replacing residues 332-367 of the $P2X_1$ C-terminus with that of $P2X_2$ also resulted in the production of a non-desensitising phenotype. However, the analogous experiment introducing $P2X_1$ (or $P2X_3$) domains into corresponding $P2X_2$ regions showed that both $P2X_1$ regions needed to be present to confer a rapidly desensitising phenotype (Werner *et al.*, 1996). Interestingly, the $P2X_{2\text{-}2}$ splice variant that has a shorter intracellular C-terminus exhibits an accelerated rate of desensitisation compared to $P2X_2$ (Brändle *et al.*, 1997). It is of note that 4 residues in this sequence that are missing in the $P2X_{2\text{-}2}$ splice variant have been demonstrated to be important in the slow desensitisation profile and therefore Ca^{2+} influx through $P2X_2$ (Koshimizu *et al.*, 1998; Smith *et al.*, 1999). However, in the human forms of the $P2X_2$ splice variants the desensitisation rates are very similar (Lynch *et al.*,1999). The human $P2X_{2\text{-}2}$ form also lacks the 4 amino acid sequence missing in the rat form, therefore indicating that other factors are involved in the desensitisation kinetics. Both the N-terminus and M1 region have also been demonstrated to have an influence on $P2X_3$ desensitisation rates. Their removal changes the fast desensitising phenotype into a slowly desensitising one (King *et al.*, 1997a). A threonine residue in the N-terminus PKC site of

$P2X_2$ has been identified to be critical for formation of a channel with a slow rate of desensitization (Boué-Grabot *et al.*, 2000). Furthermore, both N- and C- terminals have been shown to accelerate recovery from desensitisation (Werner *et al.*, 1996). Extra-receptor agents may also influence native channel kinetics, for example phosphorylation of the receptor by kinase enzymes (King *et al.*, 1997a; Boué-Grabot *et al.*, 2000), actin cytoskeleton (Parker, 1998) and cations (Li, *et al.*, 1993; Evans *et al.*, 1996; King *et al.*, 1996a; Michel *et al.*, 1997; Cook *et al.*, 1998; Wildman *et al.*, 1998, 1999). Experiments have been conducted that demonstrate allosteric modulation of $P2X_3$ and $P2X_4$ by P2 antagonists (Michel *et al.*, 1997; Miller *et al.*, 1998; Alexander *et al*, 1999). This raises the possibility of such a phenomenon occurring at native receptors through the action endogenous mediators other than those listed above.

The extracellular domain contains 10 conserved cysteine residues that have been proposed to aid stability of the loop through formation of disulphide bridges (Brake *et al.*, 1994). Furthermore, varying numbers of N-linked glycosylation sequences exist that appear to be essential to the assembly of a functional channel (Torres *et al.*, 1998a). Equally, M2 appears to be critical in P2X subunit assembly (Torres *et al.*, 1999a). Little is known about the binding site for ATP at the P2X receptor. Studies are complicated further due to the fact that often the Hill coefficient for agonist dose-response curves is greater than 1, indicating positive cooperativity of binding (Brake *et al.*, 1994). However, a recent study has highlighted a role for 3 lysine residues (68, 70 and 309) and an arginine residue (292) that appear to have a role in ATP binding and activation of the $P2X_1$ receptor (Ennion *et al.*, 2000). Similarly, two lysine residues (69 and 71) were identified to be important for the action of ATP at the $P2X_2$ receptor (Jiang *et al.*, 2000). Similar work has highlighted the role

of TM1 in determing the agonists ability to bind and gate the P2X channel. Replacement of TM1 of $P2X_2$ with that of $P2X_1$ resulted in a construct that was sensitive to αβ-meATP (Haines *et al.*, 2001). Similarly, a single point mutation (F44C) has been demonstrated to confer αβ-meATP sensitivity to $P2X_2$ (Jiang *et al.*, 2001).

1.3.2.4 Heteropolymerization of P2X receptor subunits

There is now an awareness of the increasing complexity of purinergic signalling that arises from combinations of P2X receptor subunits, such that a hetero-oligomeric assembly forms that has distinct properties from those of either individual subunit type. Two examples of co-expression of a group 1 receptor with a group 2 receptor type yields a functional recombinant channel where the group 1 receptor dominates the pharmacological properties whereas the group 2 receptor dominates the kinetics of the agonist response (Lewis *et al.*, 1995; Torres *et al.*, 1998b). The strongest correlation of this type of experimental result to a physiological response is seen with coexpression of $P2X_2$ and $P2X_3$ which yields a slowly desensitizing αβ-meATP sensitive response similar to that seen in sensory and sympathetic neurones (Lewis *et al.*, 1995; Khakh *et al.*, 1995a; Radford *et al.*, 1997; Zhong, *et al.*, 2000; Liu *et al.*, 2001). The question of subunit co-assembly was investigated in detail by examining specific protein-protein interactions (Torres *et al.*, 1999b). Table 1.3 shows a summary of the results of possible protein-protein interactions as determined by a co-immunoprecipitation assay. Of these results at least two combinations have been studied in more detail in a functional assay, $P2X_6$ coexpressed with either $P2X_2$ or $P2X_4$ (Lê *et al.*, 1998b; King *et al.*, 2000). Due to overlapping transcript expression and uncertain receptor stoichiometry, the possibility exists

Table 1.3. Biochemical association of P2X receptor subunits.

	$P2X_1$	$P2X_2$	$P2X_3$	$P2X_4$	$P2X_5$	$P2X_6$	$P2X_7$
$P2X_1$	+	+	+	-	+	+	-
$P2X_2$		+	+	-	+	+	-
$P2X_3$			+	-	+	-	-
$P2X_4$				+	+	+	-
$P2X_5$					+	+	-
$P2X_6$						+	-
$P2X_7$							+

Table 1.3. Summary of possible candidates for heteropolymerisation as determined using the co-immunoprecipitation assay. Results taken directly from Torres *et al.*, 1999b. + indicates the pairings of subunits that co-precipitate, - indicates that paired subunits do not co-precipitate.

of 3 or more distinct P2X subunits combining to further increase the number of excitatory receptors, with unique phenotypes, sensitive to ATP. It is, however, desirable to determine if the same phenotypes exhibited by recombinant heteromultimer assemblies exist in tissues where overlapping expression of P2X transcripts occur. Clearly this will require the development of new pharmacological agents possibly along with other biochemical methods that can detect specific protein-protein interactions specific to P2X receptors.

Further complexity arises from alternative splicing of P2X transcripts. Variants have been found for $P2X_1$, $P2X_2$ and $P2X_4$ (Brändle *et al.*, 1997; Dhulipala *et al.*, 1998; Troyanovskaya & Wackym 1998; Lynch *et al.*, 1999; Townsend-Nicholson *et al.*, 1999; Hardy *et al.*, 2000; Greco *et al.*, 2001). There is the possibility that the diversity of purinergic signalling can be increased through expression of homomeric populations with subtle phenotypical differences when compared to the wild type, as in the case of $P2X_2$ splice variants outlined above. Furthermore, phenotypical changes may occur in receptors that contain subunits from different isoforms (Townsend-Nicholson *et al.*, 1999). Theoretically, coexpression of different isoforms with other P2X subunits may produce heteromer receptors with different properties.

1.3.2.5 Distribution of $P2X_{1-4}$ receptor transcripts

Early attempts to determine specific ATP binding sites used radioligands such as [^{3}H]-αβ-meATP and [^{35}S]-ATPγS which were thought to be specific for the P_{2X} receptor subtype (Bo *et al.*, 1989; Michel *et al.*, 1996a). However it is clear that the binding of αβ-meATP does not necessarily indicate the presence of $P2X_1$ or $P2X_3$ as both $P2X_2$ and $P2X_4$ can bind αβ-

meATP with a high affinity (Michel *et al.*, 1996b; 1997). ATPγS is non-selective agonist and therefore these pharmacological agents, at best, can only give a general indication of P2X receptor distribution. Following the cloning of the P2X receptors it became possible to directly detect P2X receptor transcripts and localise the mature receptor in tissues using *in situ* hybridisation and polyclonal antibodies respectively.

Using *in situ* techniques, $P2X_1$ was shown to be present in smooth muscle of the vas deferens, urinary bladder, small intestine, colon and arteries (Valera *et al.*, 1994; Collo *et al.*, 1996; Longhurst *et al.*, 1996; Nori *et al.*, 1998, Lee *et al.*, 2000). Overlap of $P2X_1$, $P2X_2$ and $P2X_4$ transcripts has been detected in vascular smooth muscle (Nori *et al.*, 1998) with positive immunoreactivity seen in sensory and sympathetic ganglia (along with strong $P2X_3$ staining), as well as the inner ear (Xiang *et al.*, 1998; 1999). Interestingly, overlapping expression of $P2X_1$ and $P2X_2$ is detected in the cerebellum, hippocampal CA1-3 regions, and the dentate gyrus in the neonatal rat but not in the adult, where the $P2X_1$ transcripts were not found (Kidd *et al.*, 1995). However, immunopositive staining is detected using electron microscopy in the adult rat (Loesch & Burnstock, 1998). Immunopositive staining for $P2X_1$ has been observed in cerebral, kidney and submucosal blood vessels (Vulcanova *et al.*, 1996; Bo *et al.*, 1998; Chan *et al.*, 1998). $P2X_1$ cDNA has been isolated from human platelets (Clifford *et al.*, 2000; Scase *et al.*, 1998; Sun *et al.*, 1998; Greco *et al.*, 2001) and transcripts are detected in the spinal cord (Collo *et al.*, 1996).

Positive immunoreactivity and *in situ* results have been obtained for $P2X_2$ through-out regions of the adult rat brain and in sensory and sympathetic ganglia (Brake *et al.*, 1994; Kidd *et al.*, 1995; Lewis *et al.*, 1995; Collo *et al.*, 1996; Xiang *et al.*, 1998; Kanjhan *et al.*, 1999). Sensory neurones hold the greatest concentration of $P2X_3$ transcripts, in particular the

small and medium diameter nerve fibres of the dorsal root and trigeminal ganglia (Lewis *et al.*, 1995; Xiang *et al.*, 1998). The cDNA corresponding to $P2X_4$ was independently cloned from the brain (Bo *et al.*, 1995; Seguela *et al.*, 1996), sympathetic nerves (Buell *et al.*, 1996) and pancreatic β-cells (Wang *et al.*, 1996). Wide distribution of $P2X_4$ transcripts is seen throughout the brain, peripheral nerves, endocrine organs as well as the smooth muscle of major blood vessels, vas deferens and urinary bladder (Bo *et al.,* 1995; Buell *et al.*, 1996; Collo *et al.*, 1996; Wang *et al.*, 1996; Nori *et al.*, 1998).

1.4 History of P2X receptor antagonists

The pharmacological characterisation of native P2 purinoceptor subtypes and understanding of the role of ATP in neurotransmission continues to be hampered by the lack of potent and selective competitive antagonists. Many compounds have been tested for this purpose, most notably suramin and pyridoxal-5′-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS). However, all P2 antagonists are limited in their usefulness by their irreversibility of antagonism or by their lack of potency, selectivity and specificity for P2 purinoceptor subtypes. The majority of research into antagonist properties has been conducted in smooth muscle preparations and recombinant $P2X_{1-4}$ receptors. The discussion of the properties of the current antagonists is therefore limited to these systems.

1.4.1 $ANAPP_3$

$ANAPP_3$ is a photo-affinity derivative of ATP and was shown to be a substrate for ATPase enzymes. Exposure to visible light results in a photoactivation that causes the formation of a covalent bond and hence irreversible inhibition. The same mechanism is thought to underlie the irreversible antagonism of P2X receptors in the vas deferens and urinary bladder with this compound (Fedan *et al.*, 1985). Selectivity at the $P2X_1$-like receptor subtype in the vas deferens over non-purine receptors is exhibited by the ability of $ANAPP_3$ to antagonise responses to exogenous and endogenously released ATP (Hogaboom *et al.*, 1980, Fedan *et al.*, 1981, Sneddon *et al.*, 1982) as well as responses to more stable ATP analogues (Fedan *et al.*, 1982).

Due to the irreversible nature of antagonism produced by $ANAPP_3$ it prevented any calculation of its potency and therefore difficulty in comparison of potency between tissues. However, $ANAPP_3$ did have an important role to play in helping to distinguish P2 subtypes (Burnstock & Kennedy, 1985).

1.4.2 Suramin

Suramin is a symmetrical polysulphonated naphthylamine derivative of urea (Figure 1.2A). It was originally synthesised at the beginning of the 20^{th} century when it was tested for its trypanocidal activity. Suramin has a remarkable range of activities, aside from its pharmacological properties at P2 receptors, most notably inhibition of enzyme activity (for a detailed discussion see Voogd *et al*, 1993).

In 1988, Dunn and Blakeley were the first to show that suramin selectively antagonises $P2X_1$-like purinoceptor mediated effects in the mouse vas deferens when they showed that at a concentration of 100μM, suramin reversibly antagonises responses to αβ-meATP, a stable ATP analogue, without affecting those of carbachol and noradrenaline. Subsequently, more detailed investigations into the pharmacological properties of suramin revealed a complex form of antagonism as well as anomalous properties in some of the tissues used.

Although Dunn and Blakeley (1988) showed that suramin antagonised P2X-purinoceptor mediated responses it appeared, under the conditions used, that suramin did not show competitive antagonism as the slope of the αβ-meATP curve was increased. In the guinea-pig urinary bladder, suramin (100μM) produced a rightward shift in the dose-response curve for αβ-meATP with a suppression of the maximum. With a further 10 fold increase in suramin concentration responses to the agonist were abolished, clearly indicating non–competitive antagonism (Hoyle *et al.*, 1990). Conversely, suramin (100μM), produced an increase in efficacy to αβ-meATP in the rat vas deferens and was accompanied by a rightward shift in the dose response curve (Von Kügelgen *et al.*, 1990, Mallard *et al.,* 1992, Bültmann *et al.*, 1996a). Additional potentiating properties of suramin to αβ-meATP responses have also been revealed. Suramin (1μM) was seen to enhance the affinity of αβ-meATP by more than three fold and increase efficacy by 65% in the urinary bladder (Hoyle *et al.*, 1990). Further evidence highlighting the complicated kinetics surrounding the actions of suramin at P2 receptors is the increase in Hill slope in several smooth muscle preparations (Von Kügelgen *et al.*, 1990, Mallard *et al.*, 1992, Hourani *et al.*, 1992). A detailed investigation into the type of antagonism exhibited by suramin revealed that it could show competitive antagonism under the necessary experimental conditions. Leff and his coworkers

showed that, in the rabbit ear artery where the agonist profile of ATP and analogues is consistent with that of the P2X subtype (O'Conner *et al.*, 1990), suramin did shift the concentration response curve for αβ-meATP to the left and increase the Schild slope under conditions similar to those used by other investigators who found equivalent results. However, if each concentration of suramin was allowed to equilibrate for such a time as to theoretically allow for 95% receptor occupancy it acted in a competitive manner, shifting the concentration response curve for αβ-meATP in a parallel fashion and with a Schild plot slope of unity. The resulting pK_B was 4.79, a value similar to other estimates (Hoyle *et al.*, 1990, Von Kügelgen *et al.*, 1990, Usune *et al.*, 1996). Thus, suramin was shown to behave as a slowly equilibrating competitive antagonist in this preparation (Leff *et al.*, 1990). Further evidence for competitive antagonism is provided through binding studies where suramin was found to competitively displace the binding of tritiated αβ-meATP to P2X-like-receptors in the rat urinary bladder and vas deferens and functional studies in rabbit isolated aorta (Khakh *et al.*, 1994; Bo *et al.*, 1994; Ziyal *et al.*, 1997). Competitive antagonism has been seen with suramin against ATP-evoked responses following the activation of ATP-gated cation channels in rat pheochromocytoma PC12 cells (Nakazawa *et al.*, 1990a, Inoue *et al.*, 1991). Clearly, the antagonistic profile of suramin demonstrated thus far against P2X receptors in the urinary bladder and vas deferens is far from ideal. This profile is complicated further with the use of ATP as the agonist. Experiments clearly showed that αβ-meATP could be effectively antagonised by suramin but responses induced by high concentrations of ATP showed considerably more resistance preventing calculation of an accurate pA_2 values (von Kügelgen *et al.*, 1989, von Kügelgen *et. al.* 1990; Bailey & Hourani, 1994; Bailey & Hourani, 1995). However, in the mouse vas deferens, ATP (0.1-3μM) was antagonised by

suramin in a non-competitive manner with a resultant pA_2 of 5.36 which is in the similar range as the pA_2 values for suramin versus αβ-meATP responses. This demonstrates that ATP and αβ-meATP are acting at a common site which can be antagonised by suramin and that ATP acts at a further suramin resistant site to produce contractions in the vas deferens (Von Kügelgen *et al.*, 1990; Bültmann and Starke, 1994).

Similar to the findings of its effects on αβ-meATP responses, in the guinea-pig urinary bladder, suramin (1μM) potentiated the responses to field stimulation, with significant antagonism seen at 100μM and greater (Hoyle *et al.*, 1990). The protocol employed in these experiments was such that a secondary, tonic, response was not seen. Electrical field stimulation experiments in the vas deferens in which the tonic response was induced showed that suramin could have varying effects depending on the protocol used. To a single pulse, of such duration as to elicit the tonic response, suramin at a concentration of 10μM and greater, showed a time-dependent inhibition of the phasic response with no significant antagonism of the tonic component. Furthermore, in contrast to the results obtained in the urinary bladder, low concentrations of suramin did not potentiate the phasic component (Hoyle *et al.*, 1990; Mallard *et al.*, 1992). However, to trains of electrical stimulus, suramin again showed a time-dependence antagonism, but in this instance both components are reduced, which did not appear to be due to a reduction in transmitter release (von Kügelgen *et al.*, 1989, 1994; Mallard *et al.*, 1992; Bailey & Hourani, 1995). As both ATP and noradrenaline are known to contribute to both phases, reduction of both phases might suggest a nonspecific action. In fact, contradictory evidence has been presented regarding the specificity of suramin. It has been reported to have effects on other neurotransmitters tested on smooth muscle preparations, although only at high concentrations tested (Leff *et al.*, 1990; Hourani *et al.*,

1992; Henning *et al.*, 1992). Furthermore, antagonism of other members of a distinct superfamily has been reported. Suramin has been reported to antagonise GABA meditated currents in the rat hippocampus and NMDA and AMPA mediated currents in the hippocampus and dorsal horn of the spinal cord respectively (Nakazawa *et al.*, 1995; Gu *et al.*, 1998; Peoples & Li, 1998). In contrast suramin has been demonstrated to be selective for P2X receptors (Dunn & Blakeley, 1988; Nakazawa *et al.*, 1990a; Hoyle *et al.*, 1990; Evans & Suprenant 1992; Mallard *et al.*, 1992; Bao & Stjärne 1993; Trezise *et al.*, 1994a).

Further to its antagonistic effects at P2X receptors, suramin was shown to antagonise P2Y receptors equally effectively in the taenia coli, platelets and rat vas deferens, in a non competitive manner, generating an approximate pA_2 value in the range of 4.6-5 (Hoyle *et al.*, 1990; Hourani *et al.*, 1992, Bültmann & Starke, 1994). Competitive antagonism has been reported in aortic endothelial cells at P2Y receptors (Wilkinson *et al.*, 1993). Cloned P2Y and P_{2U} purinoceptors are also antagonised by suramin (Charlton *et al.*, 1996).

Even before the discovery of its ability to inhibit ATP responses in the vas deferens, suramin (30μM) was shown to inhibit by up to 50% ATP breakdown in human granulocytes (Smolen & Weissman, 1978). However, there is the possibility that different, pharmacologically distinct ecto-nucleotidases exist to break down extracellular ATP since suramin was shown to be less active in its ability to inhibit ecto-ATPase enzymes of the guinea-pig urinary bladder. Here, concentrations up to 10mM produced only a relatively limited inhibition (Hourani & Chown; 1989). There is now a large body of evidence showing that suramin inhibits ecto-nucleotidase (Crack *et al.*, 1994, Beukers *et al.*, 1995, Ziganshin *et al.*, 1995,

Bültmann *et al.*, 1996a; Chen *et al.*, 1996; Bültmann *et al.*, 1999). Naturally, with the slower rate of breakdown of ATP in the presence of suramin there is the possibility that there is a self-cancellation effect. That is, due to its prolonged presence, and as a result of ecto-nucleotidase inhibition, ATP can compete more effectively for the receptor-binding site, thereby, counteracting the antagonistic effect of suramin. The dual properties of suramin could therefore lead to errors in interpretation of results from experiments using this compound (Crack *et al.*, 1994).

Suramin can effectively and reversibly antagonise ATP and related nucleotide responses at four of the seven cloned rat P2X subunits ($P2X_1$, $P2X_2$, $P2X_3$ and $P2X_5$), exhibiting a potency within approximately one order of magnitude at each receptor (Valera *et al.*, 1994, Brake *et al.*, 1994, Evans *et al.*, 1995, Chen *et al.*, 1995, King *et al.*, 1997b; Bianchi *et al.*, 1999, see table 1.4). The rat $P2X_4$ receptor is relatively insensitive to suramin whereas the human orthologue is more sensitive to suramin antagonism, albeit relatively low (Garcia-Guzman *et al.*, 1997). Similarly, a difference in suramin potency is seen between the rat and human $P2X_3$ orthologues (Bianchi *et al.*, 1999) Interestingly, it was shown that suramin antagonism of ATP responses at the recombinant $P2X_2$ receptor was dependent on extracellular pH. A shift in pH from 7.4 to 5.5 increased suramin potency well over 100 fold (King *et al.*, 1997b). Extracellular Zn^{2+} also increased the affinity of suramin at $P2X_2$ (Wildman *et al.*, 1998).

1.4.2.1 Suramin derivatives

Derivatives of suramin have been synthesised in an attempt to obtain greater selectivity between P2 receptor subtypes. NF023 (figure 1.2B) and NF279 (figure 1.2C) are two

analogues that are reportedly more potent than suramin at antagonising $P2X_1$-like mediated contraction in the rat vas deferens. NF023, in common with suramin, shifts the dose response curve for αβ-meATP to the right and increases the maximum agonist response in the rat vas deferens. However, NF023 is more potent and displays a lower affinity for ectonucleotidase in this tissue (Bültmann *et al.*, 1996a). Similar results have been presented confirming that NF023 is more potent that suramin at $P2X_1$-like receptors in the rabbit vas deferens and rabbit aorta (Lambrecht, 1996; Ziyal *et al.*, 1997; Bültmann *et al.*, 1999). However, NF023 appears to be less potent than PPADS (Lambrecht, 1996; Bültmann *et al.*, 1999). Similar concentrations of NF023 that block αβ-meATP mediated currents in these preparations reversibly antagonise the recombinant rat $P2X_1$ receptor. Antagonism is also seen with NF023 at cloned $P2X_2$, $P2X_3$ and $P2X_4$ receptors but at higher concentrations, and for $P2X_3$ is dependent on the species orthologue (Soto *et al.*, 1999, see table 1.4). Similar to the results seen with suramin, ATP responses in the vas deferens are resistant to NF023 with approximately 60% residual response remaining at 100μM NF023, a concentration which abolished responses to αβ-meATP and EJPs (Bültmann *et al.*, 1999; Sneddon *et al.*, 2000). Activity at P2Y receptors is also reported but at a lower potency than suramin (Van Rhee *et al.*, 1994; Lambrecht, 1996).

NF279 was demonstrated to have a high selectivity for $P2X_1$ receptors in the rat vas deferens over P2Y in the guinea-pig taenia coli and ectonucleotidases. This compound was approximately 10 fold more potent than NF023 at antagonising αβ-meATP mediated contractions (Damer *et al.*, 1998). NF279 exhibits a high potency at recombinant human and rat $P2X_1$ receptors (Klapperstück *et al.*, 2000 and references therein, see table 1.4).

Suramin has been a welcome and useful tool for the study of native P2 receptors but, due to its wide range of actions and lack of selectivity, its use is limited. The development of NF279 may represent a step forward in the search for potent and selective antagonists due to its apparent selectivity for recombinant $P2X_1$ receptors over $P2X_2$, $P2X_3$, $P2X_4$ and $P2X_7$ (Klapperstück *et al.*, 2000).

1.4.3 Histochemical dyes

The histochemical dye class consists of a large group of chemically distinct molecules that have varying affinites for P2X and P2Y receptors. Reactive blue 2 (RB2, figure 1.2D) has been proposed to be a selective P2Y receptor antagonist (Burnstock & Warland, 1987; Reilly *et al.*, 1987). However, antagonist activity at native and recombinant P2X receptors have been demonstrated (Choo, 1981; Garcia-Guzman, 1997; Tuluc *et al.*, 1998). Furthermore, it exhibits ectonucleotidase inhibitory activity as well as non-specific actions (Tuluc *et al.*, 1998). Cibacron blue 3G is an isomer of RB2 and shows some degree of selectivity for $P2X_1$-like receptors over $P2Y_1$-like receptors (Tuluc *et al.*, 1998). Uniblue A and Reactive blue 19 are derivatives of a fragment of RB2 and show a high selectivity for the P2X receptors in the vas deferens over the $P2Y_1$-like receptors in the taenia coli. Unfortunately, all compounds demonstrate ectonucleotidase inhibitory and non-specific actions (Tuluc *et al.*, 1998). RB2 is a potent antagonist at recombinant $P2X_2$ receptors (King *et al*, 1997b). An allosteric modulatory role have been demonstrated for low concentrations of cibacron blue, which

increase the affinity and efficacy of ATP at $P2X_3$ (Alexander *et al.*, 1999) and increase the affinity of ATP at $P2X_4$ (Miller *et al.*, 1998).

Evans blue is another aromatic polysulphonic acid molecule that selectively blocks αβ-meATP responses in the rat vas deferens over ADPβS in the taenia coli. However potentiation of responses to ATP, high K^+ and exogenous noradrenaline are also found as well as ectonucleotidase inhibition (Bültmann & Starke, 1993; Wittenberg *et al.*, 1996; Bültmann *et al.*, 1999). Trypan blue lacks the non-specific and ectonucleotidase blocking actions of Evans blue but it shows little selectivity between αβ-meATP and ADPβS responses in the vas deferens and taeni coli respectively (Wittenberg *et al.*, 1996). Derivatives of these compounds have been synthesized in order to attempt to improve their pharmacological profile. Minor potency changes have been made but many problems still remain (Wittenberg *et al.*, 1996). Reactive red 2 is a potent $P2Y_1$-like antagonist with an approximately 15-fold selectivity over $P2X_1$-like receptors. However, it causes irreversible antagonism and also inhibits ectonucleotidase activity (Bültmann & Starke, 1995; Bültmann *et al.*, 1999).

1.4.4 Isothiocyanates

DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulphonate) is an ATP transport inhibitor that was initially shown to block P_{2Z} receptors on rat parotid acinar cell (McMillan *et al.*, 1988). It has been shown to cause long lasting, non-competitive block of $P2X_1$-like receptors in the vas deferens with some selectivity over ADPβS responses in the taenia coli. However, effective ectonucleotidase inhibitory activity is also seen (Bültmann *et al.*, 1996b, 1999). At

recombinant $P2X_1$ and $P2X_2$ receptors, some discrimination was seen between the level of antagonism of DIDS (Evans *et al.*, 1995, table 1.4). Other isothiocyanate compounds have shown little promise (Bültmann *et al.*, 1996b).

1.4.5 Pyridoxal phosphate derivatives

Pyridoxal 5′-phosphate (P5P), is the functional form of vitamin B_6 and a coenzyme in reactions such as nitrogen metabolism, glycogen phosphorylase reaction and transaminase reactions. P5P and its derivative PPADS (figure 1.2E) are generally nonsurmountable antagonists at $P2X_1$, $P2X_2$, and $P2X_3$. Reversibility is slow with the exception of $P2X_3$. There is the possibility that certain basic residues in the extracellular domain may have a role to play in determining the profiles of these antagonists. Formation of a Schiff base has been proposed to occur and account for the slowly reversible antagonism seen with P5P and PPADS at the Lys249 of $P2X_1$ and Lys246 at $P2X_2$ receptors (Buell *et al.*, 1996; Collo *et al.*, 1996). Indeed, replacement of Glu249 with Lysine at the recombinant $P2X_4$ receptor restored the potency of the antagonists to levels seen at recombinant $P2X_1$ and $P2X_2$ (Evans *et al*, 1995; Buell *et al.*, 1996). However, this single amino acid residue difference does not appear to account for potency differences, as the reciprocal experiment, replacing Lys249 with glutamate in $P2X_2$, does not decrease antagonist sensitivity but does, however, increase dissociation rate (Buell *et al.*, 1996). A further case for the possible involvement of Lys249 influencing the kinetic properties of PPADS is demonstrated at $P2X_3$ which recovers rapidly from PPADS block and has a threonine residue in position 249 (Buell *et al.*, 1996). As

demonstrated above and in the case of the maintenance of the potency of PPADS at $P2X_2$ following a point mutation at position 249, other residues will play a part in the binding of PPADS to the P2X receptors. The human orthologue of $P2X_4$ shows an enhanced sensitivity to PPADS. There is a 22 - 24 amino acid residue difference between the rat and the human clones which may be involved in conferring this sensitivity to PPADS in the human clone (Garcia-Guzman *et al.*, 1997). Interestingly, the point mutation Glu78lys increased the sensitivity of a rat clone to NF023 (Soto *et al*, 1999). Furthermore, the increased sensitivity to PPADS at the $P2X_4$ clone isolated from rat brain, compared to rat $P2X_4$ clones from the superior cervical ganglion and hippocampus, may relate to the two amino acid residue difference at positions 136 and 137 of the total brain clone (Bo *et al.*, 1995; Buell *et al.*, 1996; Séguela *et al.*, 1996). Mouse and human $P2X_4$ show a similar susceptibility to PPADS antagonism and have 37 amino acid residue differences in the proposed extracellular region (Jones *et al.*, 2000, table 1.4). The complexity of pharmacological interactions at the $P2X_4$ receptor is highlighted through the observations that PPADS can also potentiate ATP responses through an unknown mechanism (Bo *et al*, 1995; Buell *et al.*, 1996; Townsend-Nicholson *et al.*, 1999). In addition, PPADS appears to be a slowly equilibrating antagonist as potency can increase with longer periods of equilibration (Collo *et al.*, 1996; Jacobson *et al.*, 1998). Interestingly, it has been observed that the onset and offset of PPADS antagonism at $P2X_2$-like receptors in bullfrog DRG neurones may be affected by the conformational state of the channel at the time of exposure to the drug (Li, 2000).

P5P has been proposed to act as a specific antagonist against αβ-meATP responses in the rat vas deferens and vagus nerve (Trezise *et al*, 1994). However, the range over which it P5P antagonises αβ-meATP responses in the rat vas deferens also produced depolarisation of the

smooth muscle cells of the guinea-pig vas deferens (Sneddon *et al.*, 2000). Low activity at recombinant $P2X_1$ and $P2X_2$ has also been demonstrated (Evans *et al.*, 1995; Miller *et al.*, 1998, Table 1.4).

Depolarising activity of PPADS on guinea-pig vas deferens smooth muscle cells has also been demonstrated and is thought to be mediated through a suramin insensitive site (McLaren *et al.*, 1994). Furthermore, low concentrations of PPADS (<1μM) potentiated the initial phasic response to nerve stimulation in the guinea-pig vas deferens (McLaren *et al.*, 1994). However, PPADS (>1μM) concentration dependently inhibited αβ-meATP and initial phasic responses in the guinea-pig, rat and rabbit vas deferens (Lambrecht *et al*, 1992; McLaren *et al.*, 1994; Bültmann *et al.*, 1999). Interestingly, in the rabbit urinary bladder, PPADS does not abolish the initial phase of neurogenic contractions or αβ-meATP responses seen in the rabbit vas deferens, thus indicating a difference in the contraction mediating P2X receptors in these tissues of the rabbit. (Ziganshin *et al.*, 1993).

Generally, PPADS generates a pA_2 value of approximately 6 to 6.7 against $P2X_1$-like receptors in various in smooth muscle preparations (Ralevic and Burnstock, 1998 and references therein). Interestingly, there is a disparity in the IC_{50} values generated between the human and rat recombinant $P2X_1$ receptor orthologue, which have 34 amino acid residue differences in the extracellular region (Evans *et al.*, 1995; Jacobson *et al.*, 1998; Bianchi *et al.*, 1999, see table 1.4). PPADS exhibits a similar potency at recombinant rat $P2X_2$ and human $P2X_3$ receptors (King *et al.*, 1997b; Garcia-Guzman *et al.*, 1997, table 1.4). PPADS also blocks some recombinant and endogenous P2Y receptors (Ralevic and Burnstock, 1998 and references therein).

MRS 2220, a PPADS derivative lacking the aldehyde group, antagonises ATP responses at recombinant $P2X_1$ and $P2X_3$ receptors, but with a lower potency than the parent compound, indicating that formation of a Schiff base with the P2X receptor is not necessarily required for antagonism. In addition, MRS 2220 showed a greater selectivity for P2X over P2Y receptors and was rapidly reversible (Jacobson *et al.*, 1998).

Iso-PPADs (figure 1.2F), an isomer of PPADS, shows a similar potency to PPADS at endogenous P2X receptors. Iso-PPADS acts as a slowly equilibrating and slowly reversible antagonist at P2X receptors in rat vagus nerve and vas deferens (Khakh *et al.*, 1994; Trezise *et al.*, 1994a). At recombinant $P2X_1$ and $P2X_3$ receptors, its potency is slightly higher than that of PPADS (Jacobson *et al.*, 1998, see table 1.4).

Although PPADS and iso-PPADS are generally accepted as specific antagonists, high concentrations have been shown to have inhibitory actions at muscarinic receptors (Ziganshin *et al*, 1993; Connolly, 1995). PPNDS is a derivative of PPADS and shows a high selectivity for recombinant $P2X_1$ receptors over ectonucleotidase enzymes and $P2Y_1$ receptors (Lambrecht *et al.*, 2000, see table 1.4).

PPADS is small relative to suramin and some isothiocyanate derivatives but it does contain many groups that are open to chemical manipulation. The results seen with PPNDS and MRS 2220 lend support to the idea that modification of PPADS might lead to the development of compounds with a high degree of selectivity.

1.4.6 Other antagonists of note

Trinitrophenyl-ATP (figure 1.2G) was originally used as a fluorescent agent that could label ATP binding sites and has been used to identify the cell surface presence of P2X receptors and ecto-ATPase enzymes (Mockett *et al.*, 1994). It was found to be a potent antagonist with a high selectivity for recombinant $P2X_1$ and $P2X_3$ and heteromeric $P2X_{2/3}$ receptors over $P2X_2$ and $P2X_4$ receptors (Virginio *et al.*, 1998; see table 1.4). Other trinitrophenyl-substituted nucleosides showed similar profiles provided that there was at least one phosphate group at the 5′-position on the adenosine molecule (Virginio *et al.*, 1998). Nanomolar affinity for TNP-ATP has also been reported in isolated smooth muscle cells from the mesenteric artery (Lewis *et al.*, 1998). TNP-ATP has also been used to demonstrate the possible existance of multiple populations of P2X receptors in sensory and sympathetic neurones (Thomas *et al.*, 1998; Zhong *et al*, 2000). It is of note that the potency of TNP-ATP in whole tissue preparations is reduced approximately 15,000 fold and is thought to be accounted for by degradation by ectonucleotidase enzymes (Lewis *et al.*, 1998). Furthermore, the antagonism is non-competitive and non specific as equal concentrations of noradrenaline and αβ-meATP, that evoked contractions of similar magnitude in whole artery rings, are inhibited by approximately 40% and 50% respectively (Lewis *et al.*, 1998).

Diinosine pentaphsophate (Ip_5I) is a derivative of Ap_5A and has been shown to be a potent, selective and non competitive antagonist at recombinant $P2X_1$ receptors (King *et al.*, 1999, see table 1.4). At native $P2X_1$-like receptors in the guinea-pig vas deferens Ip_5I is

considerably less potent (pA_2 value of 6.5 ± 0.1 versus ATP responses), which may be accounted for by ecto-nucleotidase activity. However its selectivity over responses to noradrenaline and those mediated by P2Y receptors in the taenia coli and P1 receptors in the left atrium were clearly demonstrated (Hoyle *et al*, 1997).

Table 1.4. pIC_{50} values (μM) for a series of P2 antagonists at recombinant $P2X_{1-4}$ receptors[a]

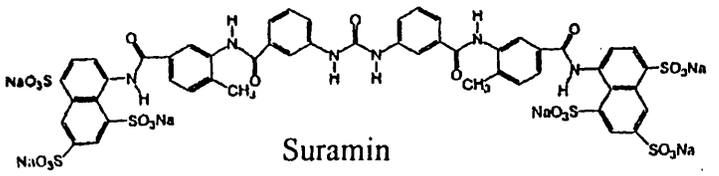
	$P2X_1$	$P2X_2$	$P2X_3$	$P2X_4$
Suramin[1,2,3,4]	0.85[b], 1[b]	10.4, 33	0.7, >100[c]	178[e]
NF023[5]	0.24	50 – 100	8.5, 28.9[c]	ia[#]
NF279[6]	0.05	nd[#]	1.6	nd[#]
P5P[1,7]	12	10[b], 40	nd[#]	219
PPADS[1,2,8,9,10]	0.098, 1[b]	1.6	0.24, 1.7[c]	10[d], 50 – 100
IsoPPADS[8]	0.042	nd[#]	0.083	nd[#]
PPNDS[11]	0.014	nd[#]	nd[#]	nd[#]
DIDS[3,12]	2[b]	>100	nd[#]	>500
TNP-ATP[13]	0.006	2	0.0009	15.2
Ip_5I[14]	0.0031	ia[#]	2.8	NA[*]

[a] Unless indicated pIC_{50} values are for rat clones
[b] Human bladder $P2X_1$
[c] Human $P2X_3$
[d] Mouse $P2X_4$
[e] Human $P2X_4$
[*] potentiated agonist responses
[#] nd = not determined, ia = inactive

[1] Bianchi *et al.*, 1999
[2] King *et al.*, 1997b
[3] Evans *et al.*, 1995
[4] Garcia- Guzman *et al.*, 1997
[5] Soto *et al*, 1999
[6] Klapperstück *et al.*, 2000
[7] Miller *et al.*, 1998
[8] Jacobson *et al.*, 1998
[9] Jones *et al.*, 2000
[10] Séguéla *et al.*, 1996
[11] Lambrecht *et al.*, 2000
[12] Wang *et al.*, 1996
[13] Virginio *et al.*, 1998
[14] King *et al.*, 1999

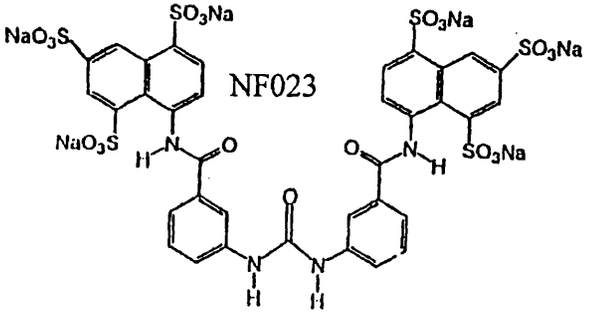
Figure 1.2 P2X antagonists

A



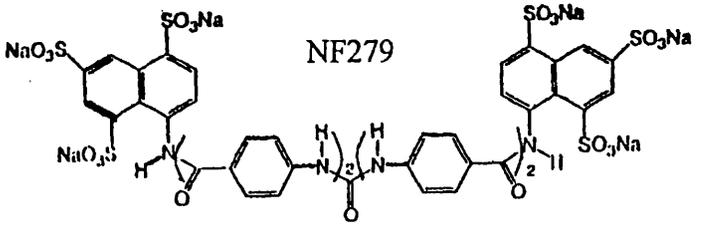
Suramin

B



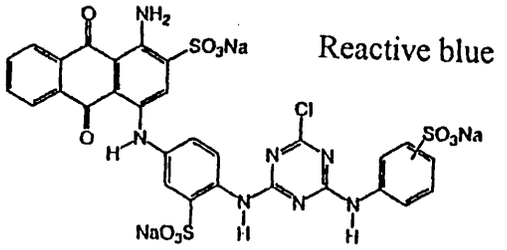
NF023

C



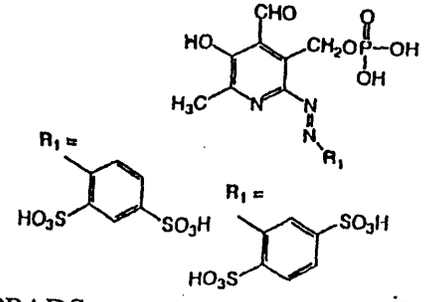
NF279

D



Reactive blue

E

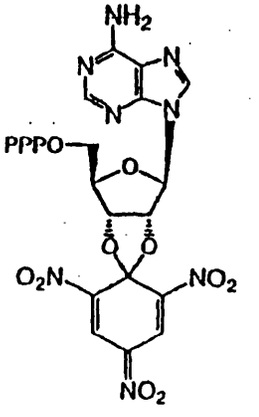


PPADS

F

iso-PPADS

G



TNP-ATP

1.5 Adenine Dinucleotides

Adenine dinucleotides can be split into two main groups. The first group consists of coenzymes such as β-nicotinamide dinucleotide and flavin adenine dinucleotide. The second group consists of α,ω-adenine dinucleotides or diadenosine polyphosphates. These molecules consist of two adenosine molecules linked via their 5'-ribose position by 2-6 phosphate groups (Ap_nA, where n = 2-6; Hoyle *et al.*, 2001).

Ap_4A was first identified as a by-product in ATP synthesis (Reiss and Moffat, 1965) and has subsequently been shown to be important in many intracellular biochemical pathways with claims for a ubiquitous presence in living cells (Ogilvie *et al.*, 1994). $Ap_{3,4,5,6}A$ have been detected in human tissues including platelets and chromaffin cells (Rodriguez del Castillo *et al.*, 1988; Pintor *et al.*, 1992b; Schlüter *et al.*, 1994). Wide ranging physiological roles have been proposed for the diadenosine polyphosphate family including haemostasis (Schlüter *et al.*, 1994; van der Giet *et al.*, 1997), control of vascular tone (Ralevic *et al.*, 1995) and neurotransmission (Pintor *et al.*, 1992a). Secretory synaptosomes from the midbrain have been shown to store and release Ap_4A and Ap_5A in a Ca^{2+} dependent manner (Pintor *et al.*, 1992a). Amphetamine induced release of diadenosine polyphosphates has been demonstrated *in vivo* from the basal ganglia of rats (Pintor *et al.*, 1993a). Furthermore, agonist induced release of Ap_4A and Ap_5A has been reported from chromaffin cells (Pintor *et al.*, 1991) and these compounds can act themselves to increase the release of catecholamines from these cells (Castro *et al.*, 1990). Demonstration of release of such compounds raises that possibility of a distinct receptor type for the diadenosine polyphosphates. Radioligand binding

conducted on rat mid brain synaptosomes revealed binding sites that had a high affinity for Ap_4A and showed a displacement order that was distinct from known P2 receptors. These authors suggested the existence of a purinoceptor with a high specificity for adenine dinucleotides, P_{2D} (Pintor *et al.*, 1993b, see table 1.1). Functional evidence has been forthcoming through experiments conducted on neurones of the locus coerulus that show a pharmacological profile consistent with that proposed for the P_{2D} receptor (Pintor *et al.*, 1997a). Claims for a P4 receptor have been made due to experiments conducted in rat brain synaptosomes. In the midbrain, Ap_4A and Ap_5A induced Ca^{2+} inward currents that were suramin insensitive and did not cross desensitize with ATP. The calcium current consisted of two components, one of which was unaffected by L or N type channel blockers (Pintor and Miras-Portugal, 1995a). These results have been confirmed and also repeated in the rat cerebellum with Ap_2A, Ap_3A and Ap_6A (Pintor *et al.*, 1997b).

Clearly however, members of the diadenosine family can act through P2X receptors to produce physiological responses. In the guinea-pig vas deferens, Ap_5A cross desensitized with ATP and αβ-meATP (MacKenzie *et al.*, 1988). Similar P2X mediated excitatory responses have also been noted for Ap_3A and Ap_4A in the vas deferens (Hoyle *et al.*, 1995; Westfall *et al.*, 1997). Ap_6A mediates contractile responses in the human urinary bladder (Hoyle *et al.*, 1989).

The ability of diadenosine polyphosphates to mediate vascular smooth muscle contraction via P2X receptors has been demonstrated. Schlüter and coworkers demonstrated that Ap_5A and Ap_6A could mediate vasoconstriction and cause an increase in perfusion pressure of the rat kidney through an increase in intracellular Ca^{2+} (Schlüter *et al.*, 1994) that appears to be P2X receptor mediated (van der Giet *et al.*, 1997). Similar results are seen in the isolated rat

mesenteric arteries (Ralevic *et al.*, 1995) and cultured vascular smooth muscle cells (Tepel *et al.*, 1996). Furthermore, diadenosine polyphosphate induced excitation of sensory neurones has also been reported (Krishtal *et al.*, 1988a).

In summary, diadenosine polyphosphates can exert effects on an array of physiological systems through direct action on P2 receptors and as such may play an important role in the maintenance of homeostasis.

CHAPTER 2

GENERAL METHODOLOGY

2.1 Organ-Bath Tissue Assays

2.1.1 General Setup and Protocol

Male Sprague Dawley rats (200-250g) were killed by CO_2 inhalation followed by cervical dislocation. The vasa deferentia were removed and subsequently cleared of connective tissue and cut (approximately 15 – 20 mm long from the prostatic end).

Tissues were mounted vertically in 10ml organ baths and 1g of resting tension was applied. The tissues were allowed to equilibrate for 30 min in oxygenated bathing solution prior to isometric recordings of mechanical activity. Electrical field stimulation was applied via two platinum-wire rings (2.5mm diameter, 10mm separation) through which the vas deferens and urinary bladder strips were threaded. The tissues were bathed in Krebs solution of the following composition (mM): NaCl 118, $NaHCO_3$ 25, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5 and glucose 11; aerated with 95% O_2/5% CO_2 (pH 7.4 – 7.5) and maintained at 37±1°C. Contractions were recorded by Grass FT03C force-displacement transducer and displayed on a Grass 79D ink-writing oscillograph. In experiments using exogenous agonists, single doses of αβ-meATP (0.1-100μM) or ATP (1-1000μM) were added directly to the organ bath and were washed out after a maximum contraction had been reached. In the case of αβ-meATP, intervals of at least 30 min, washing every 10 min, were used in order to avoid desensitization. Otherwise 20 min intervals were used between agonist additions.

2.2 Electrophysiology Experiments

2.2.1 *Xenopus Laevis*

2.2.1.1 Classification

(Kingdom: Animalia. Phylum: Chordata. Class: Lissamphibia. Order: Anura. Family: Pipidae)

The African claw-toed frog, *Xenopus laevis* (picture 1a), is a member of the tongueless frog family, Pipidae. There are 14 species of *Xenopus*, with *Xenopus laevis* being the best known and widely used laboratory animal of this species. Native to sub-Saharan Africa, these, mainly aquatic, frogs normally live in stagnant pools in grassland regions and breed from early October to late December. Contrasting with wild frogs, those that are captive bred or acclimatised frogs show little variation in their oocyte yield or quality.

2.2.1.2 Morphology

Xenopus laevis frogs have a flattened wedge-shaped appearance and lack teeth, a tongue and an inflatable-vocalising sac. They are referred to as "claw-toed" due to the presence of claws on the outer three toes on the hind legs. They are regarded as primitive frogs with the adult female of the species being considerably larger than the male, being distinguished by the presence of a tail-like bud known as cloacal lips. They are multi-coloured, generally being grey/green on the dorsal surface and creamy coloured on the ventral side. *Xenopus* frogs have

well-developed vibration-detecting tissues arranged along the sides of their bodies, called "lateral line organs" which appear as visible "stitching" marks.

2.2.1.3 *Xenopus* Husbandry

Adult female, captive bred, *Xenopus* frogs (200 – 300g) were supplied by Blade Biologicals, Edenbridge, Kent, UK. They were housed in large tanks (approximately 400-litre capacity) continuously aerated using an overflow filtration system. Temperature was constantly monitored and controlled thermostatically between 20-24°C. The tanks in which the frogs were housed were covered to prevent the entry of natural light in order to prevent seasonal variations in oocyte quality. A maximum of twelve frogs was kept in each tank with a minimum of 3 ltr^3 of space per frog. The feeding regime was of a daily meal of 300mg of trout pellets or ox liver.

2.2.2 *Xenopus Laevis* Oocytes

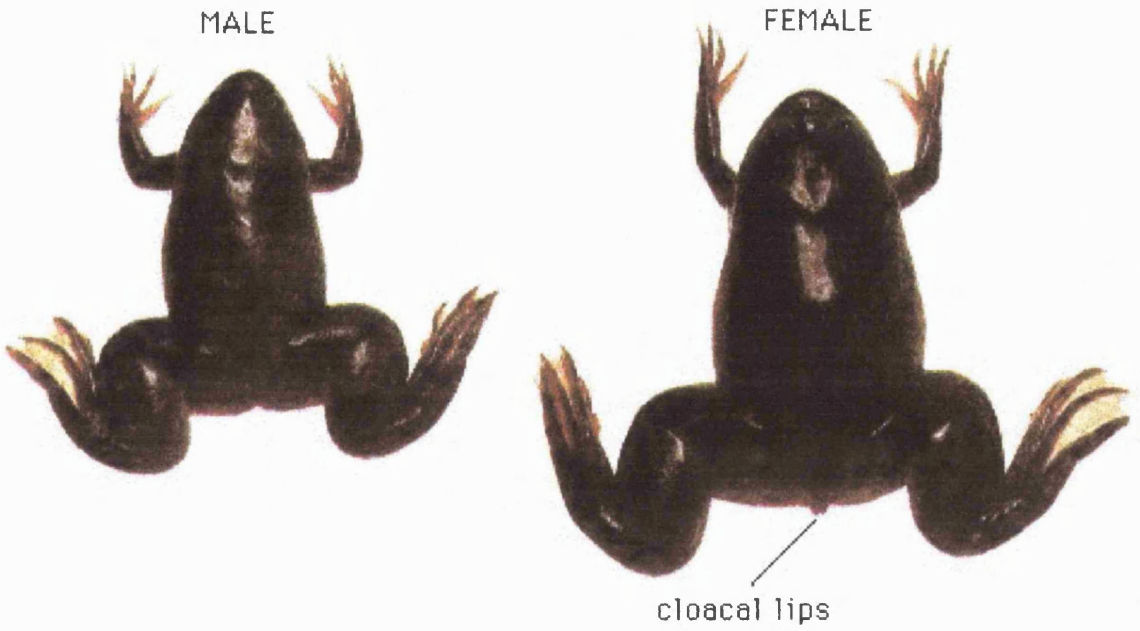
2.2.2.1 Oogenesis

A detailed account of oocyte development in laboratory maintained *Xenopus laevis* is available (Dumont, 1972). However, it is important to note that because this species is kept in such a way as to prevent normal seasonal cycles, oogenesis is continuous thus providing a constant supply of cells that are at the correct stage of development for our experiments. In 1971, Gurdon introduced the *Xenopus* oocyte expression system as a means to study aspects of control of gene expression.

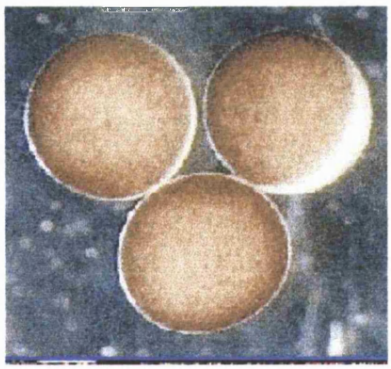
2.2.2.2 Morphology

The oocytes from *Xenopus laevis*, are germinal cells that are stored in ovarian lobes within the abdominal cavity of the frog. They consist of a dark coloured, animal, pole and a light coloured, vegetal, pole (picture 1b). Stage VI, post-vitellogenic, oocytes reach up to 1300μ in size. They are the most mature oocytes and have a clearly defined, unpigmented, equitorial band. Dumont (1972) documented the development of oocytes as occurring in 6 stages (I-VI) according to appearance and biochemical properties. Oocytes of stages V and VI are used as they exhibit the greatest rate of protein synthesis (Smith *et al.*, 1991) which permits the development of large currents and because of their size, permit the introduction of working volumes of cRNA solution. Two layers that give the cell its structural rigidity surround oocytes. A glycoprotein matrix called the vitelline membrane immediately surrounds the oocyte. Surrounding the vitelline membrane is a layer of follicular cells that are coupled to the oocyte via gap junctions. Gonadotrophin hormones influence the permeability of the gap junctions, which increases following hormonal stimulation. Such activity indicates the presence of cell surface receptors. Indeed, the follicular layer is known to possess receptors for adenosine and ATP (King *et al.*, 1995, 1996b) aswell as ecto-ATPase enzymes (Ziganshin *et al.*,1995), along with receptors for acetylcholine, noradenaline and serotonin (see Dascal, 1987 for review). Therefore it is advantageous to remove the follicular layer prior to cRNA injection. Furthermore, the rigidity that the follicular layer imparts to the cell

A



B



Picture 1. A. Photograph of an adult male and female *Xenopus Laevis* frog. Note that the fully developed female is larger than the male and that they can be further distinguished by the presence of cloacal lips on the female. B. A photograph of three stage V defolliculated oocytes. Note the contrasting pigmentation between the animal (dark) and vegetal (light) poles.

hampers penetration of the oocyte with the micropipettes. Voltage-gated ion channels exist in the cell membrane of the defolliculated oocyte but are reported to have little influence on experiments involving recombinant ligand gated ion channels. A further problem that may be encountered when using *Xenopus* oocytes as an expression system for G protein coupled receptors is that this system may lack the necessary intracellular components to link together the cascade. Clearly this is not a consideration in the function of ionotropic P2X receptors. However, a further consideration pertinent to expression of recombinant P2X receptors is that the oocyte may differ or lack the same pathways involved in synthesis of the receptor that are present in the source tissue. The possibility of such a condition is demonstrated through the different kinetic profiles for $P2X_4$ in response to ATP when expressed in Human Embryonic Kidney (HEK) 293 cells and in oocytes (Bo *et al.*, 1995; Buell *et al.*, 1996; Séguéla *et al.*, 1996). All cloned P2X receptors are known to be absent from the membranes of *Xenopus* oocytes (Dutton *et al.*, 2000). A $P2X_4$-like cDNA has been isolated from HEK 293 cells (Chang and Chang (1996), direct GenBank submission) that results in low levels of $P2X_4$ protein formation (Worthington *et al.*, 1996).

2.2.2.3 Oocyte Preparation

I. *Xenopus laevis* were anaesthetised by placing them in a closed container filled with ethyl-m-aminobenzoate, tricaine in tap water (0.4% w/v). Two basic reflexes of the frog, righting and reaction to toe pinching, were tested until the female became unresponsive. Subsequently, frogs were killed by decapitation and pithing in

accordance with Schedule-1 humane amphibia killings procedure as instructed by the Home Office. Frogs were then placed on their dorsal side and an incision made into the abdomen in order to expose the ovarian lobes. These lobes were excised and washed in Barths solution in order to remove any blood or debris from damaged oocytes. The oocytes can then be stored in Barths solution at 4°C.

II. Under a light microscope the ovarian lobes can be opened with fine tipped forceps (Dumont, 5S) to expose the folliculated oocytes. Mature oocytes at stages V and VI are plucked from the inner epithelia of the ovarian lobes and placed in a separate petri dish containing Barths solution (see **section 2.5** for solution composition)

III. Defolliculation of the oocytes is completed in two separate stages. The folliculated oocytes, isolated in step II, are then placed in Ca^{2+} free ringer solution containing collagenase Type IA (2 mg/ml) for 1 hour at 18°C. The oocytes are checked at regular intervals for damage and after 1 hour the collagenase solution is renewed and left for a further hour. Physical removal of the follicular layer can be commenced immediately following collagense treatment or the oocytes can be left overnight at 4°C in Barths solution. The cells are shrunk be placing them in double-strength, Ca^{2+}-free ringer for 20 minutes. The follicular layer is mechanically removed using the fine tipped forceps. The defolliculated oocytes are then washed in Barths solution and stored at 4°C in Barths solution, now ready for cRNA injection.

2.2.2.4 Oocyte Microinjection

The following equipment was assembled:

Absolute alcohol – Contained in a wash bottle

Alcohol burner

Boro-silicate glass – Drummon Scientific Company, PA. Outer diameter, 1.17 mm; inner diameter, 0.68 mm; Length 8 inches. Pretreated with DEPC solution for one hour (0.2% v/v). The glass is then autoclaved for 30 min (121 °C) then oven baked for 4 hours (220°C).

Glass microscope slide – Super premium, BDH, Lutterworth, Leicester.

Scalpel

Sudan IV coloured mineral oil – Both Sigma Chemical Company products. Autoclaved for 30 min (121°C)

Latex gloves

Light microscope

Microdispenser – 10µl Drummond micropipette.

Micromanipulator

Micropipettes - 10µl Gilson pipette

Nescofilm

Pipette tips – Sterile tips for micropipette

Spinal needle – Sherwood, Davis & Geck, Gosport, UK. 0.5mm inner diameter, 89mm length. Sterile.

Syringe – 1ml volume plastic sterile syringe

Vials – 10ml volume, filled with Barths solution.

The laboratory bench was cleaned with alcohol in order to provide an aseptic environment that minimises contamination with RNase enzymes. Furthermore, latex gloves were worn throughout that were also washed with alcohol. The glass slide was wiped with alcohol that was subsequently burned off with the alcohol burner. The hot slide was then covered with the Nescofilm, which was smoothed out using a sterile scalpel blade. The syringe is filled with coloured mineral oil and the spinal needle attached. Micropipettes were made be placing the boro-silicate glass in a KOPF vertical pipette puller (model 700D) and then bent mid-way along the shaft at approximately 60°. The tip of the micropipette was then broken back using fine tipped forceps to produce a micropipette with a diameter of approximately 10μM. The mineral oil can then be introduced into the micropipette by back-filling with the syringe. It is important at this stage to prevent air from entering into the micropipette. With the microdispenser firmly secured to the micromanipulator the micropipette can be placed onto the Drummond dispenser and fixed in place. Again, it is important not prevent air from entering into the micropipette. To ensure that the setup is working correctly a small drop of oil is expelled from the tip of the micropipette. Once this step is complete, an Eppendorf tube containing the required cRNA is retrieved from -80°C freezer and placed on ice. The cover is removed from the nescofilm-covered slide and placed under the light microscope. The cRNA, approximately 2μl volume, is then removed from the Eppendorf tube with the Gilson pipette and placed on the sterile nescofilm-covered slide. The cRNA can then be drawn up into the micropipette using negative-displacement that can be created by the microdispenser. Subsequently, a drop of Barths solution containing a defolliculated oocyte (section 2.2.2.3) was placed on the slide. The oocyte is positioned so that the micropipette can pierce the

surface along the equator. This was done with gentle manipulation using a Gilson pipette tip. It is important that the animal pole is not pierced as this pole contains the nucleus. The membrane of the oocyte appears as a dimple around the micropipette after the surface is pierced. To reduce this, the pipette is drawn back slightly and then cRNA (approximately 40nl) can be injected into the oocyte. After withdrawing the micropipette, the oocyte is then placed in a vial. No more than two oocytes are contained in any one vial. Prior to injecting the next oocyte it is necessary to ensure that the tip is not blocked with cytoplasmic content. Therefore, 10nl of cRNA is expelled to ensure free passage of solution. If a blockage has occurred the tip can be broken back with sterilised fine-tipped forceps.

The vials containing injected oocytes were then placed in an incubator at 19°C for 24-48 hours. Subsequently, the oocytes are stored at 4°C for up to 14 days.

2.2.3 cRNA Preparation

The cRNA was prepared courtesy of Dr. Andrea Townsend-Nicholson.

2.2.4 Electrical Recordings

2.2.4.1 Twin-Electrode Voltage Clamp

In 1982, it was first demonstrated that oocytes could be used to express functional ion channels (Barnard *et al.*, 1982). The technique used to measure currents through the expressed channels relied on adaptation of the voltage-clamp techniques developed by Cole

(1949). Broadly, the voltage-clamp technique involves clamping the cell membrane at a predetermined voltage while allowing ions to pass across the membrane by activation of ligand-gated ion channels and measuring this flow as an electric current. The twin-electrode voltage clamp technique was employed to study recombinant P2X receptors expressed in *Xenopus* oocytes.

The oocyte is impaled with two microelectrodes, a voltage recording electrode (ME_1) and current-deliverance electrode (ME_2). ME_1 is connected to a preamplifier headstage (HS-2A, Axon instruments) which records the membrane potential (V_m). V_m is calculated as the potential difference between ME_1 and the reference electrode. The reference electrode sits in a separate chamber from the oocyte in order to avoid interference from surface charges of the oocyte. V_m is compared to the command potential and the amplified difference between these signals is applied as a current through ME_2. The current that flows is proportional to the membrane conductance and therefore information can be obtained relating to the ability of compounds to affect P2X receptor activation. Two variables that must be considered when calculating transmembrane potential are tip and junction potentials. The electrolyte used to fill the electrode contributes to these values. The junction potential is the potential difference between electrode electrolyte solution and the Ringer's solution. This also contributes to the tip potential along with the resistance of the electrode. The junction potential is compensated for prior to impalement. KCl is used to fill both electrodes as K^+ and Cl^- are the most mobile of the monovalent ions and minimise the tip potential.

Mature oocytes (stages V and VI) can have large surface areas in the region of $10^6 \mu M^2$. This situation is compounded due to the invaginations that exist in the oocyte membrane thereby giving the cell membrane a high capacitance. A further consideration arises with experiments

producing currents of 10μA or greater thus potentially causing series resistance errors. Series resistance is a product of Ringer's solution, cell cytoplasm and the reference electrode. The high capacitance means that a finite time is required to maintain the voltage-clamp with the following relationship:

$$\tau = \frac{R_1 C_m}{A}$$

Were: τ = Response time

R_1 = Resistance of ME_2

C_m = Membrane capacitance

A = Gain of the amplifier

Using oocytes at a lower stage of development can reduce C_m. However, generally these cells are too small making cRNA injection difficult and have less efficient protein expression. Therefore manipulation of other variables can enable a faster response time of the clamp. Low resistance electrodes are used and the command amplifier gain is tuned. This is achieved by applying a 5mV square wave with a stimulator at the resting membrane potential and the gain increased until a square voltage-trace and a steady fast capacitative current transient were observed using an oscilloscope.

The recording equipment consisted of the following:

Amplifier – Axoclamp 2B, Axon instruments

Digital oscilloscope – Wavetek

Stimulators – Grass SD9, MP100 – Biopac systems.

MP100WSW interface – Biopac systems

Computer – Software package Acqknowledge III, Biopac systems.

The amplifier and interface allowed recordings to be made and displayed on the computer. The Grass stimulator was used to confirm impalement of the oocyte with the electrodes and to tune the amplifier with the aid of the oscilloscope. The MP100 stimulator was connected to the computer and is used to hyperpolarise the cell following impalement and to calculate input resistance.

2.2.4.2 Electrophysiological Recordings

Firstly, the flow of Ringer's solution is set at approximately 5ml/min. This is achieved by limiting the rate of gravity-driven flow from the reservoir by an adjustable clamp. Six smaller reservoirs, of 25ml volume, are washed through with Ringer's solution and are connected via an adjustable chamber selector. Perfusate is removed from the chamber in which the oocyte is placed by a suction pump. Two microelectrodes are then prepared using borosilicate glass capillaries (GC150TF-7.5. Harvard Apparatus Ltd, Kent, UK) pulled by KOPF vertical pipette puller (model 700D). The puller is set such that the resulting microelectrodes have a tip resistance of 1-2MΩ. The microelectrodes are then backfilled with 3M KCl and clamped securely into two micromanipulators (Prior) on the left and right hand sides of the oocyte chamber. Two silver-silver chloride electrodes, each attached to a preamplifer headstage, are then placed in separate microelectrodes and a seal is made using a small drop of vaseline. The tip of the electrode is then placed in the bath and the tip potential of the electrodes is compensated for using the bridge "offset" control. A cRNA injected oocyte is removed from the incubator and placed in a sygard-based chamber (0.5ml volume), held in position by a

basket arrangement of pins. Hyperpolarising current is passed (40nA, 20ms duration, 5Hz) as the oocyte is impaled along the equatorial band. Following successful impalement, the hyperpolarising current is then changed (40nA, 1000ms duration, 0.2 Hz for 20 minutes) in order to aid cell recovery and to calculate input resistance. The oocytes vary in their susceptibility to damage through the preparation process, injection and impalement. This results in variability in the input resistance of the cells. Experiments were conducted on cells that has a resistance of >1 MΩ resistance. Oocytes were clamped at a membrane potential of between –60 to –90mV prior to experimentation.

2.2.4.3 Electrophysiology experimental protocol

All drugs solutions were made up in Ringer's solution at pH 7.5 unless stated. Agonists were perfused over the voltage-clamped oocyte for a period of 60s or until measured currents reached a plateau. The agonists were then washed off for a period of 20 minutes for $P2X_1$, $P2X_3$ and $P2X_4$ and for 5 minutes for $P2X_2$.

Two different protocols were used to examine antagonist activity at recombinant P2X receptors. For generation of antagonist IC_{50} values, firstly, consistent ATP responses to an approximate EC_{70} concentration are obtained (values for P2X receptor subtypes stated in results text). Subsequently, antagonist is added to the perfusate and the response to the EC_{70} concentration of ATP is repeated in the presence of the antagonist. During each subsequent wash out period an increasing concentration of antagonist is added to the perfusate and is continued until the maximal activity of the antagonist is seen. To investigate the

pharmacological properties of the more potent antagonists concentration response curves to ATP were constructed alone and in the presence of increasing concentrations of antagonist.

2.3 Statistical Analysis

Data are presented as means±S.E.M. Number of experiments (n) are indicated in the text. Data are analysed using a commercially available software package Graphpad, Prism version 2.0. For concentration response curves to the agonist alone and in the presence of antagonist, results were expressed as a percentage of the maximum control response. The agonist concentration and antagonist concentration evoking (EC_{50}) and inhibiting (IC_{50}) half maximal responses respectively are calculated from Hill plots constructed using the formula:

$\log I/I_{max}-I$ Were I = Current evoked by each concentration of agonist

I_{max} = Maximum current

The Hill coefficient (n_H) was taken directly from the slope of the Hill plots as calculated by the statistical package.

2.4 Immunohistochemistry

2.4.1 Tissue Preparation

Male Sprague-Dawley rats (200-250g) were killed by cervical dislocation and the vasa deferentia were removed and subsequently cleared of connective tissue. The tissue was then embedded in OCT compound and frozen in isopentane pre-cooled in liquid nitrogen. The tissues were sectioned at 12μm on a cryostat (Reichert Jung CM1800), collected on gelatin-coated slides and air-dried at room temperature. The slides are stored at –20°C.

2.4.2 Antibody Preparation

Antibodies against $P2X_{1-7}$ were supplied by Roche Bioscience, Paolo Alto, CA, USA.

2.4.3 Immunolocalisation

The avid-biotin technique has been previously described (Llewellyn-Smith *et al.*, 1992, 1993). The sections were left at room temperature for 10 minutes and then fixed in 4% formaldehyde (in 0,1M phosphate buffer) containing 0.03% picric acid (pH 7.4) for 2 minutes. Endogenous peroxidase was blocked by applying 50% methanol containing 0.4% hydrogen peroxide for 10 minutes. Non-specific binding sites were blocked by a 20 minute incubation with 10% normal horse serum in phosphate-buffered saline (PBS) containing 0.05% merthiolate.

Primary antibody solution was diluted with 10% normal horse serum (NHS) to 2.5μg/ml. The sections were then incubated overnight with the diluted antibody solution. The sections are

then washed thoroughly with phosphate-buffered saline solution and incubated for 1 hour at room temperature with the secondary antibody. The secondary antibody used was a biotinylated donkey anti-rabbit IgG (Jackson Immunoresearch, PA, USA. Diluted to 1:500). Another wash step with phosphate buffer was followed by the ExtrAvidin-horseradish peroxidase conjugate diluted to 1:1500 for 1 hour. To visualise the immunoreaction a nickel-diaminobenzidine enhancement technique was used. The freshly prepared reaction mixture is placed drop-wise onto the sections until they are covered. The reaction is left to complete then the DAB is shaken off and the slides immersed in PBS to stop the reaction. The sections are then washed thoroughly with PBS and are dehydrated by placing them in increasing concentrations of alcohol for 3 minutes each time. The sections were dehydrated with xylene and mounted in Eukitt.

Two control experiments were also conducted to rule out non-specific immunoreactivity. In one experiment, the primary antibody was absent. In another, the primary antibody was replaced with rabbit pre-immune IgG.

The results were photographed using Kodak TMX 100 black and white film along with Edge R400 light microscope (Edge Scientific Instrument Company, Santa Monica, CA, USA).

2.5 Drugs, Solutions and Abbreviations

Adenosine 5′-diphosphate disodium salt (ADP): Sigma

Adenosine-5′-*O*-(α-thiotriphosphate) (ATPαS): Boehringer Mannheim, Sigma-RBI

Adenosine 5′-triphosphate disodium salt (ATP): Boehringer Mannheim, Sigma-RBI

Ammonium chloride: Sigma

Cytosine-5′-triphosphate (CTP): Sigma

P^1, P^2-Di(adenosine-5′) pyrophosphate, sodium salt (Ap_2A): Sigma

P^1, P^3-Di(adenosine-5′) triphosphate, ammonium salt (Ap_3A): Sigma

P^1,P^4-Di(adenosine-5′) tetraphosphate, ammonium salt (Ap_4A): Sigma

P^1, P^5-Di(adenosine-5′) pentaphosphate, sodium salt (Ap_5A): Sigma

P^1, P^6-Di(adenosine-5′) hexaphosphate, ammonium salt (Ap_6A): Sigma

Diaminobenzidine tetrahydrochloride: Sigma

Diethyl Pyrocarbonate (DEPC): Sigma

Eukitt: BDH, Merck

D-glucose: Sigma

Glucose oxidase solution: Sigma

Hydrogen peroxide: Sigma

i.a. : intra-arterially

i.v. : intravenous

Merthiolate: Sigma

Methanol: A.R. quality, Hayman

α,β-Methylene ATP lithium salt (αβ-meATP): Sigma

β,γ-Methylene ATP (βγ-meATP)

2-Methylthio-ATP (2-MeSATP)

Nickel ammonium sulfate: Sigma

OCT – Tissue Tek

Phosphate buffer – Sigma

Pyridoxal-5'-phosphate-6-azophenyl-2′,4′-disulphonic acid tetrasodium (PPADS): RBI

Reactive blue 2 (RB-2)

Suramin hexasodium: Sigma

Sudan IV - Sigma

Trinitrophenol-ATP (TNP-ATP)

All common salts used in the preparation of solutions were AnalaR grade (Aldrich Chemicals, UK.

The pH level of all the drugs and solutions stated in the text was achieved by the use of 1N HCl or 1N NaOH where appropriate.

Barth's solution: (pH 7.5) containing (mM): NaCl 110, KCl 1, Tris-HCl 7.5, $Ca(NO_3)_2$ 0.33, $CaCl_2$ 0.41, $MgSO_4$ 0.82, autoclaved then further addition of $NaHCO_3$ 2.4 and gentamycin sulphate 50$\mu g\ l^{-1}$.

Ba^{2+} Ringer's solution: (pH 7.5) containing (mM): NaCl 110, KCl 2.5, HEPES 5, $BaCl_2$ 1.8.

DAB reaction mixture: 5mg/ml DAB in 0.1M phosphate buffer, pH 7.4; 0.2M sodium phosphate buffer, pH 7,4. 0.4% NH_4Cl in distilled water; 20% glucose solution containing 0.05% sodium azide; distilled water; 1% Nickel ammonium sulfate in distilled water. Immediately before addition to sections glucose oxidase is added.

Formaldehyde solution: 10ml, 40% formaldehyde solution stabilised with 10% methanol (Analar, BDH); 0.2ml, saturated picric acid solution (Sigma); 50ml, 0.2M sodium phosphate buffer, pH 7.4.

Methanol/Hydrogen peroxide solution: 50ml, Absolute methanol; 50ml distilled water; 15ml 3% H_2O_2.

Krebs solution (mM): NaCl 118, $NaHCO_3$ 25, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5 and glucose 11; aerated with 95% O_2/5% CO_2.

NHS (10%)-PBS: 5ml NHS; 45ml PBS merthiolate.

PBS-merthiolate: 100ml PBS; 0.05g Thimerosal.

Ringer's solution: (pH 7.5) containing (mM): NaCl 110, KCl 2.5, HEPES 5, $CaCl_2$ 1.8.

GENERAL RESULTS SECTION

CHAPTER 3 – CHAPTER 8

CHAPTER 3

A COMPARISON OF RECOMBINANT $P2X_1$ AND NATIVE $P2X_1$-LIKE RECEPTORS

3.1 Summary

1. The pharmacology of recombinant $P2X_1$ receptors ($rP2X_1$) expressed in *Xenopus* oocytes and that of $hP2X_1$ expressed in 1321-N1 human astrocytoma cells, was compared with native $P2X_1$-like receptors in the isolated rat vas deferens and rat mesenteric artery smooth muscle cells.
2. ATP was approximately equipotent at $rP2X_1$ and $hP2X_1$ receptors (EC_{50} values of 98 and 58nM respectively). ATP was 6-fold more potent at $rP2X_1$ receptors than at $rP2X_1$-like receptors in mesenteric artery smooth muscle cells (EC_{50} = 591nM).
3. The agonist potency orders for $rP2X_1$ and $hP2X_1$ were the same: ATP=2-MeSATP > αβ-meATP. However, αβ-meATP was nearly 17-fold more potent at $hP2X_1$ receptors (EC_{50} values of 190nM and 3,185nM for $hP2X_1$ and $rP2X_1$ receptors respectively). In contrast, the antagonist potency orders differed at $rP2X_1$ receptors and $hP2X_1$ receptors. PPADS was over 30-fold more potent at $rP2X_1$ receptors than $hP2X_1$ receptors (IC_{50} values of 68nM and 2,086nM respectively). Suramin was approximately 2-fold more potent at $hP2X_1$ receptors than $rP2X_1$ receptors (IC_{50} values of 951nM and 1697nM respectively).
4. The general agonist potency orders were identical for $rP2X_1$ and $rP2X_1$-like receptors, ATP = 2-MeSATP > αβ-meATP > βγ-meATP >> CTP. However, there were several notable differences in potency values between agonists and the efficacy of βγ-meATP was higher at $rP2X_1$ receptors. PPADS was equipotent against ATP mediated and αβ-meATP mediated responses at $rP2X_1$ and $rP2X_1$-like responses in the mesenteric artery respectively.
5. All comparable diadenosine polyphosphates (Ap_nAs) were more potent and more active at $rP2X_1$ receptors than $rP2X_1$-like receptors in mesenteric artery smooth muscle cells.

6. Quarter-log point additions of agonists were used to construct agonist concentration response curves in the isolated rat vas deferens. A high-affinity component of the resultant concentration-response curves most resembled $P2X_1$ data. Here, both ATP and Ap_5A were marginally less potent than at $rP2X_1$ receptors and $rP2X_1$-like responses in mesenteric artery smooth muscle cells. This was probably due to the action of ecto-ATPase enzymes since the potency of the stable agonist αβ-meATP was close to that seen at $rP2X_1$-like receptors in the mesenteric artery and similar to $rP2X_1$ receptors (EC_{50} values of 705nM, 1,072nM and 3,185nM respectively).
7. The comparison of $rP2X_1$ receptors with native $P2X_1$-like receptors in rat mesenteric artery smooth muscle cells and rat isolated vas deferens has revealed a number of pharmacological similarities and differences between the recombinant $P2X_1$ and native $P2X_1$-like. Possible explanations for these discrepancies are discussed. The occurrence of pharmacological differences between $P2X_1$ receptor orthologues stresses the need for caution when extrapolating results between isoforms.

3. 2 Introduction

It is widely accepted that ATP acts as a fast excitatory neurotransmitter throughout the central, peripheral and enteric nervous systems (Brake and Julius, 1996; Ralevic and Burnstock, 1998; Burnstock, 1999b; Furness, 2000; Khakh and Henderson, 2000; Khakh 2001a,b). Currently, seven P2X receptor subtypes have been cloned and many mediate the fast excitatory responses to ATP (King, 1998; Ralevic and Burnstock, 1998; North and Surprenant, 2000; Khakh *et al.*, 2001a,b). Furthermore, it has been proposed that P2X receptor subunits can form at least 11 different heteromeric receptors (Torres *et al.*, 1999b). Of these multimers, at least 4 assemblies have been described functionally (North & Surprenant, 2000; Khakh *et al.*, 2001a).

The cloning of P2X receptors and their study in isolation in receptor expression systems has permitted significant advances in the field of purinergic signalling by allowing researchers to relate the properties of recombinant P2X-receptors back to the tissues from which they were cloned, or to attempt to account for specific types of ATP-responses in tissues. For example, taking the data from recombinant studies in conjunction with molecular biological and immunohistochemical results, it is clear that $P2X_2$, $P2X_3$ and $P2X_{2/3}$ receptors have a prominent role in ATP-mediated sensory and sympathetic transmission (see Dunn *et al.*, 2001 for review). Of particular interest in this thesis are the pharmacological properties of rat $P2X_1$ receptors ($rP2X_1$). $P2X_1$-like responses have been well documented in the smooth muscle of the vasculature, vas deferens and urinary bladder of the rat. The pharmacological comparison between recombinant $P2X_1$ receptors and excitatory ATP responses in these preparations has relied on a limited number of investigative tools. $P2X_1$ was cloned from the

rat vas deferens and was demonstrated to broadly have the same operational profile as P2X-mediated responses from the vas deferens tissue preparation (Valera *et al.*, 1994; Evans *et al.*, 1995; Khakh *et al.*,1995b; Evans and Surprenant, 1996). Subsequently, transcripts and positive immunoreactivity for $P2X_1$ have been found throughout the vasculature and in the urinary bladder (Valera *et al.*, 1994; Collo *et al.*, 1996; Longhurst *et al.*, 1996; Vulcanova *et al.*, 1996; Bo *et al.*, 1998; Chan *et al.*, 1998; Nori *et al.*, 1998, Lee *et al.*, 2000) thereby suggesting that this receptor subtype contributes in part, to the nucleotide mediated excitatory responses in these tissues (Evans and Surprenant, 1996; Gitterman & Evans, 2000; Lewis and Evans, 2000a; Sneddon, 2000; Burnstock, 2001). Despite the limited choice of selective pharmacological agents that can distinguish between P2X receptor subtypes, it is clear that there are functional subpopulations of native P2 receptors in tissue preparations. Perhaps the clearest demonstration of the presence of multiple contraction mediating P2 receptors has been in the vas deferens. The largest body of evidence points to a receptor that is sensitive to ATP but insensitive to suramin (Von Kügelgen *et al.*, 1990; Bailey and Hourani, 1994, 1995; Reilly and Hirst, 1996; Bültmann *et al.*, 1999). Bültmann and Starke (1994) demonstrated that the rat vas deferens contained three contractile mediating P2 receptors: P2X, P2Y and a non-P2X-non-P2Y receptor that is sensitive to PPADS (pyridoxal-5′-phosphate-6-azophenyl-2′,4′-disulphonic acid) but not suramin or Reactive blue-2. Similar findings confirmed the presence of a PPADS sensitive, suramin insensitive component to the ATP-mediated response in the rat vas deferens (Bültmann *et al.*, 1999). Furthermore, in vascular smooth muscle preparations a number of P2X receptor transcripts have been reported to overlap (Nori *et al.*, 1998; Lewis *et al.*, 2000a) and anomalous properties have been presented which may point to functional populations of P2X receptors existing along side $P2X_1$-like receptors

(Van Der Giet *et al.*, 1999). Indeed, heteromeric $P2X_{1/5}$ receptors have been proposed to be present in submucosal arterioles of the guinea-pig (Surprenant *et al.*, 2000), thereby demonstrating that presence of another contraction-mediating P2X receptor in smooth muscle is clearly viable.

The aim of this preliminary study is to investigate the pharmacological phenotype of rat $P2X_1$ ($rP2X_1$) receptors expressed in *Xenopus* oocytes and compare these results with similar studies conducted on the human orthologue ($hP2X_1$) and two tissues thought to exhibit a $P2X_1$-like pharmacology. The results demonstrate a phenotypical difference between receptor orthologues, recombinant receptor and tissue preparations and also between tissues.

3.3 Methods

3.3.1 The general methodology has been described in detail for electrophysiological studies conducted on *Xenopus* oocytes and organ bath studies using the rat vas deferens (see section 2.1.1).

3.3.2 Data for human $P2X_1$ and second/third order rat mesenteric artery cells were measured directly from the published work of the authors (Bianchi *et al.*, 1999 for human orthologue ($hP2X_1$); Lewis & Evans, 2000b for general nucleotide and antagonist data; Lewis *et al.*, 2000b for diadenosine polyphosphate data). Original graphs were photocopied and enlarged. Measurements were taken using a ruler. Data was redrawn relative to the desired maximum. Graphs were plotted using commercial software (Prism v2.0, Graphpad; San Diego, CA). Analysis parameters were as described previously in section 2.3.

3.4 Results

3.4.1 *Agonist and antagonist activity at rP2X$_1$ in Xenopus oocytes and P2X$_1$-like receptors in rat mesenteric artery smooth muscle cells.*

At rP2X$_1$ receptors expressed in *Xenopus* oocytes, ATP (1 – 10 000nM) was a potent agonist with an EC_{50} of 98nM (Figure 3.1 A.i and see Table 3.1 for EC_{50} values, confidence intervals and Hill co-efficients for all concentration-response curves at both preparations). ATP (10 – 30 000nM) was 6 fold less sensitive at rat P2X$_1$-like receptors giving an EC_{50} value of 591nM (figure 3.1B.i). Similarly, 2-MeSATP was over 4 fold more potent at rP2X$_1$ receptors than at the rat P2X$_1$-like receptors (EC_{50} value 113nM and 479nM respectively). Conversely, the stable analogue αβ-meATP is approximately 3 fold less potent at rP2X$_1$ receptors compared with rat P2X$_1$-like receptors (EC_{50} value 3185nM and 1072nM respectively). The agonist potencies for other analogues were similar with the exception of UTP (1mM), which produced an inward current approximately 10% of the ATP maximum at rP2X$_1$ receptors but was inactive at rat P2X$_1$-like receptors. Furthermore, the maximum activities of both 2-MeSATP and βγ-meATP were noticeably greater at rP2X$_1$ receptors than at rat P2X$_1$-like receptors. Also, the Hill coefficients of all the agonists at rP2X$_1$ are less than unity. With the exception of ATP, the Hill slope is greater than unity for each nucleotide at rat P2X$_1$-like receptors. GTP, ITP, and TTP were inactive at recombinant and native P2X receptors. PPADS was a potent antagonist of ATP responses at rP2X$_1$ receptors (IC_{50}, 68nM) and of αβ-meATP responses at rat P2X$_1$-like receptors (IC_{50}, 72nM; compare Figure 3.1 A.ii and B.ii). Suramin was substantially less potent against agonist responses in these preparations being 25 fold less potent than PPADS at rP2X$_1$ receptors and 66 fold less potent

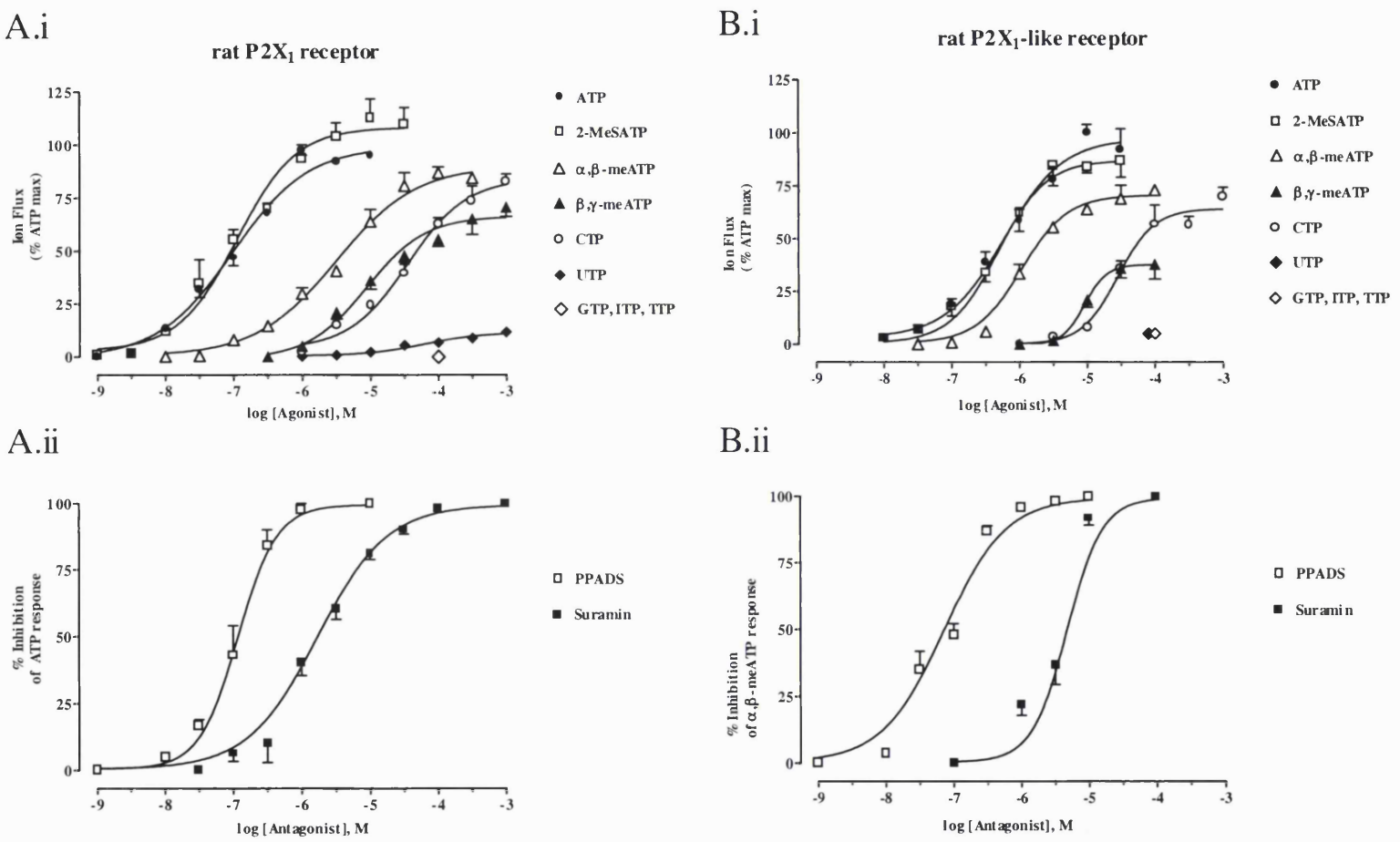


Figure 3.1 Comparison of common agonist and antagonist profiles at recombinant $rP2X_1$ receptors expressed in *Xenopus* oocytes (A.i and A.ii) and rat $P2X_1$-like receptors in rat mesenteric artery smooth muscle cells (B.i and B.ii). See table 3.1 for potency indices. $P2X_1$-like data taken from Lewis and Evans 2000b, see section 3.3.

than PPADS at rat $P2X_1$-like receptors (see figure 3.1 A.ii and B.ii and table 3.1 for IC_{50} values, confidence limits and Hill slopes for both antagonist inhibition curves).

3.4.2 Agonist and antagonist activity at rP2X$_1$ receptors expressed in Xenopus oocytes and hP2X$_1$ receptors expressed in 1321-N1 human astrocytoma cells.

Figure 3.2 A.i and B.i compare concentration-response curves for three agonists at the rat and human $P2X_1$ orthologues expressed in *Xenopus* oocytes and 1321-N1 cells respectively. ATP was approximately equipotent at $hP2X_1$ and $rP2X_1$ receptors (EC_{50} values 58nM and 98nM respectively, see Table 3.2 for EC_{50} values, confidence limits and Hill slope for each concentration response curve). 2-MeSATP was two fold more potent at $hP2X_1$ receptors than $rP2X_1$ receptors (EC_{50} values 55nM and 113nM respectively). The potency of αβ-meATP was considerably higher at $hP2X_1$ receptors compared with its activity at $rP2X_1$ receptors. The EC_{50} value generated for αβ-meATP at $hP2X_1$ receptors was 190nM which is approximately 17-fold more potent than the EC_{50} value of 3,185nM calculated for αβ-meATP at $rP2X_1$ receptors. Interestingly, the Hill slope values for the agonist concentration-response curves at $hP2X_1$ receptors are closer to 2 and, therefore, contrast with those values for these agonists at $rP2X_1$ which, as described above, are less than unity.

Bianchi and co-workers have reported that 1321-N1 astrocytoma cells lack endogenous P2 receptors (Bianchi *et al.*, 1999; Yu *et al.*, 1999). However, it appears in Figure 2 B.ii that the inhibition curves, for suramin and PPADS, are of a biphasic nature. The first phase of the antagonist inhibition curves, against an approximate EC_{75} concentration of ATP, is shallow and appears to occur between 1nm – 100nM. Thereafter, the inhibition is distinct and most

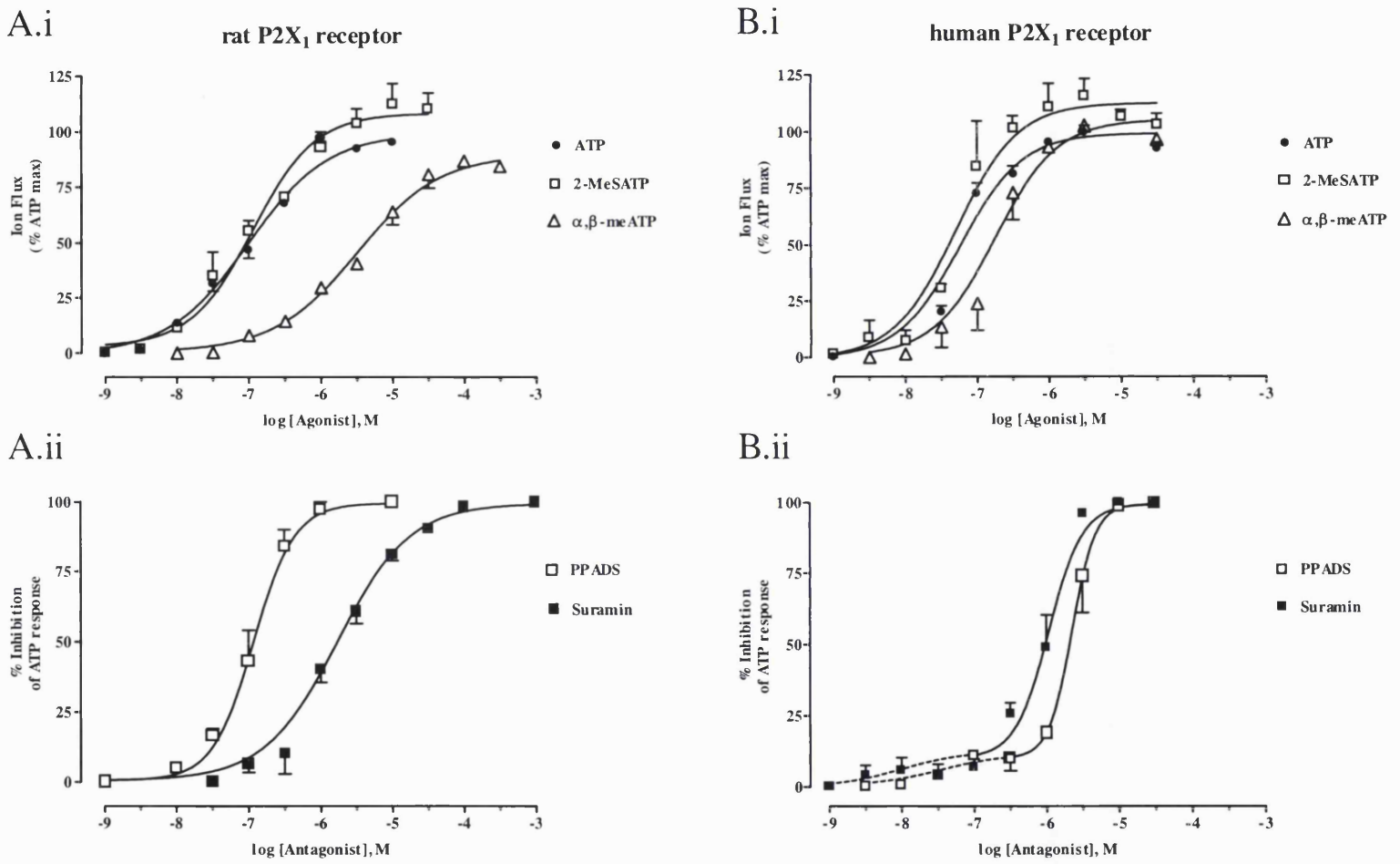


Figure 3.2 Comparison of a selection of P2X receptor agonists and antagonists profiles at recombinant $rP2X_1$ (A.i and A.ii) and $hP2X_1$ (B.i and B.ii) receptors expressed in *Xenopus* oocytes and 1321-N1 human astrocytoma cells respectively. See table 3.2 for potency indices. 1321-N1 data taken from Bianchi *et al.*, 1999, see s ection 3.3.

probably reflects the effects of the antagonist on ATP responses at $hP2X_1$ receptors. The nature of the inhibition curves casts doubt upon the homogenous nature of the P2 receptor population in this cell line. Aside from the mixed population issue in the human cell line, there are clear differences in antagonist potencies between the receptor orthologues. Most notably, PPADS is over 30 times less effective at antagonising ATP-mediated responses at $hP2X_1$ receptors than at $rP2X_1$ receptors (IC_{50} value 2086nM and 68nM respectively, see Table 3.2 for confidence limits and Hill slope values). In contrast, suramin was virtually equipotent against ATP-mediated responses at $hP2X_1$ receptors than $rP2X_1$ receptors, generating IC_{50} values of 951nM and 1,697nM respectively and, thus, making suramin more potent than PPADS at the human $P2X_1$ clone (compare Figure 3.2 A.ii and B.ii).

3.4.2 Comparison of the activity of ATP, αβ-meATP and the adenine dinucleotide series (Ap_nA, n = 2-6) between recombinant $rP2X_1$ receptors and rat $P2X_1$-like receptors in the isolated vas deferens preparation and mesenteric artery smooth muscle cells.

Figure 3.3 highlights the partial agonism of Ap_5A at $rP2X_1$ receptors. Although Ap_5A has low efficacy it is approximately 8-fold more potent than αβ-meATP (EC_{50} values are 388nM and 3185nM respectively (see Table 3.3 for EC_{50} values, confidence limits and Hill co-efficients). As noted previously, agonist concentration-response curves in the rat vas deferens do not plateau (Bailey & Hourani, 1995; Khakh *et al.*, 1995). However, constructing concentration-response curves using quarter log point increments in agonist concentration revealed a biphasic nature to the curves (Figure 3.3.B.). Analyses of the first, high affinity, phase of these curves showed αβ-meATP to be the most potent agonist at the rat vas

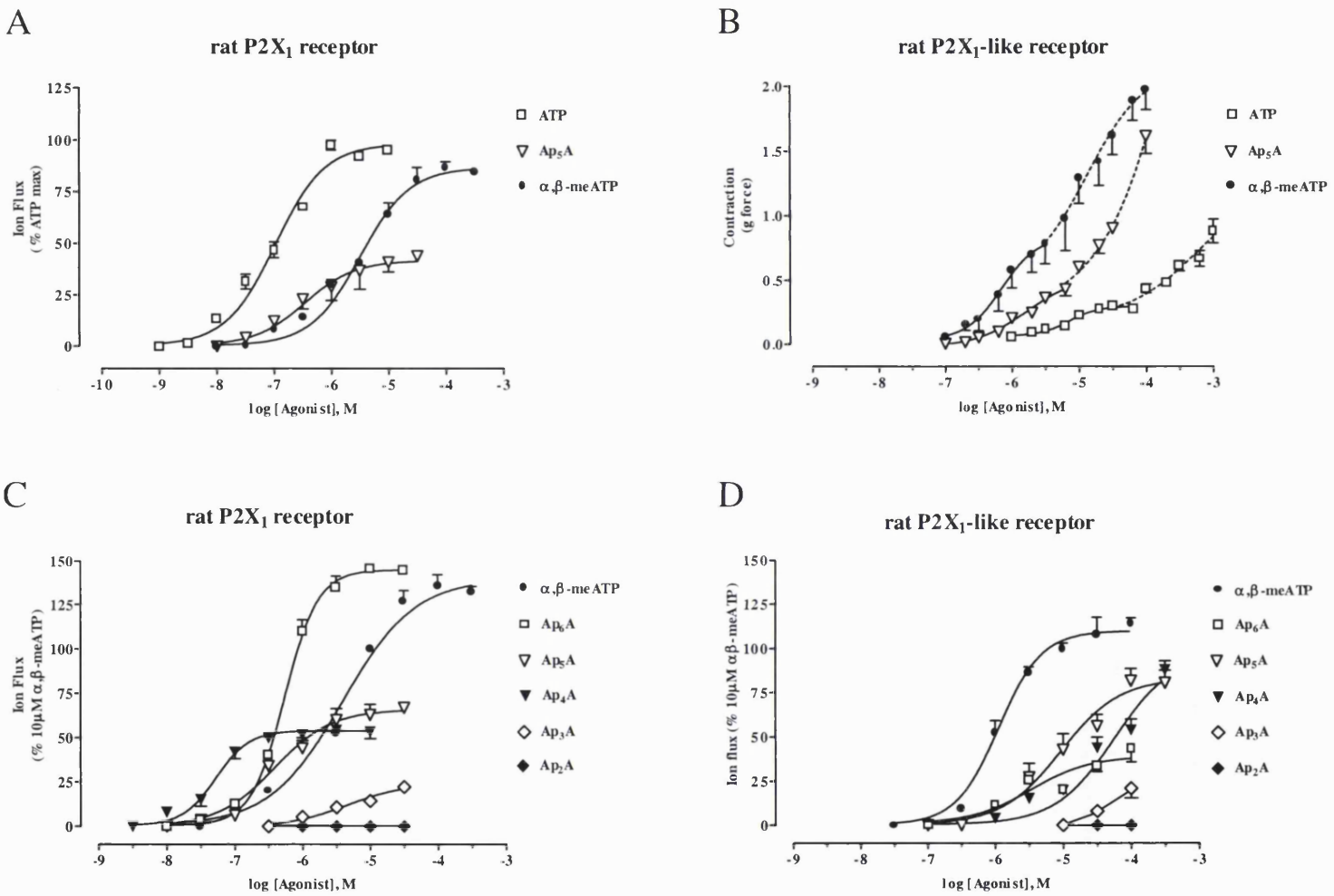


Figure 3.3 Agonist profiles at recombinant rat $P2X_1$ receptors expressed in *Xenopus* oocytes (A, C) and $P2X_1$-like receptors in the rat vas deferens (B) and rat mesenteric artery smooth muscle cells (D). See table 3.3 for potency indicies. Data in D taken from Lewis *et al.*, 2000b, see section 3.3).

deferens, generating an EC_{50} value over 4 fold lower than the EC_{50} value for αβ-meATP at recombinant $rP2X_1$ receptors (EC_{50} values 705nM and 3185nM respectively). Interestingly, the calculated potency of αβ-meATP at this high affinity component was close to the EC_{50} value of αβ-meATP at the rat $P2X_1$-like receptor in mesenteric artery smooth muscle cells (see Table 3.3). In contrast, the potency of ATP at the high affinity site was over 73 fold less at the isolated rat vas deferens than at recombinant $rP2X_1$ receptors (EC_{50} value 7,178nM and 98nM respectively). The potency of Ap_5A at the high affinity site in the isolated rat vas deferens was over 4-fold less than at $rP2X_1$ receptors (EC_{50} values 1,682nM and 388nM respectively).

There were several marked differences between the efficacy and potency of the adenine dinucleotide series at $rP2X_1$ receptors and rat $P2X_1$-like receptors in the mesenteric artery. At $rP2X_1$ receptors, Ap_4A, Ap_5A, Ap_6A all produced concentration-response curves with clearly definable maximum values. In contrast, Ap_5A was the only dinucleotide that appeared to give a definable maximum response at $P2X_1$-like receptors in mesenteric artery smooth muscle cells (compare Figure 3.3.C. and 3.3.D.). However, the maximum activity of Ap_5A in both preparations is similar (approximately 70% and 80% of 10μM αβ-meATP for $rP2X_1$ and rat $P2X_1$-like receptors respectively). The most notable difference in activity of dinucleotides between preparations is seen with Ap_4A and Ap_6A. The maximum response for Ap_4A at $rP2X_1$ receptors was approximately 50% of the response to 10μM αβ-meATP. The concentration response curve for Ap_4A did not plateau. However, 300μM Ap_4A elicited a response that was equivalent to approximately 90% of the response to 10μM αβ-meATP. Conversely, Ap_6A had a greater efficacy at $rP2X_1$ than rat $P2X_1$-like receptors (approximately, 140% and 40% of the response to 10μM αβ-meATP respectively). Notably,

Ap_4A, Ap_5A and Ap_6A are all considerably more potent at $rP2X_1$ receptors than rat $P2X_1$-like receptors in the mesenteric artery (see Table 3.3). The potency difference between the two systems for Ap_4A and Ap_6A was estimated by matched responses, that is, EC_{50} values for rP2X and estimated EC_{50} values for rat $P2X_1$-like receptors. As a result, Ap_4A is approximately 1062-fold, Ap_5A is nearly 25-fold and Ap_6A is over 6-fold more potent at $rP2X_1$ receptors than rat $P2X_1$-like receptors. Ap_3A has low activity and Ap_2A is inactive in both systems.

Table 3.1. *Activity of agonists and antagonists at recombinant and native forms of rat $P2X_1$ receptor.*

LIGAND	RAT $P2X_1$ RECEPTOR			RAT $P2X_1$-like RECEPTOR		
	EC_{50} (nM)	*CI-95% (nM)*	*n_H*	*EC_{50} (nM)*	*CI-95% (nM)*	*n_H*
ATP	98	76-127	0.77	591	273-1,280	0.83
2-MeSATP	113	69-186	0.74	479	322-691	1.22
α,β-meATP	3,185	2,324-4,365	0.75	1,072	627-1,833	1.07
β,γ-meATP	8,714	5,282-14,380	0.55	9,160	6,416-13,090	2.61
CTP	35,110	22,950-53,710	0.64	28,620	13,460-60,840	1.61
UTP	61,300	27,880-134,800	0.31	ia	-	-
GTP, ITP, TTP	ia	-	-	ia	-	-
PPADS	68	46-102	1.87	72	27-188	0.95
Suramin	1,697	1,165-2,473	0.85	4,746	4,177-53,930	1.65

Values given as the mean, and confidence intervals at the 95% level (CI-95%), of at least four determinations of the 50% effective concentration (EC_{50}) for agonism and antagonism of recombinant $P2X_1$ receptors (rat isoform) expressed in *Xenopus* oocytes and native $P2X_1$-like receptors in rat mesenteric artery. Data for the native $P2X_1$-like receptor were taken from Lewis and Evans (2000), then redrawn and reanalysed in parallel with data for rat $P2X_1$ receptor using *Prism v2.0* (GraphPad). The Hill co-efficient (n_H) is also given for concentration-response curves for each agonist and antagonist.

Table 3.2. *Comparison of agonist and antagonist activity at species orthologues of $P2X_1$ receptors.*

LIGAND	RAT $P2X_1$ RECEPTOR			HUMAN $P2X_1$ RECEPTOR		
	EC_{50} (nM)	*CI-95% (nM)*	*n_H*	*EC_{50} (nM)*	*CI-95% (nM)*	*n_H*
ATP	98	76-127	0.77	58	32-103	1.96
2-MeSATP	113	69-186	0.74	55	43-71	1.96
α,β-meATP	3,185	2,324-4,365	0.75	190	135-268	1.74
PPADS	68	46-102	1.87	2,086	1,648-2,640	2.36
Suramin	1,697	1,165-2,473	0.85	951	526-1,720	1.40

Values given as the mean, and confidence intervals at the 95% level (CI-95%), of at least four determinations of the 50% effective concentration (EC_{50}) for agonism and antagonism of rat and human orthologues of recombinant $P2X_1$ receptors. Rat $P2X_1$ receptors were expressed in *Xenopus* oocytes and human $P2X_1$ receptors in 1321-N1 human astrocytoma cells. Data for the human $P2X_1$ receptor were taken from Bianchi *et al.* (1999), then redrawn and reanalysed in parallel with data for rat $P2X_1$ receptor using *Prism v2.0* (GraphPad). The Hill co-efficient (n_H) is also given for concentration-response curves for each agonist and antagonist.

Table 3.3. *Mono- and dinucleotide activity at recombinant and native forms of rat $P2X_1$ receptor.*

LIGAND	RAT $P2X_1$ RECEPTOR			RAT $P2X_1$-like RECEPTOR (m.art.)			RAT $P2X_1$-like RECEPTOR (v.d.)		
	EC_{50} (nM)	*CI-95% (nM)*	*n_H*	*EC_{50} (nM)*	*CI-95% (nM)*	*n_H*	*EC_{50} (nM)*	*CI-95% (nM)*	*n_H*
ATP	98	76-127	0.77	591	273-1,280	0.83	7,178*	5,222-9,811	2.03
α,β-meATP	3,185	2,324-4,365	0.75	1,072	627-1,833	1.07	705*	285-1,748	1.61
Ap_6A	546	466-640	1.73	~3,310	nd	nd	nd	nd	nd
Ap_5A	388	183-815	1.02	9,251	3,904-21,930	0.68	1,682*	703-4,024	1.11
Ap_4A	53	37-76	1.76	~56,320	nd	nd	nd	nd	nd
Ap_3A	4,521	2,780-7,341	1.00	nd	nd	nd	nd	nd	nd
Ap_2A	ia	-	-	ia	-	-	nd	nd	nd

Values given as the mean, and confidence intervals at the 95% level (CI-95%), of at least four determinations of the 50% effective concentration (EC_{50}) for activation of recombinant $P2X_1$ receptors (rat isoform) expressed in *Xenopus* oocytes and native $P2X_1$-like receptors in rat mesenteric artery (m.art.) and vas deferens (v.d.). Data for the $P2X_1$-like receptor in mesenteric artery taken from Lewis *et al.* (2000), then redrawn and reanalysed in parallel with other $P2X_1$ receptor data using *Prism v2.0* (GraphPad). Determinations for $P2X_1$-like receptor in vas deferens (marked by asterisk) were based on data for the high affinity component of biphasic concentration-response curves. The Hill co-efficient (n_H) is also given for concentration-response curves for each agonist.

3.5 Discussion

3.5.1 General Summary

The pharmacological properties of recombinant $rP2X_1$ receptors expressed in *Xenopus* oocytes were compared to those of the human orthologue expressed in human astrocytoma 1321-N1 cells, and to native rat $P2X_1$-like receptors in the isolated rat vas deferens and in acutely dissociated rat mesenteric smooth muscle cells. Close comparison of like data reveals a number of distinct differences between data sets. Most notably, the potency of ATP is higher at $rP2X_1$ receptors than at rat $P2X_1$-like receptors in mesenteric artery smooth muscle cells. Also, comparing these two investigations revealed that βγ-meATP has a lower efficacy at rat $P2X_1$-like receptors in mesenteric artery smooth muscle cells than at $rP2X_1$ receptors. The potency of αβ-meATP at $hP2X_1$ receptors is considerably higher than that at $rP2X_1$ receptors. Conversely, the potency of the antagonist PPADS is much lower at $hP2X_1$ receptors, such that suramin has a higher potency index than PPADS and, therefore, is in stark contrast with $rP2X_1$ receptors. All comparable diadenosine polyphosphate concentration response curves in rat mesenteric artery smooth muscle cells were to the right of those at $rP2X_1$ receptors. Ap_4A and Ap_6A were less active at rat mesenteric artery smooth muscle cells than at $rP2X_1$ receptors. Quarter log point addition of agonist revealed a biphasic nature to the concentration response curves in the rat vas deferens. ATP and Ap_5A had a lower potency at the high affinity component compared with $rP2X_1$ receptors. Conversely, αβ-meATP had a higher potency at the high affinity component in the rat vas deferens than at $rP2X_1$ receptors. The potency of αβ-meATP is similar at $P2X_1$-like receptors in mesenteric

smooth muscle cells and the high affinity component of the agonist concentration-response curves in the vas deferens. However, Ap_5A has a higher potency at the latter.

3.5.2 Comparison recombinant rP2X$_1$ and rat P2X$_1$-like receptors

The potency order for nucleotides at $rP2X_1$ receptors and rat $P2X_1$-like receptors in mesenteric artery smooth muscle cells is ATP = 2-MeSATP > αβ-meATP > βγ-meATP >> CTP. This potency order is in agreement with previous determinations (Khakh *et al.*, 2001b). It is noticeable that there are a number of agonists that have lower efficacy values at rat $P2X_1$-like receptors in mesenteric artery smooth muscle cells when compared with $rP2X_1$ receptors. In particular, 2-MeSATP, βγ-meATP and Ap_6A. Interestingly, each of these agonists also has a lower potency at the $P2X_1$-like receptors in the mesenteric artery smooth muscle cells when compared with $rP2X_1$ receptors. In a similar vein, subtle variations between the pharmacological phenotypes in these two experimental setups are highlighted with ATP, UTP, Ap_4A and Ap_5A which all are more potent at $rP2X_1$ receptors than $P2X_1$-like receptors in the mesenteric artery smooth muscle cells. These observations are compounded by the lower potency of suramin at the latter. The purity of ATP used in the study of the mesenteric artery $P2X_1$-like receptors may have resulted in an apparent reduction in potency (see Appendix 2 for fuller analysis and discussion). However, ATP used in the study of $hP2X_1$ was obtained from same company (Research Biochemicals International) as the commercial source of ATP used by Evans and coworkers and, moreover, ATP was found to be of similar potency at $rP2X_1$ and $hP2X_1$ receptors. As there are a number of clear discriminating results between the studies on $rP2X_1$ receptors and rat $P2X_1$-like receptors, a

number of possible explanations may be put forward to explain these differences. Firstly, the $P2X_1$ receptors in the mesenteric artery are not a homogenous population. Indeed, positive immunoreactivity has been detected for $P2X_4$ and $P2X_5$ (Gitterman & Evans, 2000, Lewis & Evans, 2000b). As αβ-meATP is virtually inactive at $rP2X_4$ receptors, no activation and therefore functional presence would be detected despite effective blockade of $P2X_1$-like receptors with antagonist challenges. Therefore it is not possible to fully exclude the possibility of discrete populations of $P2X_4$ receptors. However, the presence of small populations of $P2X_4$ receptors is unlikely to explain the reduced potency and activity of the agonists mentioned above. The presence of discrete populations of homomeric $P2X_5$ or heteromeric $P2X_{1/5}$ receptors is also unlikely (Lewis and Evans, 2000b). However, the existence of a heteromeric channel consisting of combinations of P2X receptors, for which positive immunoreactivity was detected for, cannot be conclusively ruled out (Lewis and Evans, 2000b). P2Y receptors have been proposed to be present in mesenteric arteries (Gitterman and Evans, 2000) and several subtypes of the cloned P2Y receptors are sensitive to diadenosine polyphosphates (Hoyle *et al.*, 2001). Therefore, it may be possible that the apparent lower potency of Ap_nAs at $rP2X_1$-like receptors in mesenteric artery smooth muscle cells is due to the presence of P2Y receptors that bind, but are not activated by or have a low sensitivity to, Ap_nAs. Another possibility may be that the $P2X_1$-like receptor present in the mesenteric artery is a variant of the $P2X_1$ receptor cloned from the rat vas deferens. Indeed, a splice variant of the $P2X_1$ receptors have been found in megakaryocytic cell lines and platelets (Greco *et al.*, 2001). The isoform in human blood cells has been characterised functionally and termed by the authors as P_{2X1del} due to the deletion of a 17-amino acid extracellular sequence that corresponds to alternative splicing of exon 6 (Greco *et al.*, 2001).

Remarkably, the sensitivity of the receptor to ATP is decreased to the extent that ADP is a potent agonist and αβ-meATP and βγ-meATP are inactive (Greco *et al.*, 2001). If the population of these $P2X_1$ variants, or other $P2X_1$ receptors with altered pharmacology, were large enough in a tissue then it is probable that the pharmacological phenotype would be different from a homogenous wild-type $P2X_1$ receptor population. Another intriguing possibility is that the post-translational modifications and subsequent tertiary structures of the rat $P2X_1$ receptors studied are different in each case. The 17-amino acid deletion results in the loss of an N-linked glycosylation site. The resulting mature protein is 60kd which is 7kd less than the wild-type (67kd) $P2X_1$ protein found in the blood cells examined (Greco *et al.*, 2001). Previously, 2 groups independently cloned $P2X_1$ receptors with molecular masses of 60kd and 70kd from platelets (Scase *et al.*, 1998; Sun *et al.*, 1998). Only the pharmacology of the latter was investigated and it was found to be similar to the 67kd $P2X_1$ receptor isolated by Greco and colleagues. As noted, it is possible that the 60kd $P2X_1$ receptor isolated by Scase *et al.*, is in fact P_{2X1del} (Greco *et al.*, 2001). Therefore, small differences in the tertiary structure of P2X receptors may result in subtle but distinct pharmacological differences.

As the $P2X_1$ receptor has a high sensitivity for agonists, it would be reasonable to suggest that the first, high affinity, component to the concentration-response curves for vas deferens is largely due to $P2X_1$-like receptor activation. It has been demonstrated that the presence and actions of ecto-ATPase enzymes on the cell surface of smooth muscle cells significantly alters the potency of agonists susceptible to breakdown (Khakh *et al.*, 1995b). Therefore, it would be expected that ATP and Ap_5A have a low potency at the $P2X_1$-like receptors in the rat vas deferens. However, the potency of αβ-meATP approximates that seen in the rat

mesenteric artery which is higher than the potency of this agonist at $rP2X_1$ receptors. Yet, the native $P2X_1$-like receptors are dissimilar in their sensitivity to Ap_5A. Clearly, there is some unknown factor, be it in receptor processing or receptor population, which results in phenotypic differences between recombinant $rP2X_1$ and rat $P2X_1$-like receptors in different systems.

3.5.3 Comparison of rat and human $P2X_1$ orthologues.

There is a high degree of homology (approximately 90%) between the amino acid sequences of the rat and human $P2X_1$ isoforms (Valera *et al.*, 1995). However, there are two distinct pharmacological differences, namely the potencies of the agonist αβ-meATP and the antagonist PPADS. Pharmacological differences between P2X receptor orthologues are not limited to $P2X_1$. Differences in phenotype between rat and human clones have been noted for $P2X_2$, $P2X_3$ and $P2X_4$ receptors (see general Introduction, Table 1.2 and general Discussion table 9.1). Such differences highlight the need for caution when extrapolating results between recombinant rat and human P2X receptors.

3.5.4 Conclusions

Pharmacological anomalies have been highlighted in this investigation that occur between recombinant $rP2X_1$ receptors and native rat $P2X_1$ receptors and may be explained in one of several ways. Either, the native populations of receptors are not homogeneous, that is mixed with other homo- or heteromeric P2 receptors, or that there are variants of the $P2X_1$ receptor

be it in primary sequences (i.e. splice variant) or in tertiary structure. The occurrence of pharmacological differences between species homologue is an important consideration when conducting experiments on human tissues.

CHAPTER 4.

SELECTIVITY OF DIADENOSINE POLYPHOSPHATES FOR RAT P2X RECEPTOR SUBUNITS.

4.1 Summary

1. The activity of diadenosine polyphosphates was studied at three recombinant P2X receptors ($rP2X_1$, $rP2X_3$, $rP2X_4$) expressed in *Xenopus* oocytes and compared to known data for $hP2X_1$ and $rP2X_2$ receptors.
2. At the $rP2X_1$ receptor, only Ap_6A was a full agonist (EC_{50} value, 0.7±0.1μM; n=4) yet 2-3 fold less potent than ATP (EC_{50} value, 0.3±0.01μM; n=4). Ap_3A, Ap_4A and Ap_5A were all partial agonists (respective EC_{50} values, >100μM; 0.04±0.01μM; 0.9±0.1μM; all n=4).
3. At the $P2X_3$ receptor, Ap_4A, Ap_5A and Ap_6A were full agonists (respective EC_{50} values, 0.8±0.1μM; 1.3±0.3μM; 1.6±0.4μM; all n=4) and more potent than ATP (EC_{50} value, 1.8±0.3μM; n=4). Ap_2A potentiated (175±26%) control responses to submaximal ATP (EC_{25}).
4. Ap_4A (EC_{50} value, 3.0±0.4μM; n=4) alone was equipotent with ATP (EC_{50} value, 4.1±1.0μM; n=4) at the $rP2X_4$ receptor, but only a partial agonist. Both Ap_2A and Ap_3A maximally potentiated submaximal ATP responses (EC_{40}) by 146±7% and 154±13% respectively.
5. These findings contrast with $rP2X_2$ receptors where only Ap_4A is a full agonist (EC_{50} value, 15.2±1.0μM; n=4) yet 4-fold less potent than ATP (EC_{50} value, 3.7±0.7μM; n=4) and Ap_5A potentiates submaximal ATP activity.
6. These data provide a useful basis for selective agonists and modulators of P2X receptor subunits.

4.2 Introduction

Diadenosine polyphosphates (Ap_nA, n = 2-6) are naturally-occurring adenine dinucleotides, in which two adenine molecules are linked at the 5′ position of the ribose moiety by a chain of phosphates varying from 2 to 6 in length. These adenine dinucleotides possess both intracellular and extracellular actions, are concentrated in central synaptosomes, and are released in a Ca^{2+}-dependent process from brain slices after nerve stimulation (for a review, see: Hoyle *et al.,* 2001).

Extracellularly, diadenosine polyphosphates bind to and activate both P2X and P2Y receptor subtypes in a number of tissues and with a variety of effects (Hoyle, 1990; Abbracchio & Burnstock, 1995; Pintor & Miras-Portugal, 1995b; Ogilvie *et al.,* 1996). For the P2X receptor family, seven subtypes ($P2X_{1-7}$) have been cloned so far (North & Barnard, 1997), although the pharmacological activity of the diadenosine polyphosphate family has only been studied at the human $P2X_1$ ($hP2X_1$) expressed in *Xenopus* oocytes and HEK 293 cells (Evans *et al.,* 1995) and rat $P2X_2$ ($rP2X_2$) receptors expressed in *Xenopus* oocytes (Pintor *et al.,* 1996).

In the present study, the pharmacological survey of adenine dinucleotide activity has been extended to include three recombinant P2X receptors ($rP2X_1$, $rP2X_3$ and $rP2X_4$).

4.3 Methods

Xenopus oocytes were harvested and prepared, as previously described (Wildman *et al.*, 1998). Defolliculated oocytes were injected cytosolically with cRNA encoding either rat $P2X_1$ (Valera *et al.*, 1994), rat $P2X_3$ (Chen *et al.*, 1995) or rat $P2X_4$ (Bo *et al.*, 1995) receptors, incubated for 48 h at 18°C in Barth's solution and kept for 5-10 days at 4°C until used in electrophysiological experiments. ATP-activated inward-currents (I_{ATP}) were recorded from injected oocytes using a twin-electrode voltage-clamp amplifier (Axoclamp 2B; V_h = -60mV to -90mV). Voltage-recording and current-recording microelectrodes (1-5MΩ tip resistance) were filled with 3.0M KCl. Oocytes were placed in a Perspex recording chamber and superfused with modified Ringer's solution containing (mM) NaCl, 110; KCl, 2.5; HEPES, 5; $BaCl_2$, 1.8, adjusted to pH7.5.

All solutions were made up in modified Ringer's solution which was nominally Ca^{2+}-free. Omission of extracellular Ca^{2+} prevented the activation of Ca^{2+}-dependent Cl^--channels in *Xenopus* oocytes secondary to Ca^{2+} influx through the ion-channel gated by $P2X_1$ (Valera *et al.*, 1994), $P2X_3$ (Chen *et al.*, 1995) and $P2X_4$ (Bo *et al.*, 1995) receptors. ATP and the adenine dinucleotides (Ap_nA series) were prepared in Ca^{2+}-free Ringer's solution (at the concentrations stated in the text). Agonists were added for 120s or until membrane currents peaked, then washed out for a period of 20min. This extended washout period was necessary to ensure successive ATP-responses (applied at the EC_{50} value) were of constant amplitude. Adenine dinucleotides, when used as modulators, were superfused for 20 minutes prior to (and during) superfusion of submaximal concentrations of ATP ($rP2X_1$, 0.1μM; $rP2X_3$, 1μM; $rP2X_4$, 3μM). The pH of the Ringer's solution, and all drugs used, was adjusted to pH 7.5 by adding either 1.0N HCl or 1.0N NaOH, to take into

account that agonist activity at P2X receptors is sensitive to changes in pH (King *et al.*, 1996a, 1997b; Stoop *et al.*, 1997).

Data are presented as mean±s.e.mean of 4 sets of data from different oocyte batches. Significant differences were determined by Student's *t*-test, using a commercial software package (*Instat*, v2.05A; GraphPad).

All common salts were AnalaR grade (BDH, UK). ATP disodium salt was purchased from Boehringer Mannheim (Germany) while all adenine dinucleotides (Ap_3A, Ap_4A and Ap_6A ammonium salts, Ap_2A and Ap_5A sodium salts) were purchased from Sigma Chemical Co. (Poole, Dorset UK). The purity of commercially-prepared diadenosine polyphosphates has been assessed beforehand and no significant contamination (<1%) with ATP seen (Pintor *et al.*, 1996).

Results

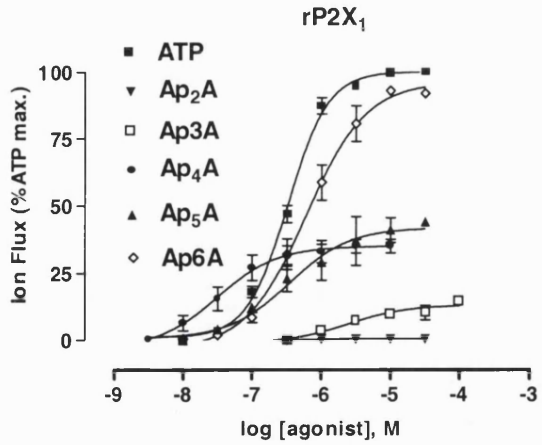
4.4.1 Agonist activity of adenine dinucleotides at rP2X$_1$ receptor

ATP (0.01-30μM) evoked inward membrane currents in defolliculated *Xenopus* oocytes expressing rP2X$_1$ receptors (EC_{50} value, 0.30±0.01μM; Hill co-efficient (n_H), 1.5±0.1; n=4). Of the dinucleotide series tested, only Ap_6A was a full agonist (EC_{50}, 0.9±0.1μM; n_H, 1.2±0.2; n=4) but 2-3 fold less potent than at ATP at the rP2X$_1$ receptor. Both Ap_4A and Ap_5A were partial agonists with maximal activity as low as 40% of the ATP maximum (Fig. 4.1A). However, Ap_4A (EC_{50}, 38±11nM; n_H, 1.2±0.1; n=4) was 8-fold more potent than ATP, whereas Ap_5A (EC_{50}, 0.90±0.1μM; n_H, 1.0±0.1; n=4) was 2-3 fold less potent. Ap_2A (0.1-30μM) was inactive as an agonist and, furthermore, neither antagonised nor potentiated ATP-responses.

4.4.2 Agonist activity of adenine dinucleotides at rP2X$_3$ receptor

ATP (0.01-100μM) evoked inward membrane currents in oocytes expressing rP2X$_3$ receptors (EC_{50}, 1.8±0.3μM; n_H, 0.7±0.05; n=4). Three dinucleotides were full agonists at rP2X$_3$ and were as potent as ATP, or more so: Ap_4A (EC_{50}, 0.80±0.12μM; n_H, 0.9±0.1; n=4); Ap_5A (EC_{50}, 1.3±0.3μM, n_H, 0.7±0.1; n=4); Ap_6A (1.6±0.4μM; n_H, 0.8±0.1; n=4). Ap_3A was a partial agonist with maximal activity as low as 60% of the ATP maximum (Fig. 4.1B), although this dinucleotide (EC_{50}, 1.0±0.5μM; nH, 0.8±0.1; n=4) was as potent as ATP. Ap_2A (0.1-30μM) was inactive as an agonist. However, Ap_2A (100μM) caused a modest potentiation (175±26%) of control responses (taken as 100%) to

A



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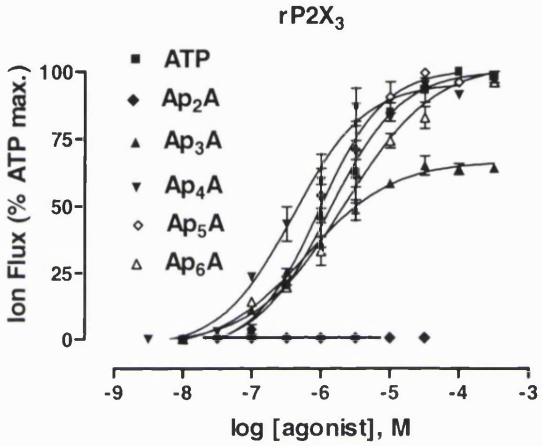

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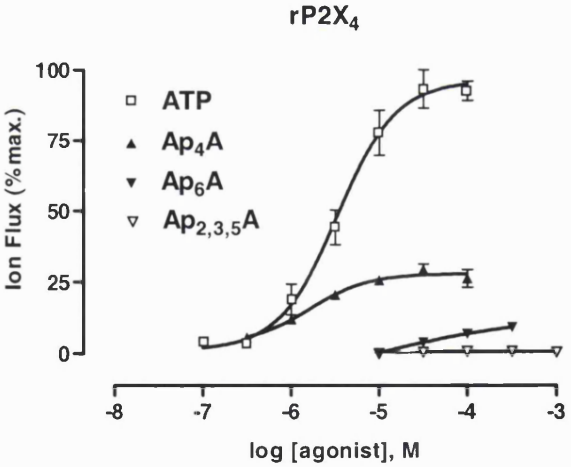


Figure 4.1 Adenine dinucleotide activity at rat $P2X_1$, $P2X_3$ and $P2X_4$ receptors. Concentration-response curves for ATP and the adenine dinucleotide series (Ap_nA, n = 2-6) at $P2X_1$ (A), $P2X_3$ (B) and $P2X_4$ (C). Agonist activity was normalized to the maximal response to ATP in each experiment. Agonist potency was determined as the EC_{50} value for each curve, for four determinations per agonist. EC_{50} vaues are given in Table 4.1 and in the text. Hill coefficients for agonists are also given in the text. Curves were fitted using the Hill equation, as defined by Prism v2.0 (Graphpad).

submaximal ATP (0.3μM, approximately EC_{25}) and this effect was reversed on washout. The EC_{50} value for this potentiation was 8.3±0.7μM (n=4).

7.4.3 Agonist activity of adenine dinucleotides at rP2X$_4$ receptor

ATP (0.1-100μM) evoked inward membrane currents in oocytes expressing rP2X$_4$ receptors (EC_{50}, 4.1±1.0μM; n_H, 1.2±0.1; n=4). Ap_4A was as potent as ATP at rP2X$_4$ (EC_{50}, 3.0±0.4μM; n_H, 1.1±0.2; n=4), although a partial agonist with a maximal activity as low as 30% of the ATP maximum (Fig. 4.1C). Ap_6A (30-300μM) was much less active than either ATP or Ap_4A, evoking maximal responses as low as 10% of the ATP maximum (n=4). Ap_2A, Ap_3A and Ap_5A (10-300μM) were inactive as agonists. However, Ap_2A and Ap_3A (1-100μM) potentiated ATP-activated currents at rP2X$_4$ receptors in a concentration-dependent manner. ATP-responses (to 3μM, approximately EC_{40}) were maximally increased by 146±7% (Ap_2A: EC_{50}, 1.6±0.8μM) and 154±13% (Ap_3A: EC_{50}, 0.93±0.12μM). The potentiating effects of Ap_2A and Ap_3A were reversed on washout. Ap_5A (1-30μM) was inactive, either as a modulator or antagonist, against ATP-activated currents.

4.5 Discussion

In the present study, it was found that the diadenosine polyphosphate series (Ap_nA, n=2-6) showed different patterns of activity at three recombinant P2X receptors ($rP2X_1$, $rP2X_3$, $rP2X_4$) (see Table 4.1), than at recombinant $hP2X_1$ (Evans *et al.*, 1995) and $rP2X_2$ receptors (Pintor *et al.*, 1996).

Only Ap_6A was as active as ATP at $rP2X_1$, although the potency order (based on EC_{50} values) was Ap_4A>ATP>Ap_6A=Ap_5A. Ap_4A and Ap_5A were partial agonists at $rP2X_1$; in the same vein, Ap_5A is a partial agonist at the human orthologue ($hP2X_1$) (Evans *et al.*, 1995). Four dinucleotides were as active as ATP at $rP2X_3$ and showed a potency order $Ap_4A \geq Ap_3A > Ap_5A = Ap_6A \geq ATP$. However, Ap_3A was a partial agonist while the other three dinucleotides were full agonists. Ap_4A and Ap_6A activated the $rP2X_4$ receptor with a potency order $Ap_4A \geq ATP >> Ap_6A$, although neither dinucleotide was a full agonist. Ap_4A was about 30% as active as ATP, while Ap_6A was a relatively weak (10% maximal activity). These results for $rP2X_1$, $rP2X_3$ and $rP2X_4$ contrast with data for $rP2X_2$, where Ap_4A alone is as active as, but less potent than, ATP (Pintor *et al.*, 1996). However, a consistent finding with all four P2X subunits was an inability by Ap_2A to act as an agonist. $P2X_{1-4}$ subunits are relatively insensitive to ADP and adenine diphosphates (*e.g.*, ADPβS, 2-MeSADP) compared to ATP and adenine triphosphates (e.g., ATPγS and 2-MeSATP) (King, 1998), and this trend appears to hold true for the diphosphate of Ap_2A.

The combined results shown in Table 4.1 reveal the potential to discriminate between P2X subunits on the basis of the activity and potency of diadenosine polyphosphates relative to ATP. For fast desensitizing P2X subunits, Ap_6A is a full agonist at $rP2X_1$ and $rP2X_3$, whereas the latter is also fully activated by Ap_4A and Ap_5A and the former is not.

Table 4.1 Agonist activity of ATP and adenine dinucleotides at recombinant $P2X_{1-4}$ receptors

	$rP2X_1$	**$rP2X_2$**	**$rP2X_3$**	**$rP2X_4$**
ATP	0.30±0.01μM	3.7±0.7μM	1.8±0.3μM	4.1±1.0μM
Ap_2A	inactive 0.1-30μM	inactive 1-100μM	***Potentiator*** 8.3±0.7μM	***Potentiator*** 1.6±0.5μM
Ap_3A	***partial agonist*** EC_{50}>100μM	inactive 1-100μM	***partial agonist*** 1.0±0.5μM	***Potentiator*** 0.9±0.1μM
Ap_4A	***partial agonist*** 0.04±0.01μM	***full agonist*** 15.2±1.0μM	***full agonist*** 0.80±0.12μM	***partial agonist*** 3.0±0.4μM
Ap_5A	***partial agonist*** 0.9±0.1μM	***Potentiator*** 3.0±0.7μM	***full agonist*** 1.3±0.3μM	Inactive 0.1-30μM
Ap_6A	***full agonist*** 0.72±0.08μM	inactive 0.1-100μM	***full agonist*** 1.6±0.4μM	***partial agonist*** EC_{50}>100μM

Activity and potency of ATP and the adenine dinucleotide series (Ap_nA, n=2-6) at recombinant homomeric $P2X_1$-$P2X_4$ receptors expressed in *Xenopus* oocytes. Potency indices are expressed as EC_{50} values (mean±SEM; n=4). Full agonists matched the maximal activity of ATP, whereas partial agonists failed to do so, potentiators were tested on a submaximal concentration of ATP (approx. EC_{40}). Data for $rP2X_2$ taken from Pintor *et al.* (1996). Ap_5A is a partial agonist (~50% of maximal ATP activity, with an EC_{50} value of 0.8μM) at $hP2X_1$ receptors (Evans *et al.*, 1995).

Also, Ap_3A is reasonably potent at $rP2X_3$ but not at $rP2X_1$. For the slowly-desensitizing P2X subunits, Ap_4A is a full agonist at $rP2X_2$ but not at $rP2X_4$. These two P2X subunits are found throughout the PNS and CNS (Vulchanova *et al.*, 1996; Lê *et al.*, 1998) and their different pharmacological profiles for dinucleotides may provide the means to identify these functional P2X subunits in P2X receptors in neural tissue.

Transcripts for $rP2X_2$ and $rP2X_4$ also co-localise with $rP2X_1$ in blood vessels (Nori *et al.*, 1998). Potentially, the relative activities of Ap_6A and Ap_4A at these three P2X subunits may help reveal their presence in P2X receptors in different vascular beds. Transcripts for $rP2X_2$ and $rP2X_4$ also co-localise with $rP2X_3$ in neurons of sensory ganglia (Collo *et al.*, 1996). It will be interesting to see the relative activities of diadenosine polyphosphates at native P2X receptors in rat sensory (DRG) ganglia where, it is believed, the pharmacological and biophysical profiles of this P2X receptor change with age, from the $P2X_3$ phenotype in neonatal tissue (Robertson *et al.*, 1996) to the $P2X_{2/3}$ heteromultimer in older sensory (DRG) ganglia (Evans & Surprenant, 1996). The activity of diadenosine polyphosphates has been studied for P2X receptors in sensory neurons of rat nodose ganglia, where Ap_4A was reported to be a partial agonist and Ap_2A, Ap_3A and Ap_5A were considered to be antagonists (Krishtal *et al.*, 1988a).

It has also been shown that several adenine dinucleotides devoid of agonist activity were modulators of ATP-activity at P2X subunits. Ap_2A was not an agonist at the $rP2X_3$ receptor but, instead, reversibly potentiated ATP-responses. Similarly, Ap_2A and Ap_3A were not agonists of $rP2X_4$ yet reversibly potentiated ATP-activated currents. These modulatory actions are reminiscent of the actions of Ap_5A which selectively potentiates ATP-responses at $rP2X_2$ receptors by shifting the concentration-response curve for the agonist leftwards (Pintor *et al.*, 1996). Neither Ap_2A nor Ap_3A has agonist or modulatory activity at the $rP2X_2$ receptor (Pintor *et al.*, 1996). The degree of Ap_2A potentiation at

$rP2X_3$ and $rP2X_4$ receptors was modest, increasing the amplitude of ATP-responses by 1-2 fold. Ap_5A potentiation at $rP2X_2$ receptor also was modest, showing a 1-2 fold increase in agonist activity (Pintor *et al.*, 1996). Recently, Ap_5A has been shown to potentiate ATP-responses in rat cerebellar astrocytes (Jimenez *et al.*, 1998), although such ATP-responses appear be mediated by metabotropic ATP receptors. If true, these data indicate that some diadenosine polyphosphates have the capacity to potentiate ATP responses at both P2X (ionotropic) and P2Y (metabotropic) receptor subtypes.

In conclusion, it is evident that there is some selectivity in the actions of the adenine dinucleotides at the P2X subunits tested so far ($hP2X_1$, $rP2X_1$, $rP2X_2$, $rP2X_3$ and $rP2X_4$ receptors). A picture is emerging that these P2X subunits are affected by certain adenine dinucleotides in different ways. Some adenine dinucleotides are either selective agonists or modulators of P2X subtypes. Accordingly, these compounds might prove to be useful tools in identifying P2X subtypes in endogenous P2X receptors in whole tissues when, currently, there is a lack of selective agonists and antagonists for these ligand-gated ion channels.

CHAPTER 5

ACTIVITY OF NOVEL ADENINE NUCLEOTIDE DERIVATIVES AS AGONINSTS AND ANTAGONISTS AT RECOMBINANT P2X RECEPTORS

5.1 Summary

1. The effects of structural modifications of adenine nucleotides previously shown to enhance either agonist (2-thioether groups) or antagonist (additional phosphate moieties at the 3′- or 2′-position) properties at $P2Y_1$ receptors were examined at recombinant rat $P2X_1$, $P2X_2$, $P2X_3$, and $P2X_4$ receptors expressed in *Xenopus* oocytes.
2. The potency of $P2Y_1$ agonists HT-AMP (2-(hexylthio)adenosine-5′-monophosphate) and PAPET (2-[2-(4-aminophenyl)ethylthio]adenosine-5′-triphosphate) was examined at P2X receptors. Both nucleotides showed a preference for the Group I ($\alpha\beta$-meATP-sensitive, fast-inactivating) P2X subunits. HT-AMP was 5-fold more potent than ATP at $P2X_3$ receptors and a partial agonist at all except $P2X_2$ receptors, at which it was a full agonist. The efficacy of HT-AMP was as low as 23% at $P2X_4$ receptors. PAPET was a weak partial agonist at rat $P2X_4$ receptors and a nearly full agonist at the other subtypes. At rat $P2X_3$ receptors, PAPET was more potent than any other known agonist (EC_{50} = 17 ± 3 nM).
3. MRS 2179 (N^6-methyl-2′-deoxyadenosine 3′, 5-bisphosphate, a potent $P2Y_1$ receptor antagonist) inhibited ATP-evoked responses at rat $P2X_1$ receptors with an IC_{50} value of 1.15 ± 0.21 μM. MRS 2179 was a weak antagonist at rat $P2X_3$ receptors, with an IC_{50} value of 12.9 ± 0.1 μM, and was inactive at rat $P2X_2$ and $P2X_4$ receptors. Thus, MRS 2179 was 11-fold and 130-fold selective for $P2Y_1$ receptors versus $P2X_1$ and $P2X_3$ receptors, respectively. MRS 2209, the corresponding 3′-deoxy-2′-phosphate isomer, was inactive at rat $P2X_1$ receptors, thus demonstrating its greater selectivity as a $P2Y_1$ receptor antagonist.
4. Various adenine bisphosphates in the family of MRS 2179 containing modifications of either the adenine ($P2Y_1$ antagonists with 2- and 6-substitutions), the phosphate (a 3′,5′-cyclic diphosphate, inactive at $P2Y_1$ receptors), or the ribose moieties (antagonist

carbocyclic analogue), were inactive at both rat $P2X_1$ and $P2X_3$ receptors. An anhydrohexitol derivative (MRS 2269) and an acyclic derivative (MRS 2286) proved to be selective antagonists at $P2Y_1$ receptors, since they were inactive as agonist or antagonist at $P2X_1$ and $P2X_3$ receptors.

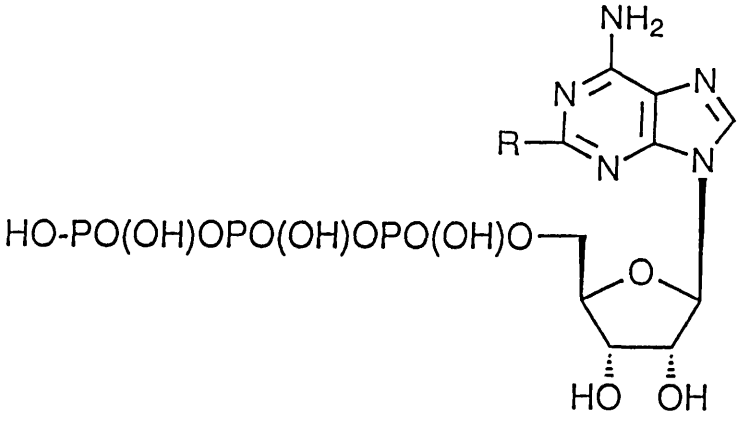
5.2 Introduction

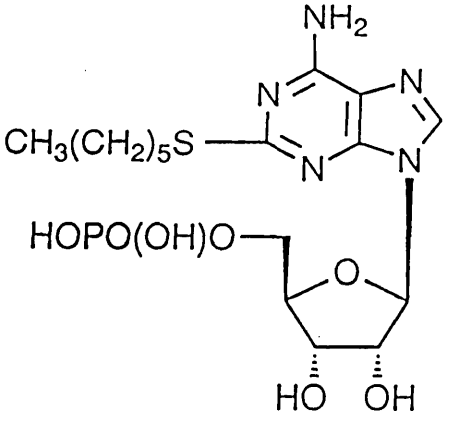
Extracellular ATP and other adenine nucleotides play an important physiological role in the central and peripheral nervous systems and in the immune, cardiovascular, renal, and musculoskeletal systems through the activation of P2 receptors (North and Barnard, 1997; Burnstock, 1996). Two families of P2 receptors have been defined (Abbracchio and Burnstock, 1994): ligand-gated cation channels (P2X subtype), activated by adenine nucleotides, and G-protein coupled receptors (P2Y subtype), most of which are activated by both adenine and uracil nucleotides. Several of the P2Y subtypes are activated exclusively by uracil nucleotides (Communi and Boeynaems, 1997). $P2X_{1\text{-}7}$ and $P2Y_{1,2,4,6,11,12}$ designations have been unambiguously assigned to mammalian nucleotide receptors (Burnstock and King, 1996; Fredholm et al., 1997; Communi et al., 1997; King *et al.*, 2001), although there is still uncertainty about the correspondence of these cloned sequences to the pharmacological phenotypes of native P2 receptors. The $P2X_3$ receptors found in dorsal root ganglion neurons are a therapeutic target for pain control (Burgard et al., 1999). Synthetic ligands which display high potency and/or selectivity at various subtypes of P2 receptors are currently being designed (Lambrecht et al., 1996; Williams and Bhagwat, 1996; Jacobson et al., 1997; Fischer, 1999). One of the first advances in the design of highly potent P2 receptor agonists was the introduction of 2-thioether groups, i.e. analogues of 2-MeSATP. For example, PAPET (2-[2-(4-aminophenyl)ethyl-thio]adenosine-5′-triphosphate) and 2-cyanohexylthioATP (Figure 5.1) were shown to activate turkey erythrocyte $P2Y_1$ receptors with EC_{50} values of 2.5 and 1.0 nM, respectively (Fischer et al., 1993). The linkage of ATP with bulky 2-thioether groups not only enhanced affinity, but also decreased susceptibility to degradation by nucleotidases (Zimmet et al., 1993). In the series of 5′-monophosphates, certain 2-thioether groups enhanced potency as P2 receptor agonists. Thus, Boyer et al. (1996) found that HT-AMP

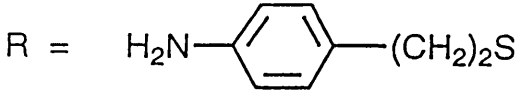
(2-(hexylthio)adenosine-5′-monophosphate) (Figure 5.1) was 72-fold more potent than ATP in activating turkey P2Y 1 receptors, with an EC_{50} of 59 nM. Compound 3 (2-(5-hexenyl)thioadenosine-5'-triphosphate, MRS 2055), is a moderately potent agonist at the adenylate cyclase-coupled P2Y receptor [$P2Y_{AC}$, Boyer et al., 1996] and inactive at $P2Y_1$ receptors.

Selective antagonists have been designed for $P2Y_1$ receptors (Camaioni et al., 1998; Boyer et al., 1998; Nandanan et al., 1999) and the newly cloned $P2Y_{12}$ receptors (Ingall et al., 1999). MRS 2179 (N^6-methyl-2′deoxyadenosine 3′,5′-bisphosphate), 4, a bisphosphate derivative (Figure 5.1), was found to be a pure, competitive antagonist at turkey and human $P2Y_1$ receptors, with a K_B value of 100 nM (Boyer et al., 1998). Before such ligands can be used as definitive pharmacological probes, for example in platelets, in which $P2X_1$, $P2Y_1$, and $P2Y_{12}$ receptors coexist (Jin et al, 1998; Hechler et al., 1998; Fagura et al., 1998), it is necessary to explore their activity at the full range of P2 receptors (Bianchi et al, 1999). This is the first study of the activity of these novel agents at recombinant P2X receptors.

Figure 5.1 Structure of adenosine-5′-triphosphate and monophosphate 2-thioester derivatives previously found to be potent P2 receptor agonists.





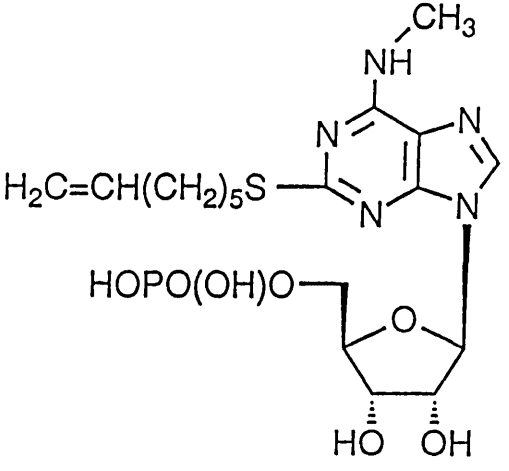
R = 

1a, 2-(4-aminophenylethylthio)-ATP

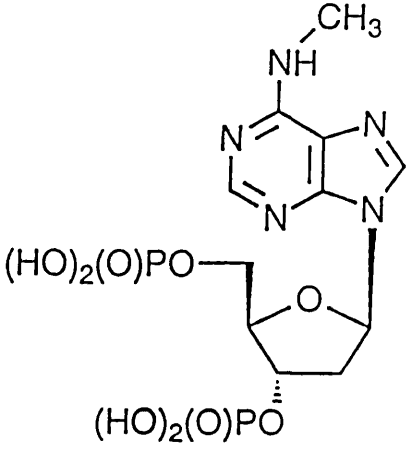
R = $CN(CH_2)_6S$

1b, 2-cyanohexylthio-ATP

2, 2-hexylthio-AMP



3, MRS 2055



4, MRS 2179

5.3 Methods

Adenosine nucleotide derivatives were synthesized as reported [Fischer et al., 1993; Boyer et al., 1996; Nandanan et al., 1999; Kim et al., 2000].

5.3.1 Antagonist activity at recombinant P2X receptors

Xenopus oocytes were harvested and prepared as previously described [King et al., 1997]. Defolliculated oocytes were injected cytosolically with 40 nl of a solution of cRNA of rat $P2X_1$, $P2X_3$, or $P2X_4$ receptors (1 μg/ml) or rat $P2X_2$ receptors (0.002 μg/ml) incubated for 24 h at 18°C in Barth's solution and kept for up to 12 days at 4°C until used in electrophysiological experiments. ATP-activated membrane currents (V_h = -90 mV) were recorded from cRNA-injected oocytes using the twin-electrode voltage-clamp technique (Axoclamp 2B amplifier). Voltage recording (1-2 ΩM tip resistance) and current-recording microelectrodes (5 ΩM tip resistance) were filled with 3.0 M KC1. Oocytes were held in an electrophysiological chamber and superfused with Ringer's solution (5 ml/min, at 18°C) containing (mM) NaCl, 110; KCI, 2.5; HEPES, 5; $BaC1_2$, 1.8, adjusted to pH 7.5. ATP was superfused over oocytes for 120 sec then washed out for a period of 20 min. The agonist concentration (1 μM for $P2X_1$, 10 μM for $P2X_2$, 3 μM for $P2X_3$, or 30 μM for $P2X_4$) was approximately equal to the EC_{70} value at each subtype. For inhibition curves, data were normalized to the current evoked by ATP at pH 7.5. Test substances were added for 5 min prior to ATP exposure; all compounds were tested for reversibility of their effects. The concentration required to inhibit the ATP-response by 50% (IC_{50}) was taken from Hill plots constructed using the formula $\log(I/I_{max} - I)$, where I

was the current evoked by ATP in the presence of an antagonist. Data are presented as mean ± s.e.mean (n = 4) for data from different batches of oocytes.

5.4 Results

ATP agonists were tested in functional assays of recombinant rat $P2X_{1,2,3,4}$ receptors expressed in *Xenopus* oocytes (Figure 5.2). Values for potency (EC_{50}) in activating membrane currents and maximum efficacy (as %) are reported (Table 5.1). HT-AMP was 5-fold more potent than ATP at $P2X_3$ receptors and a partial agonist at all except $P2X_2$ receptors, at which it was a full agonist. The efficacy of HT-AMP was as low as 23% at $P2X_4$ receptors. PAPET was a weak partial agonist at rat $P2X_4$ receptors and a nearly full agonist at the other subtypes. At rat $P2X_3$ receptors, PAPET was more potent than any other known agonist (EC_{50} = 17 ± 3 nM). Various adenine bisphosphates (Figure 5.3) in the family of MRS 2179 (a potent $P2Y_1$ receptor antagonist) containing modifications of either the adenine (2- and 6-substitutions), the phosphate (a 3′,5′-cyclic diphosphate), or the ribose moieties (carbocyclic and anhydrohexitol analogues), were examined for the ability to interact with P2X receptors. MRS 2179 (Figure 5.1), itself, inhibited ATP-evoked responses at rat $P2X_1$ receptors with an IC_{50} value of 1.15 ± 0.21μM (Figure 5.4A). MRS 2179 was a weak antagonist at rat $P2X_3$ receptors, with approximately 40% inhibition occurring at 10 μM, and was inactive at rat $P2X_2$ and $P2X_4$ receptors (Figure 5.4B). The IC_{50} value of MRS 2179 at rat $P2X_3$ receptors was determined to be 12.9 ± 0.1 μM. At rat $P2X_{1,2,3,4}$ receptors, MRS 2179 displayed no agonist properties in the absence of ATP. Figure 5.3 shows the structures of a variety of bisphosphate derivatives that have been tested at turkey erythrocyte $P2Y_1$ receptors as antagonists of the effects of 2-MeSADP. These compounds were tested at recombinant rat $P2X_I$ and $P2X_3$ receptors.

All of the bisphosphate analogues shown in Figure 5.3, *i.e.* compounds 5 - 10, were demonstrated to be inactive at $P2X_1$ and $P2X_3$ receptors, either as agonists (in the absence of ATP) or antagonists (at 1 or 10 μM for rat $P2X_1$ and at 1 μM for rat $P2X_3$ receptors, versus either 1 or 3 μM ATP, respectively). Compounds 5a - 5c, which are 2-substituted analogues of MRS 2179, and MRS 2209 (compound 6) the 3′-deoxy-2′-phosphate isomer of MRS 2179, were inactive at rat $P2X_1$ and $P2X_3$ receptors, thus demonstrating their selectivity as $P2Y_1$ receptor antagonists. Similarly, other ribose-modified bisphosphate antagonist of $P2Y_1$ receptors, in which the ribose ring was replaced with either cyclopentane (compound 7) or anhydrohexitol, (compound 9), were found to be inactive at both rat $P2X_1$ and $P2X_3$ receptors. Compounds 7, a cyclic diphosphate corresponding to an anhydride of MRS 2179, and 10, a six-membered ring derivative having an ethyl phosphonate on a ring nitrogen, were inactive at $P2Y_1$ receptors as either agonists or antagonists and were found to be similarly inactive at $P2X_1$ and $P2X_3$ receptors. The acyclic nucleotide analogue MRS 2286 [Kim et al., 2000], 11, is an antagonist at turkey $P2Y_1$ receptors, but is inactive at both rat $P2X_1$ and $P2X_3$ receptors.

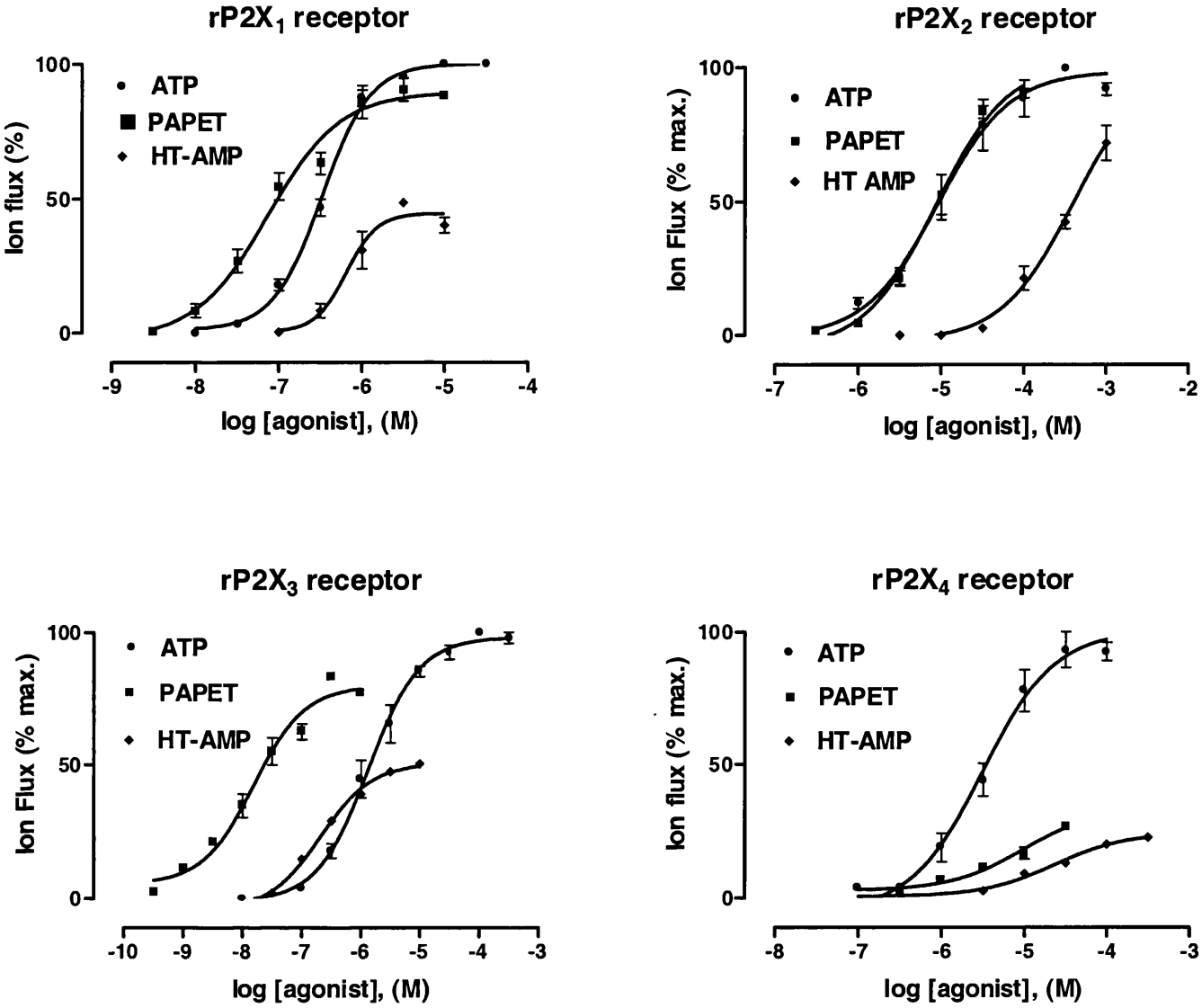


Figure 5.2 Effect of various analogues as indicated on current induced at recombinant rat P2X receptors, expressed in *Xenopus* oocytes (n = 4). The twin electrode voltage clamp technique was used. The medium consisted of Ba^{2+} Ringer's buffer at pH 7.50. Results summarised in table 5.1.

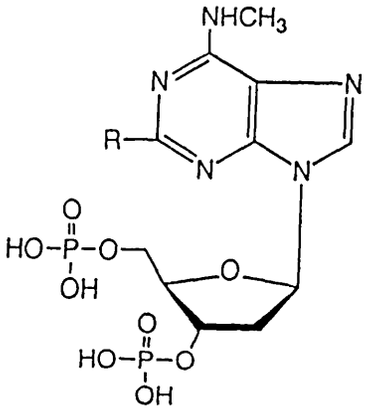
Table 5.1 Agonist potency and efficacy at rat $P2X_{1,2,3,4}$ receptors.

Receptor	ATP	PAPET	HT-AMP
EC_{50} Value (μM)			
$P2X_1$	0.30 ± 0.01	0.098 ± 0.010	0.84 ± 0.11
$P2X_2$	11.0 ± 3.2	10.2 ± 1.0	180 ± 60[a]
$P2X_3$	1.85 ± 0.37	0.017 ± 0.003	0.35 ± 0.22
$P2X_4$	4.14 ± 1.34	15.3 ± 5.3	20.4 ± 0.31
Efficacy (% maximum)			
$P2X_1$	100	91 ± 5	49 ± 2
$P2X_2$	100	91 ± 2	≤100[b]
$P2X_3$	100	83 ± 2	50 ± 2
$P2X_4$	100	27 ± 2	23 ± 1

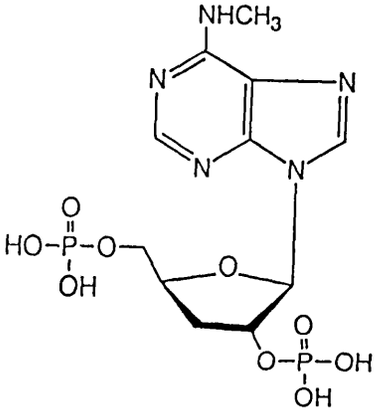
[a]Estimated: the log concentration-response curve for HT-AMP at $P2X_2$ receptors did not reach a definable maximum count and accordingly, the stated EC_{50} value is an estimate.

[b] The maximum was not determined over the concentration range studied.

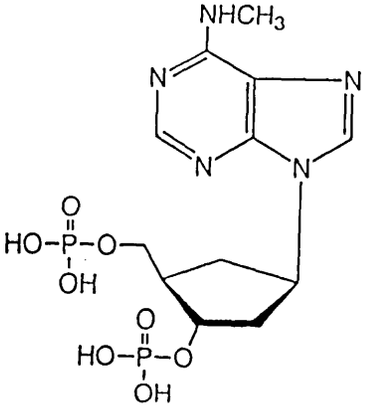
Figure 5.3 Structures of deoxyadenosine bisphosphate derivatives previously tested as $P2Y_1$ receptor agonists/antagonists (Camaioni *et al.*, 1998; Nandanan *et al.*, 1999; Kim *et al.*, 2000). IC_{50} values (μM) for inhibition of phospholipase C elicited by 10nM 2-MeSADP at the turkey erythrocyte $P2Y_1$ receptors were found to be: **5a**, 0.206; **5b**, 0.362; **5c**, 1.85; **6**, 0.324; **7**, 2.53; **8**, >50; **9**, 1.64; **10**, no effect; **11**, 0.84.



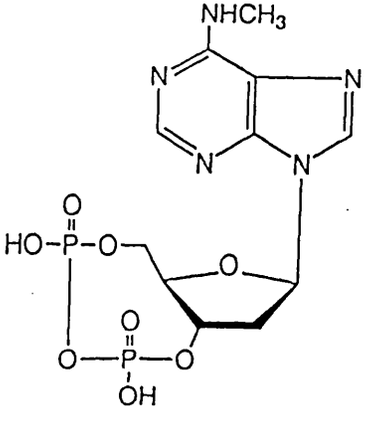
5a, R = Cl MRS 2216
5b, R = SCH_3 MRS 2217
5c, R = NH_2 MRS 2248



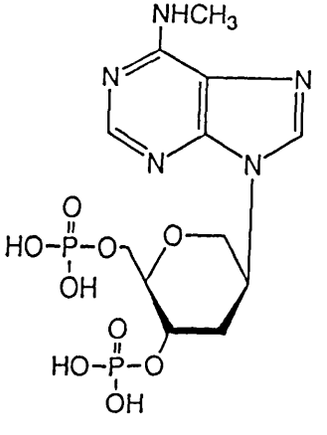
6, MRS 2209



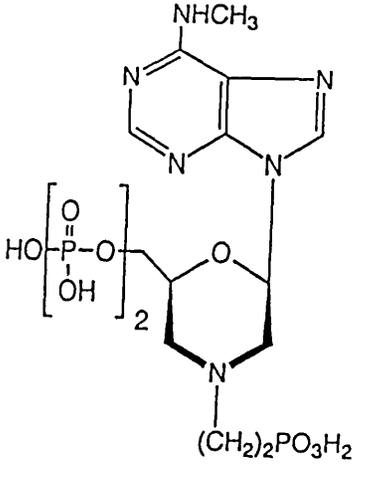
7, MRS 2267



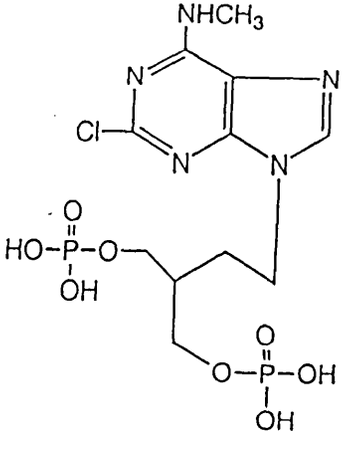
8, MRS 2233



9, MRS 2269



10, MRS 2271



11, MRS 2286

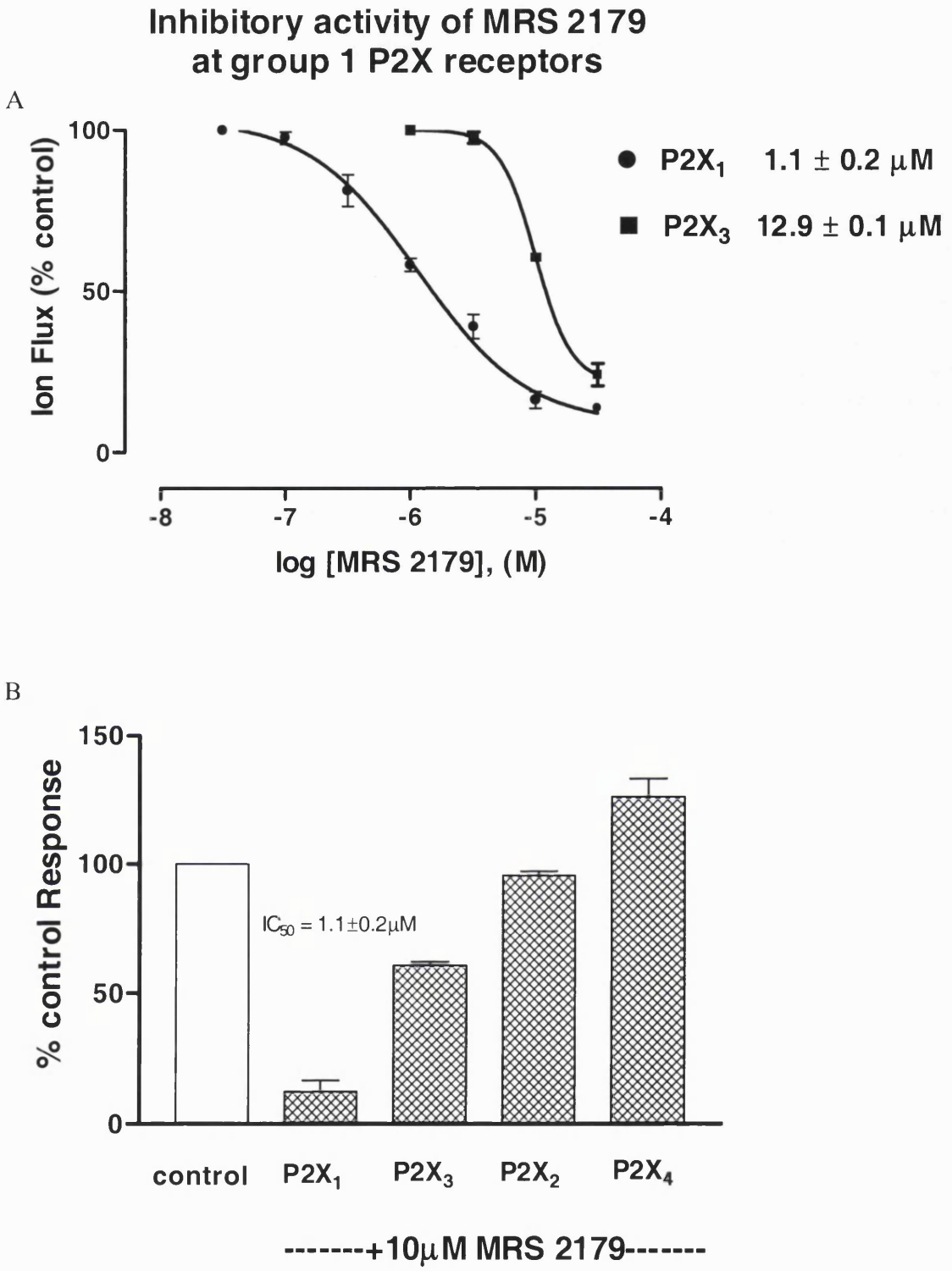


Figure 5.4 Effects of MRS 2179 on current induced by activation of recombinant rat $P2X_1$ and $P2X_3$ receptors (concentration-response curve, A) and rat $P2X_{1-4}$ receptors (at 10μM, B), expressed in *Xenopus* oocytes (n = 8). The IC_{50} at $P2X_1$ receptors was found to be 1.15 ± 0.21 μM. The twin electrode voltage clamp technique was used. The medium consisted of Ba^{2+} Ringer's buffer at pH 7.5.

5.5 Discussion

Since agonist potency at $P2X_1$ and $P2X_3$ receptors is often linked, an aim of this study has been to distinguish these two subtypes with selective ligands. At rat $P2X_3$ receptors, PAPET (compound 1) was more potent than any other known agonist, with an EC_{50} value of 17 nM. This derivative was a full agonist at $P2X_1$, $P2X_2$, and $P2X_3$ receptors and a partial agonist at $P2X_4$ receptors. The monophosphate derivative, HT-AMP (compound 2) appeared to be a partial agonist at $P2X_1$, $P2X_3$, and $P2X_4$ receptors, and a weak full agonist at $P2X_2$ receptors. Both of these 2-alkylthio nucleotide derivatives showed a preference for the Group 1 ($\alpha\beta$meATP-sensitive, fast-inactivating) P2X subunits, *i.e.* $P2X_1$ and $P2X_3$ receptors. Thus, PAPET was 600- and 900- fold selective for $P2X_3$ versus $P2X_2$ and $P2X_4$ receptors, respectively. HT-AMP was 510- and 58- fold selective for $P2X_3$ versus $P2X_2$ and $P2X_4$ receptors, respectively. The preference for Group 1 was much more pronounced for the 2-thioether derivatives, including 2-MeSATP (King, 1998), than for ATP, itself. 2-MeSATP was reported to have the following EC_{50} values (μM): 0.4 ($P2X_1$), 7.1 ($P2X_2$), 0.2 ($P2X_3$), and 74 ($P2X_4$) (King, 1998). Thus, PAPET appears to be more potent than 2-MeSATP at recombinant $P2X_1$ and $P2X_3$ receptors expressed in oocytes. The utility of PAPET and HT-AMP and similar 2-thioethers derivatives as potent P2 agonists *in vivo* may be further enhanced due to increased resistance to nucleotidases (Zimmet et al., 1993).

Among bisphosphate derivatives tested, only MRS 2179 (compound 4) showed any antagonist properties at P2X receptors. Thus, MRS 2179 remained a moderately selective $P2Y_1$ receptor antagonist, with ratios of selectivity versus $P2X_1$ and $P2X_3$ receptors of 11- and 130-fold, respectively, and, as reported, is inactive at $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors (Boyer et al., 1998). MRS 2179 has also been found to be inactive at $P2Y_{AC}$ (now $P2Y_{12}$)

receptors in platelets (Boyer et al., 1998) and at $P2Y_{11}$ receptors (B. Torres, A. Zambon, and P. Insel, unpublished observations), as well as $P2X_2$ and $P2X_4$ receptors. MRS 2179 (100 μM) is also inactive as either substrate or inhibitor of recombinant rat ecto-ATPase and ecto-apyrase expressed in CHO cells (C. Hoffmann, K. Jacobson, and H. Zimmermann, unpublished observations). Nucleotides that distinguish between $P2X_1$ and $P2Y_1$ receptors may be useful pharmacological probes to study aggregation effects in platelets. Among the most important findings of the present study are that compounds 5 - 7, 9, and 11, which bind to $P2Y_1$ receptors in the micromolar range appear to be very selective for that subtype. The substitution of MRS 2179 at the 2-position with a chloro-, methylthio-, or amino- group (compounds 5a - 5c) abolished activity at $P2X_{1-4}$ receptors, and similarly, the 2', 5'-isomer of MRS 2179, 6, was inactive at P2X receptors. The carbocyclic derivative, 7, was a moderately potent $P2Y_1$ receptor antagonist (IC_{50} 2.53 μM) and inactive at $P2X_{1,3}$ receptors. Thus, compounds 5a – 5c, 6, and 7, which are relatively potent as antagonists at $P2Y_1$ receptors ($IC_{50} < 3$ μM), were highly selective at that subtype. The anhydrohexitol derivative 9, an antagonist at $P2Y_1$ receptors, appeared to be a selective antagonist of $P2Y_1$ receptors, i.e. to the extent that is has no effect at $P2X_{1,3}$ receptors. The corresponding 6-NH_2 derivative (MRS 2255), a pure agonist at $P2Y_1$ receptors with an EC_{50} value of 2.99 μM (Nandanan et al., 1999), for stimulation of phospholipase C in turkey erythrocytes, should also be evaluated at P2X receptors and may prove to be a selective $P2Y_1$ agonist. A selective $P2Y_1$ receptor agonist may be useful as a hypotensive agent or in the treatment of diabetes, while a selective $P2Y_1$ receptor antagonist may be useful as an antithrombotic agent.

CHAPTER 6

STRUCTURE ACTIVITY RELATIONSHIPS OF PYRIDOXAL PHOSPHATE DERIVATIVES AS POTENT AND SELECTIVE ANTAGONISTS OF $P2X_1$ RECEPTORS

6.1 Summary

1. Novel analogues of the P2 receptor antagonist pyridoxal-5'-phosphate 6-azophenyl-2′,5′-disulphonate (iso-PPADS), were synthesized and studied as antagonists in functional assays at recombinant rat $P2X_1$, $P2X_2$, and $P2X_3$ receptors expressed in *Xenopus* oocytes (ion flux stimulation) and at turkey erythrocyte $P2Y_1$ receptors (phospholipase C activation).
2. Modifications were made in the 4-aldehyde and phosphate groups of the pyridoxal moiety, i. e. a CH_2OH group at the 4-position in pyridoxine was either condensed as a cyclic phosphate or phosphorylated separately to form a bisphosphate, which reduced potency at P2 receptors. 5-Methylphosphonate substitution, anticipated to increase stability to hydrolysis, preserved P2 receptor potency.
3. At the 6-position, halo-, carboxylate, sulphonate, and phosphonate variations made on the phenylazo ring modulated potency at P2 receptors. The *p*-carboxyphenylazo analogue of iso-PPADS displayed an IC_{50} value of 9 nM at recombinant $P2X_1$ receptors, and was 1300-, 16-, and > 10,000-fold selective for $P2X_1$ versus $P2X_2$, $P2X_3$, and $P2Y_1$ subtypes, respectively. The corresponding 5-methylphosphonate was equipotent at $P2X_1$ receptors.
4. The 5-methylphosphonate analogue containing a 6-[3,5-bis(methylphosphonate)]phenylazo moiety, 9, had IC_{50} values of 11 nM and 25 nM at recombinant $P2X_1$ and $P2X_3$ receptors, respectively.
5. The analogue containing a phenylazo 4-phosphonate group was also very potent at both $P2X_1$ and $P2X_3$ receptors. However, the corresponding 2,5-disulphonate analogue was 28-fold selective for $P2X_1$ versus $P2X_3$ receptors. None of the analogues were more

potent at $P2Y_1$ receptors than iso-PPADS, which acted in the micromolar range at this subtype.

6.2 Introduction

Adenine and uracil 5'-nucleotides act in cellular signalling in the nervous, muscular, cardiovascular, renal, and immune systems, through the activation of P2 receptors (North and Barnard, 1997; Ralevic and Burnstock, 1998; Abbrachio and Burnstock, 1998). These nucleotide receptors consist of two families of distinct structure and function: P2X ligand-gated cation channels and G protein-coupled P2Y receptors (Communi & Boeynaems, 1997). $P2X_{1\text{-}7}$ and $P2Y_{1,2,4,6,11,12}$ designations have been unambiguously assigned to mammalian nucleotide receptors (Burnstock and King, 1996; Fredholm *et al.*, 1997; Communi & Boeynaems, 1997; King *et al.*, 2001) although there is still uncertainty about the correspondence of these sequences to the pharmacological phenotypes described prior to P2 receptor cloning (King *et al.*, 1998). The therapeutic potential of potent and selective agonists and antagonists of P2 receptors has been discussed, although in mainly hypothetical terms, due to the current lack of such agents. For example, activation of the $P2X_1$ subtype mediates vasoconstriction at vascular smooth muscle, thus selective antagonists may be antihypertensive. The $P2Y_1$ subtype participates in blood platelet aggregation, thus selective antagonists may be useful in regulating haemostasis. It appears that activation of the $P2X_3$ receptor subtype mediates nociception via the dorsal root ganglia, thus a selective antagonist may be anti-nociceptive (Burnstock and Wood, 1996; Cook *et al.*, 1997).

Derivatives of pyridoxal-5'-phosphate in which an azoaryl group is present at the 6-position were shown to be non-selective P2 receptor antagonists (Lambrecht *et al.*, 1996). PPADS (Compound 1, pyridoxal-α^5-phosphate-6-azophenyl-2′,4′-disulphonic acid), and iso-PPADS (Compound 2, the 2,5-disulphonate isomer) (figure 6.1) have been studied as ATP

antagonists at the turkey erythrocye $P2Y_1$ receptor (Jacobson *et al.*, 1998; Boyer *et al.*, 1994) and at $P2X_1$-like receptors in the rabbit vas deferens (Lambrecht *et al.*, 1992) urinary bladder (Ziganshin *et al.*, 1993) isolated blood vessels (Ziganshin *et al.*, 1994b) guinea-pig isolated vas deferens (McLaren *et al.*, 1994) and perfused rat mesenteric arterial bed (Windschief *et al.*, 1994). PPADS was also found to be a weak antagonist at the $P2X_7$ receptor in mouse microglial cells (Chessell *et al*, 1998). PPADS and isoPPADS are ATP antagonists at $P2X_3$-like receptors in sensory neurons (Trezise *et al.*, 1994a; Rae *et al.*, 1998).

The present study demonstrates that both subtype-selectivity and high affinity can be achieved through structural modification of the pyridoxal-5'-phosphate series of antagonists. Recently we reported (Jacobson *et al.*, 1998) that the cyclic pyridoxine-$\alpha^{4,5}$-monophosphate corresponding to iso-PPADS (MRS 2220), compound 3, was a weak, but selective antagonist of ATP-evoked responses at rat $P2X_1$ receptors. Furthermore MRS 2220 was inactive at phospholipase C-coupled P2Y receptors, at the adenylate cyclase-coupled P2Y receptors in rat C6 glioma cells, and at adenosine receptors. In the present study, additional analogues of both pyridoxal and pyridoxine are explored, resulting in enhanced potency and/or selectivity at $P2X_1$ receptors. In certain analogues, the polyanionic nature of the derivatives is diminished, and in other analogues the stability is potentially enhanced through the introduction of a phosphonate linkage. Many of the compounds were found to be more freely reversible as antagonists, in comparison to PPADS and iso-PPADS. Several of the present 5'-phosphate and phosphonate derivatives were reported in a preliminary study (Kim, *et al.*, 1998) and are now characterized more fully at a broader range of recombinant P2X receptor subtypes.

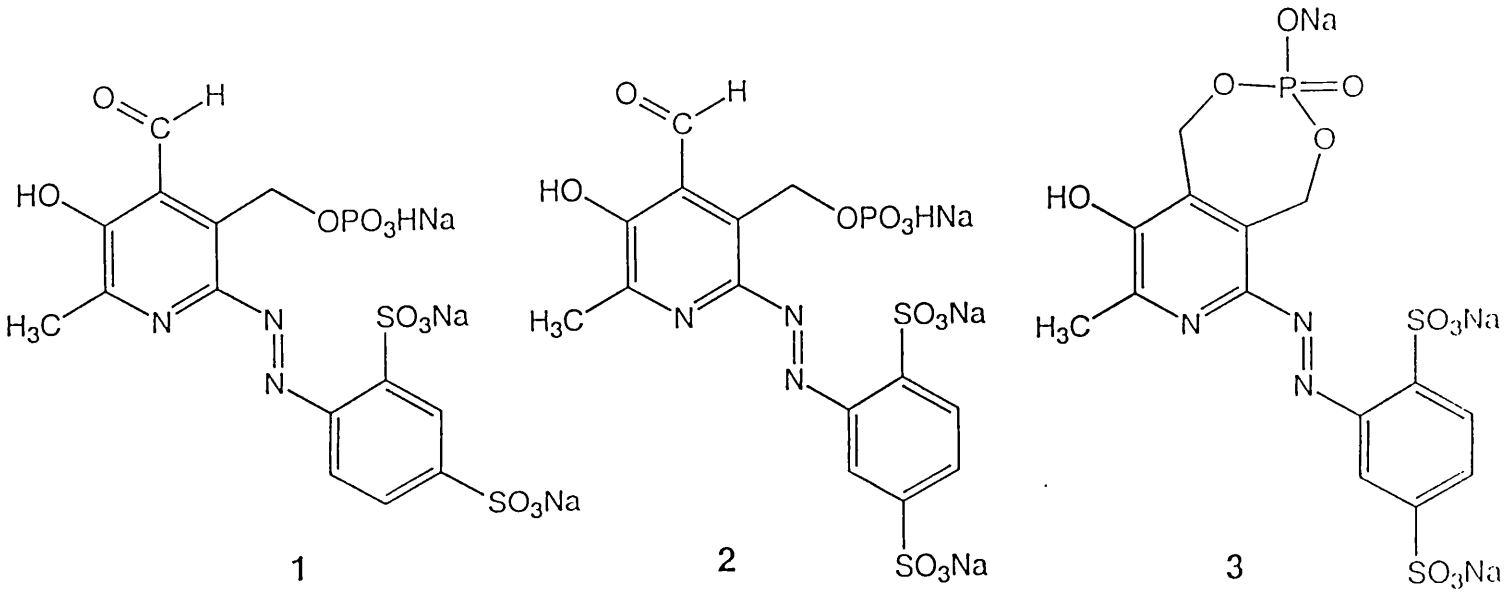


Figure 6.1 Structures of azo derivatives of pyridoxal-5'-phosphate (**1** and **2**) and a cyclic pyridoxine-$\alpha^{4,5}$-monophosphate derivatives (**3**), all of which act as P2 receptor antagonists.

6.3 Methods

Xenopus oocytes were harvested and prepared as previously described (King *et al.*, 1997). Defolliculated oocytes were injected cytosolically with rat $P2X_1$, $P2X_2$ or $P2X_3$ receptor cRNA (40nL, 1μg/ml), incubated for 24hr at 18°C in Barth's solution and kept for up to 12 days at 4°C until used in electrophysiological experiments.

ATP-activated membrane currents (V_H= -90mV) were recorded from cRNA-injected oocytes using the twin-electrode voltage-clamp technique (Axoclamp 2B amplifier). Voltage recording (1 – 2 MΩ tip resistance and current-recording microelectrodes (5 MΩ tip resistance) were filled with 3.0M KCl. Oocytes were held in an electrophysiological chamber and superfused with Ringer's solution (5 mL/min at 18°C) containing (mM) NaCl, 110; KCl, 2.5; HEPES (N-[2-hydroxyethyl]piperazine-N-[3-propanesulfonic acid]), 5; $BaCl_2$, 1.8, adjusted to pH 7.5.

ATP(at the EC_{70} value in μM for respective subtypes: $P2X_1$, 1; $P2X_2$, 10; $P2X_3$, 3) was superfused over the oocytes for 60-120s then washed out for a period of 20 min. For inhibition curves, data were normalized to the current evoked by ATP, at pH 7.5. Test substances were added for 20 min prior to ATP exposure; all compounds were tested fro reversibility of their effects. The concentration required to inhibit the ATP response by 50% (IC_{50}) was taken from Hill plots constructed using the formula: $\log(I/I_{max}-1)$, where I is the current evoked by ATP in the presence of an antagonist. Data are presented as mean±SEM (n=4) for data from different batches of oocytes.

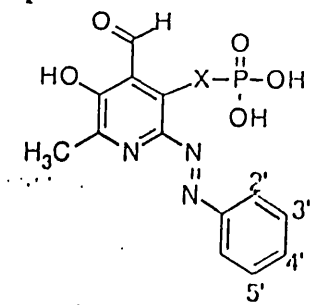
6.4 Results

We have introduced 5′-phosphonate linkages (Kim *et al.*, 1998), cyclic phosphate diesters (Jacobson *et al.*, 1998), *bis*phosphates, and modified functional groups on the phenylazo moiety (Table 6.1) were introduced with the aim of developing more potent and selective antagonists for P2 receptor subtypes. Halo-, carboxylate, sulphonate, and phosphonate (data not shown) variations were made on the phenyl ring (compounds 4 - 11). 5-Methylphosphonate and ethylphosphonate substitutions of the methylphosphate moiety were introduced. Analogues of the P2 receptor antagonists pyridoxal-5′-phosphate and 6-azophenyl-2′,5′-disulphonate derivative (iso-PPADS), in which the phosphate group was cyclized by esterification to a CH_2OH group at the 4-position in pyridoxine, were synthesized (compounds 13 - 16). Finally, bisphosphate derivatives of pyridoxine (compounds 18 - 20) were prepared (data not shown). The new derivatives were characterized using NMR and high resolution mass spectroscopy, and purity of 95-98% was demonstrated using high pressure liquid chromatography (HPLC) in two different solvent systems (data not shown). The compounds were tested in ion channel assays (King *et al.*, 1997) of ATP-induced current at recombinant rat $P2X_1$, $P2X_2$, and $P2X_3$ receptors, expressed in *Xenopus* oocytes, using the twin electrode voltage clamping technique. Several compounds (4, 8, 10) were previously reported to antagonize agonistinduced cation flux at recombinant $P2X_2$ receptors, with the IC_{50} values of 1 - 10 μM. Most of the compounds were weak or inactive as antagonists at the turkey erythrocyte $P2Y_1$ receptor, in activation of phospholipase C activity (Harden *et al.*, 1988; Boyer *et al.*, 1989) induced by 10 nM 2-MeSADP. Compound 11 displayed an IC_{50} value of 27 μM. In inhibition of the inward current elicited by ATP at recombinant $P2X_1$ receptors, compounds 4 - 11, with IC_{50} values of 9 - 42 nM, were found to be more potent

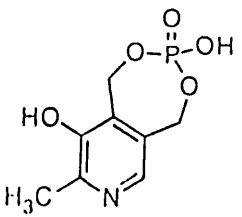
than 1 and 2 (IC_{50} values of 99 and 43 nM, respectively). The *p*-carboxylate analogue (compound 4) having a phosphate linkage similar to compound 1, selectively inhibited ATP-evoked responses at $P2X_1$ receptors with an IC_{50} value of 9 nM and was 1300-fold and 16-fold selective versus $P2X_2$ and $P2X_3$ receptors, respectively. The corresponding 5-methylphosphonate analogue (compound 5) was equipotent to compound 4 at $P2X_1$ receptors (Figure 6.2). Thus, compound 5 was 16-fold selective for $P2X_1$ versus $P2X_3$ receptors and 19-fold selective versus $P2X_2$ receptors. The monocarboxylic acid 5-ethylphosphonate analogue (compound 6) and the dicarboxylic acid derivative (compound 7) were slightly less potent than 5 at $P2X_1$ receptors. Phosphonate substitution of the arylazo ring proved to have distinctive effects on potency and selectivity. The IC_{50} values for the *bis*(methylphosphonate), (compound 9) at $P2X_1$ and $P2X_3$ receptors were 11 and 25 nM, respectively, in the presence of 1 μM and 3 μM ATP, respectively, thus the $P2X_3$ receptor potency was enhanced. Compound 10, a 5-methyl phosphonate having the same arylazo sulphonate substitution as compound 2, antagonized responses at $P2X_1$ receptors with an IC_{50} value of 12 nM. Compound 10 was 28-fold selective for $P2X_1$ versus $P2X_3$ receptors and 92-fold selective versus $P2X_2$ receptors. A *p*-phenylphosphonate derivative, (compound11, Figure 6.3), was nearly as potent as compound 9 at both $P2X_1$ and $P2X_3$ receptors. Thus, 11 was 79-fold selective for $P2X_1$ versus $P2X_2$ receptors. The time course of washout at $P2X_1$ and $P2X_3$ receptors of various potent antagonists was examined (Figure 6.4). Ion channel activity was more rapidly restored, following a 20 minute washout, for several active compounds than for PPADS. The percent of control activity recovered for PPADS (compound 1) following washout was only 30-40%. isoPPADS (compound 2) was fully reversible at both receptors, and most other analogues (compounds 4, 8 -11), except for

compounds 5 and 7 with 20-30%, displayed ≥ 75% recovery. The cyclic phosphates and bisphosphates derived from pyridoxine (compound 3, 13 - 20) were much less potent than the pyridoxal derivatives in antagonizing the activation of recombinant P2X receptors expressed in oocytes. Compound 15, a cyclic phosphate in which the 3-OH group was acetylated, was completely inactive at $P2X_1$ receptors, which in comparison to compound 3 demonstrates the importance of the hydroxyl group for receptor recognition.

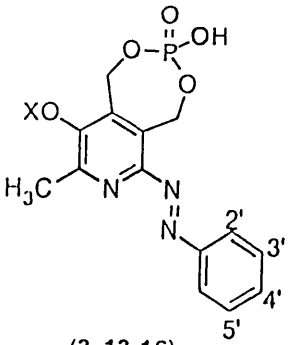
Table 6.1 The structure and pharmacological activities of pyridoxal and pyridoxine derivatives (phosphates and phosphonates) at P2 receptors



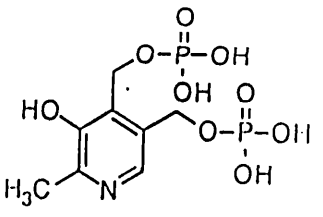
(1, 2, 4-11)



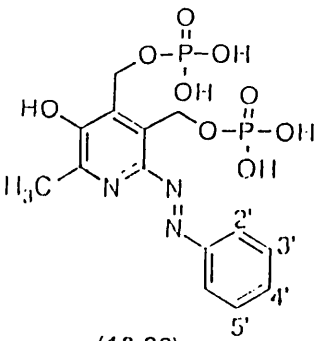
(12)



(3, 13-16)



(17)



(18-20)

Compound	Positions					$P2X_1$[a] recombinant	$P2X_2$[a] recombinant	$P2X_3$[a] recombinant	$P2Y_1$[c] PLC assay
	2'	3'	4'	5	X	IC_{50} (μM) or % inhibition	IC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)
1 (PPADS)	SO_3H	H	SO_3H	H	CH_2O	0.099 ± 0.006	1.6 ± 0.1	0.240 ± 0.038	16.6 ± 2.5
2 (IsoPPADS)	SO_3H	H	H	SO_3H	CH_2O	0.043 ± 0.018	0.398 ± 0.125	0.084 ± 0.004	21.4 t 9.0
4[d,f]	H	H	COOH	H	CH_2O	0.009 ± 0.002	11.9 ± 1.4	0.140 ± 0.011	~100
5	H	H	COOH	H	CH_2	0.008 ± 0.002	0.150 ± 0.020	0.128 ± 0.019	
6[d]	H	H	COOH	H	CH_2CH_2	0.020 ± 0.003	2.4 ± 0.3	0.145 ± 0.057	~100
7	COOH	H	H	COOH	CH_2	0.037 ± 0.008	0.486 ± 0.023	0.330 ± 0.014	

8[d]	H	H	H	H	CH_2O	0.042 ± 0.006	1.2 ± 0.2	0.480 ± 0.090	54 ± 3
9[f]	H	$CH_2P(O)(OH)_2$	H	$CH_2P(O)(OH)_2$	CH_2	0.011 ± 0.005	0.280 ± 0.030	0.025 ± 0.007	14.5 ± 2.1
10[d,f]	SO_3H	H	H	SO_3H	CH_2	0.012 ± 0.003	1.1 ± 0.2	0.340 ± 0.040	46 ± 21
11[f]	H	H	$P(O)(OH)_2$	H	CH_2	0.012 ± 0.008	0.948 ± 0.019	0.036 ± 0.010	27 ± 3
12[e]	-	-	-	-	-	5.9 ± 1.8 (EC_{50})[g]	inactive	inactive	inactive
3[e]	SO_3H	H	H	SO_3H	H	10.2 ± 2.6	inactive	58.3 ± 0.1	inactive
13	Cl	H	H	SO_3H	H	43 ± 4%[b]			
14	H	H	COOH	H	H	39 ± 10%[b]			
15	SO_3H	H	H	SO_3H	CH_3CO	inactive			
16	H	H	H	H	H	inactive			inactive
17	-	-	-	-	-	inactive			inactive
18	SO_3H	H	H	SO_3H	-	27 ± 7%[b]			-100
19	Cl	H	H	SO_3H	-	78 ± 7%[b]		30 ± 10%[b]	-100
20	H	H	H	H	-	50 ± 4%[b]			>100

[a]Inhibition of ion current (mean ± s.e.m., n = 3), unless noted, induced by ATP (at the EC_{70} values in μM for respective subtypes: $P2X_1$ 3, $P2X_2$ 10, and $P2X_3$ 1) at each recombinant P2X receptors expressed in Xenopus oocytes (b; % inhibition at 10 μM).
[c]Inhibition of 10nM 2-MeSADP-stimulated phospholipase C in turkey erythrocyte membranes (mean ± s.e.m., n = 3), labeled using [^{3}H]inositol.
[d]Compounds reported in Kim *et al.*, 1998
[e]Compounds and values reported in Jacobson *et al.*, 1998
[f]Compound 4, MRS 2159; compound 9, MRS 2257; compunds 10, MRS 2191; compound 11, MRS 2273.
[g]Compound 12 potentiates the effect of ATP at P2X receptors, Jacobson *et al.*, 1998.

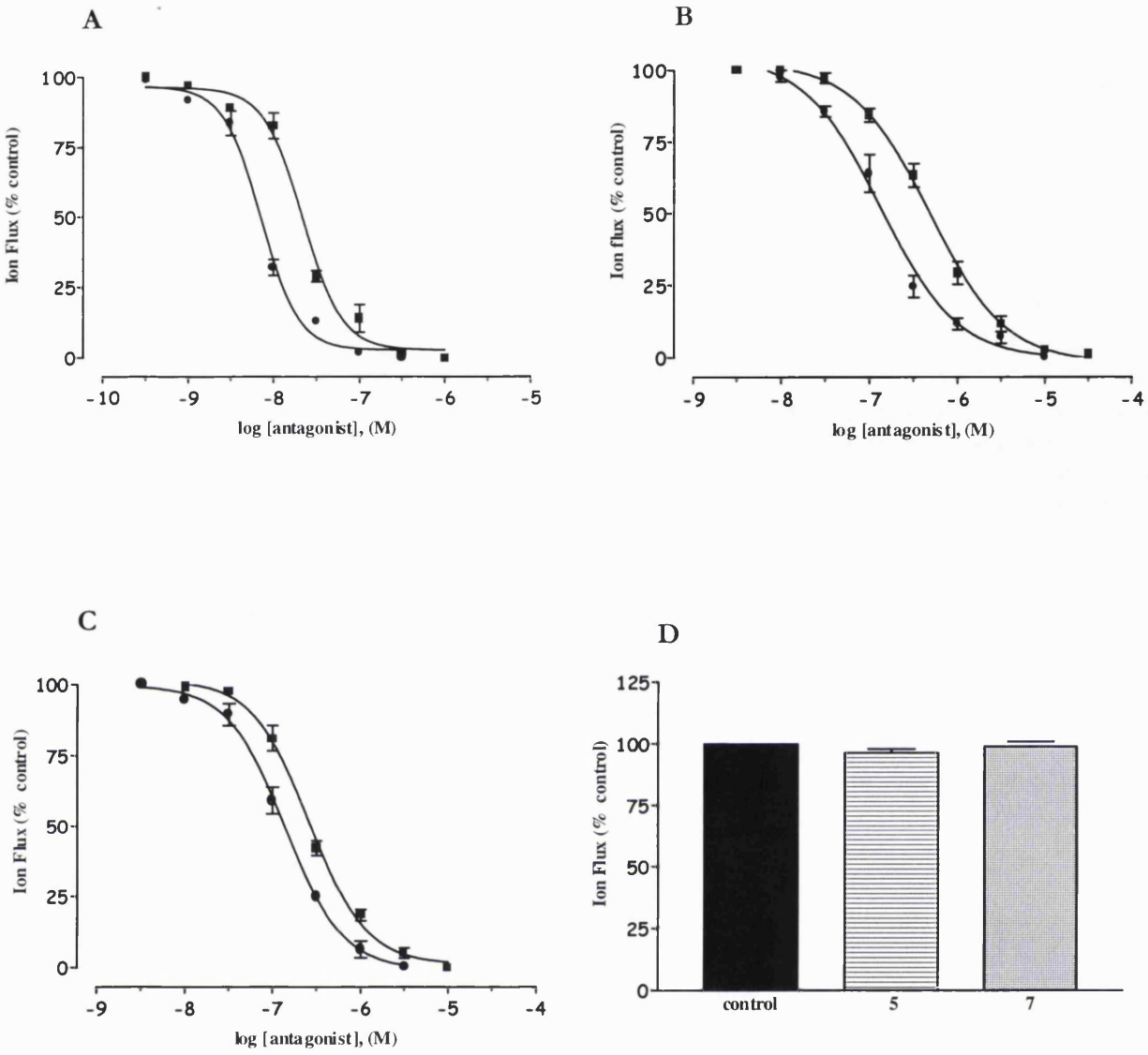


Figure 6.2 Potent effects of compounds 5 (•) and 7 (■) on inward current induced by ATP at recombinant $P2X_1$ receptors (A), intermediate potency at rat $P2X_2$ (B) and rat $P2X_3$ (C) receptors. Compounds 5 and 7 (10mM) were inactive at rat $P2X_4$ receptors (D). Receptors were expressed in Xenopus oocytes and current measured using the twin electrode voltage clamp technique (pH 7.5 Ba^{2+} Ringer's solution). All data points were mean ± S.E.M. of 4 observations. ATP concentrations were as in material and methods. IC50 values given in table 1. Hill coefficients (nH) were: (A) -1.20 ± 0.07 (5) and -1.19 ± 0.08 (7); (B) -1.11 ± 0.009 (5) and -0.95 ± 0.15 (7); (C) -0.87 ± 0.04 (5) and -0.89 ± 0.05 (7).

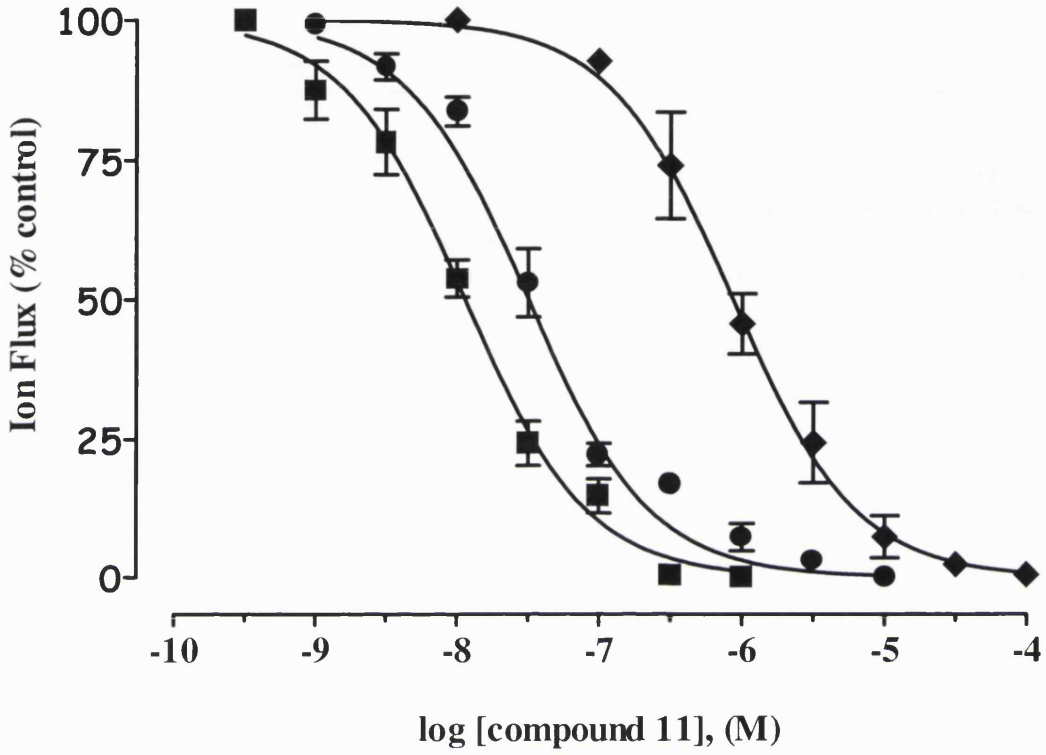
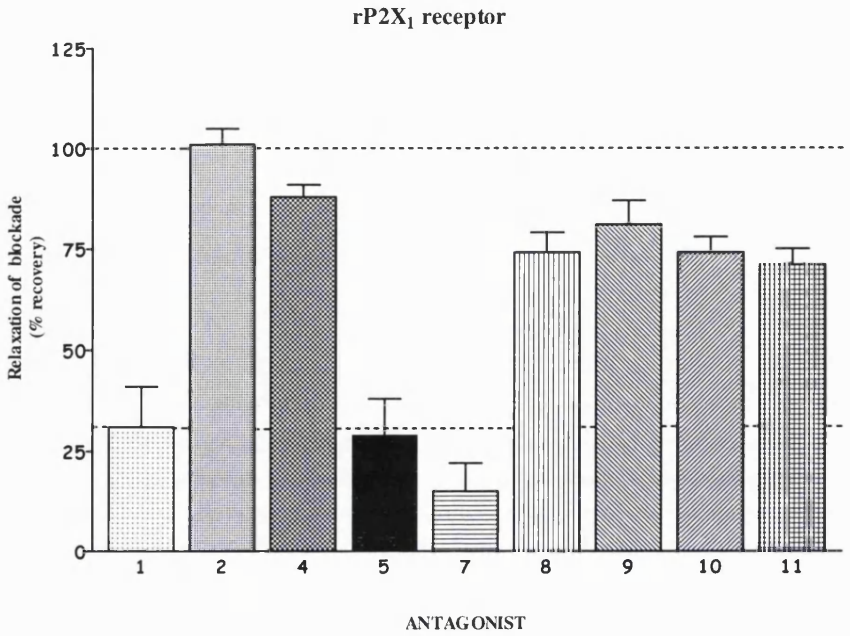


Figure 6.3 Selectivity of compound **11** on inward current induced by ATP at recombinant $P2X_1$ (■) and rat $P2X_3$ receptors (●) ("Group 1") versus rat $P2X_2$ receptors (◆) ("Group 2"), expressed in *Xenopus* oocytes, using the twin electrode voltage clamp technique (pH 7.5, Ba^{2+} Ringer's solution). All data points were mean ± S.E.M. of 4 observations. ATP concentrations were as in the materials and methods. IC_{50} values are given in table 1. Hill coefficients (n_H) were: ($P2X_1$) –1.05 ± 0.05, ($P2X_2$) –1.11 ± 0.06 and ($P2X_3$) –1.03 ± 0.04.



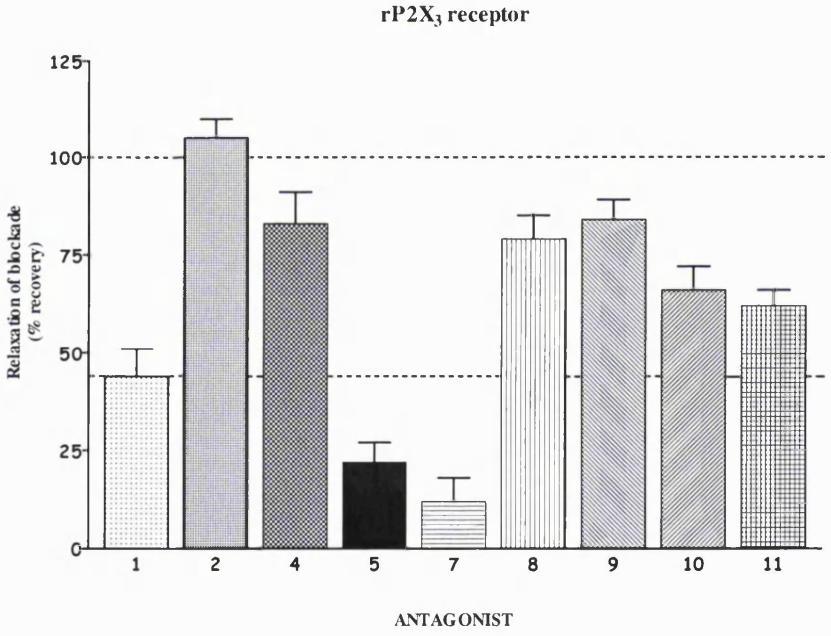


Figure 6.4 Histogram showing the degree of recovery of rat $P2X_1$ (A) and rat $P2X_3$ (B) receptors from full blockade by various antagonists, 20 min after drug removal. Receptors were expressed in *Xenopus* oocytes and current measured using the twin electrode voltage clamp technique (pH 7.5, Ba^{2+} Ringer's solution). ATP concentrations were as in materials and methods.

6.5 Discussion

PPADS and its analogues have been studied at a variety of P2 receptor subtypes. At $P2Y_1$ receptors, a range of potencies of PPADS has been reported from approximately 1 μM (Charlton *et al.*, 1996) to 16.6 μM in the present study. Brown *et al.*, 1997, also showed that PPADS had a curious potentiating effect at turkey $P2Y_1$ receptors, (which may be stimulated by washing cells). Nevertheless, the potency of PPADS, iso-PPADS, and analogues prepared in the present study generally tends to be greater at $P2X_{1-3}$ subtypes than at $P2Y_1$ receptors. Furthermore, among the present set of analogues in which the aldehyde group remained, potencies often increased over 2 at the $P2X_1$ and $P2X_3$ subtypes. 5-Methylphosphonate substitution of the methylphosphate moiety of pyridoxal phosphate-related P2 receptor antagonists, anticipated to increase stability of the analogues to hydrolysis, was found to be well-tolerated at P2X receptor subtypes. At $P2X_1$ receptors a methylphosphonate (compound 5) was more potent than the corresponding ethylphosphonate, (compound 6). However, the reduction of the 4-aldehyde group, that apparently results in molecules that exhibit high selectivity for P2X over P2Y receptors, and its phosphoryation reduced the potency at all P2 receptors examined. For example, the cyclic phosphate analogue of iso-PPADS (compound 3) previously reported to display selectivity for the $P2X_1$ subtype (Jacobson *et al.*, 1998) and the corresponding bisphosphate (compound 18) were relatively weak, The cyclic phosphate analogue unsubstituted on the arylazo ring (compound 16) was inactive. Thus, cyclic phosphate and bisphosphate analogues were both less potent at P2 receptors than the corresponding 4-aldehyde derivatives. Functional group variation on the 6-(phenylazo) substituent was found to greatly modulate the potency as P2 receptor antagonists. The

6-[3,5-di(methylphosphonate)]-phenylazo and the 6-[4-(phosphonate)phenylazo] groups resulted in enhanced potency at both $P2X_1$ and $P2X_3$ receptors. The *p*-carboxyphenylazo analogue of PPADS (compound 4) displayed an IC_{50} value of 9 nM at recombinant $P2X_1$ receptors with 1300-/16-/ >10,000-fold selective selectivity versus $P2X_2/P2X_3/P2Y_1$ receptors, respectively. The 5-methylphosphonate analogue containing a 6-[3,5-di(methylphosphonate)]-phenylazo moiety (compound 9) had an IC_{50} of 11 nM and 25 nM at recombinant $P2X_1$ and $P2X_3$ receptors, respectively, and was approximately 25- and 1300-fold selective for the $P2X_1$ subtype, versus $P2X_2$ and $P2Y_1$ receptors, respectively. The phenylazo analogue containing a 4-phosphonate group (compound 11) was also very potent at $P2X_1$ and $P2X_3$ receptors. However, the corresponding 2,5-disulphonate analogue, 10, was 28-fold selective for $P2X_1$ versus $P2X_3$ receptors. Thus, compounds 9 and 11 were selective for "Group 1" ($P2X_1$ and $P2X_3$ receptors) versus "Groups 2-4" ($P2X_{2,4}$) receptors. The view that PPADS is an irreversible antagonist stemmed originally from pharmacokinetic data on blockade of P2X receptors in whole tissues. PPADS was noted as having a slow on-rate (slow equilibration) and slow off-rate (slow reversibility) at native P2X receptors in vascular and visceral smooth muscle. The fact that antagonism does eventually reverse resulted in the term *"pseudo-irreversible"* to describe the slow kinetics of these blocking agents (Soto *et al.*, 1997). Nonetheless, problems associated with diffusion, surface adhesion, uptake, compartmentalization and re-release of PPADS have never been clearly established where whole tissue assays are employed to test their activity. Most of these problems are avoided if recombinant P2X receptors are used instead, since the antagonist has direct access to the cell surface and receptor and drug concentration can be controlled by fast application and washout. The slow onset and slow reversal of PPADS blockade at the recombinant $P2X_1$,

$P2X_2$ and $P2X_5$ receptors of rat clones led to the suggestion that the aldehyde group on the pyridoxal moiety formed a Schiff's base with a strategic lysyl residue at equivalent extracellular positions in P2X subunits (K249 on $rP2X_1$, K246 on $rP2X_2$. K251 on $rP2X_5$; Buell *et al.*, 1996). However, evidence for this assumption is far from clear. Point mutation of the lysine in the $rP2X_2$ subunit (K246E) did increase the rate of recovery from PPADS blockade but it did not significantly affect the potency of the antagonist (Buell *et al.*, 1996). On the other hand, substitution of glutamate for lysine at the rat $P2X_4$ subunit (E249K) increased the potency of PPADS (IC_{50}, >500 μM (wt) and 2.6 μM (mutant)), yet did not noticeably accelerate recovery from blockade (Buell *et al.*, 1996). A similar introduction of lysine in the $P2X_6$ subunit (L251K) increased PPADS potency (IC_{50} >500 p,M (wt) and 2.0 μM (mutant)) without affecting the rate of recovery (Collo *et al.*, 1996). Human and mouse forms of $P2X_4$ do not possess a lysine residue at position 249 yet are significantly more sensitive to PPADS (27.5 μM and 21 μM, respectively) than $rP2X_4$ (Garcia-Guzman *et al.*, 1997; Townsend-Nicholson *et al.*, 1999). Although sensitive to the antagonist, human and mouse $P2X_4$ receptors also recover very slowly from PPADS blockade. Mutation of $rP2X_4$ (N127K) to provide a lysine unique to $hP2X_4$ did not enhance the sensitivity of the rat orthologue to PPADS (Garcia-Guzman *et al.*, 1997). Furthermore, $mP2X_4$ only possesses extracellular lysyl residues that are common to $rP2X_4$ (Townsend-Nicholson *et al.*, 1999) and, consequently, the heightened PPADS sensitivity of the former cannot be ascribed to a strategic lysine. The $P2X_3$ subunit lacks a lysine residue at the equivalent position, at which a threonine residue is found (T235), and yet PPADS is potent (IC_{50} ~l μM) and recovery from blockade is rapid for both rat and human orthologues (Garcia-Guzman *et al.*, 1997; Lewis *et al.*, 1995). Thus, the critical role of a strategic lysyl residue in condensation with the

aldehyde of PPADS is difficult to reconcile given the available data. In the present study, the IC_{50} value for PPADS at the $rP2X_1$ receptor was lower than at $P2X_3$ receptor (0.099 vs 0.240 μM; see Table 6.1), although the rate of recovery was faster at the $rP2X_3$ receptor. Compound 2 proved to be more potent at both $rP2X_1$ and $rP2X_3$ receptors (0.043 vs 0.084 μM, see Table 6.1), yet full recovery occurred at these receptors within 20 minutes of drug washout. Blockade by compound 2 at the $P2X_1$-like receptor in rat vas deferens was considered surmountable and a slow recovery reported (Khakh *et al.*,1994). Similarly, blockade by compound 2 was reversible at P2X receptors of rat vagus nerve bundle (Trezise *et al.*, 1994a) in which $P2X_3$ mRNA is present. Furthermore, it was shown that reversal of PPADS blockade at human $P2X_3$ receptors is accelerated by Cibacron blue which, of itself, potentiates ATP-responses at this P2X receptor (Buell *et al.*, 1996). Some of the PPADS derivatives tested in the present study *(e.g.* compounds 4, 8 - 11) showed a level of recovery of about 75% and greater at $rP2X_1$ and $rP2X_3$ receptors within 20 minutes of washout. Thus, it now seems unnecessary to alter the aldehyde on the pyridoxal moiety, as with MRS 2220 a pyroxidine cyclic phosphate (Jacobson *et al.*, 1998) to make allowances for Schiff's base formation and obtain reversible antagonism at these P2X receptors. Recently, a naphthyl derivative of PPADS that also retains the aldehyde group, was shown to be a potent selective and reversible antagonist of $P2X_1$ receptors (Lambrecht *et al.*, 2000). Some P2 receptor antagonists also may inhibit these ecto-nucleotidases, making these studies even more complex (Hoffman *et al.*, 1999). We are currently studying ectonucleotidase inhibition by selected iso-PPADS analogues. Preliminary results with the recombinant enzymes expressed in CHO cells indicate that most of the PPADS analogs were better inhibitors of ecto-apyrase than of ecto-ATPase. The aldehyde group appears to be required for ectoapyrase inhibition.

However, the efficacy of PPADS derivatives as inhibitors of ectoenzymes had no bearing on their efficacy as antagonists at $P2X_{1-3}$ receptors expressed in *Xenopus* oocytes, since defolliculated oocytes are largely devoid of surface enzymes that degrade ATP (Ziganshin *et al.*, 1996).

In conclusion, this study has identified highly potent and selective antagonists of $P2X_1$ receptors and antagonists of combined $P2X_{1,3}$ receptor selectivity. Damer et al. have also reported an antagonist with high affinity and apparent $P2X_1$ receptor-selectivity, the suramin analogue NF279 (8,8′-(carbonylbis(imino-4,1-phenylenecarbonylimino-4,1-phenylenecarbonyl-imino))bis(1,3,5-naphthalenetrisulphonic acid). A selective $P2X_1$ receptor antagonist may have potential utility in controlling receptor-mediated contraction of visceral and vascular smooth muscle *(e.g,* vascular hypertension and instability of the urinary bladder detrusor muscle). A selective $P2X_3$ receptor antagonist may be useful in pain control. The properties of selective P2 receptor antagonists in the central nervous system remain to be examined.

CHAPTER 7

ACTIONS OF A SERIES OF PPADS ANALOGUES AT $P2X_1$ AND $P2X_3$ RECEPTORS

7.1 Summary

We have recently manufactured an extensive series of PPADS-based antagonists that show a marked selectivity for Group I P2X receptors over other P2X and P2Y receptor subtypes (Kim *et al.*, Structure-activity relationships of pyridoxal phosphate derivatives as potent and selective antagonists of P2X receptors. *J. Med. Chem.*, **44**: 340-349, 2001). Here, the actions of seven PPADS (**P**yridoxal-5′-**P**hosphate 6-**A**zophenyl 2′,4′-**DiS**ulfonate) analogues were investigated further at Group 1 P2X receptors expressed in X*enopus* oocytes. All seven analogues potently inhibited $P2X_1$ (IC_{50} range, 5-32 nM) and $P2X_3$ (IC_{50} range, 22-345 nM), the two Group I P2X receptor subtypes. Analogues showed greater inhibitory activity where the pyridoxal moiety of PPADS contained a 5′-phosphonate group, rather than a 5′-phosphate group. Analogues also showed greater potency where disulfonate groups were removed from, or substituted at, the azophenyl moiety. The most active analogue was MRS 2257 (pyridoxal-5′-phosphonate 6-azophenyl 3′,5′-bismethylenephosphonate) at $P2X_1$ (IC_{50}, 5 nM) and $P2X_3$ (IC_{50}, 22 nM) receptors, being 14-fold and 10-fold more potent than PPADS itself. MRS 2257 produced a nonsurmountable inhibition when tested against a range of ATP concentrations, although blockade was reversed by about 85% after 20 minutes of washout. TNP-ATP and Ip_5I were equipotent with MRS 2257 at $P2X_1$ receptors, whereas TNP-ATP was 64-fold more potent than MRS 2257 at $P2X_3$ receptors. In conclusion, the PPADS template can be altered at the pyridoxal and phenyl moieties to produce $P2X_1$ and $P2X_3$ receptor antagonists showing higher potency and greater degree of reversibility than the parent compound at these Group I P2X receptors.

7.2 Introduction

Adenosine 5′-triphosphate (ATP) is widely regarded as a major signalling molecule in the peripheral nervous system, where exocytotically released ATP can act on a plethora of P2 purinoceptor subtypes (Ralevic and Burnstock, 1998). Purinergic receptors can be subdivided into two families: *i) P2X receptors* gated principally by ATP, and incorporating an intrinsic ion-channel and *ii) P2Y receptors* activated by either purine or pyrimidine nucleotides, and coupled to heterotrimeric G proteins. The P2X receptor subfamily has been further subdivided into functional groups with distinct operational profiles (Khakh *et al.*, 2001a). The first of these groups, Group I, includes the $P2X_1$ and $P2X_3$ receptors which are characterised by exceedingly fast kinetics for ion channel activation and inactivation, an acute sensitivity to the agonist α,β-methylene-ATP as well as ATP itself, and blockade by suramin and PPADS. $P2X_1$ receptors are abundant in vascular smooth muscle, whereas $P2X_3$ receptors are concentrated in nociceptive sensory nerve fibres and their cell bodies (King, 1998; Ralevic and Burnstock, 1998; Khakh *et al.*, 2001a).

An awareness of the therapeutic importance of $P2X_1$ and $P2X_3$ receptors as drug targets has given impetus to the development of selective antagonists for these receptor subtypes (Burnstock, 1998; Williams and Jarvis, 2000). PPADS was originally proposed to be a selective blocker of P2X receptors in vascular smooth muscle (Lambrecht *et al.*, 1992; Ziganshin *et al.*, 1993, 1994b; McLaren *et al.*, 1994). Further studies revealed that, at high concentrations, PPADS blocked both native $P2Y_1$-like and recombinant $P2Y_1$ receptors (Boyer *et al.*, 1994; Windscheif *et al.*, 1994, 1995a,b; Brown *et al.*, 1995; Schachter *et al.*, 1996) and, at best, PPADS showed only a modest selectivity for P2X *versus* P2Y receptors. PPADS was also found to inhibit the hydrolytic activity of ecto-

ATPase, thus enhancing ATP potency at both P2X and P2Y receptors and complicating the pharmacological analysis of PPADS antagonism in assay systems (Windscheif *et al.*, 1995a; Chen *et al.*, 1996; Ziganshin *et al.*, 1996). Such complications notwithstanding, PPADS does not noticeably interact with α_1 and β_1 adrenoceptors, muscarinic (M_{1-4}), histamine (H_1), serotonin (5-HT_3) or adenosine (A_1 and A_{2B}) receptors (Lambrecht *et al.*, 1992, 2000; Boyer *et al.*, 1994; Trezise *et al.*, 1994c; Ziganshin *et al.*, 1994b), and, therefore, remains a useful template to design chemical derivatives with improved potency and selectivity at P2X receptor subtypes.

The first PPADS derivative investigated for altered potency was pyridoxal-5′-phosphate (P5P), which is a derivative of pyridoxine (vitamin B_6) and was reported to block P2X receptors in rat vagus nerve (pK_B, 5.4) and rat vas deferens (pK_B, 5.3) in a competitive manner (Trezise *et al.*, 1994c). Pyridoxine $\alpha^{4,5}$-monophosphate (MRS 2219, a cyclized form of P5P) lost antagonist activity at $P2X_{1-4}$ receptors and potentiated ATP-responses at $P2X_1$ receptors (Jacobson *et al.*, 1998). IsoPPADS (pyridoxal-5′-phosphate 6-azophenyl 2′,5′-disulfonate) was found to have similar potency to PPADS at P2X receptors in rat vagus nerve (pK_B, 6.0), although blockade by isoPPADS but not PPADS was reversible upon washout (Trezise *et al.*, 1994a). IsoPPADS potently inhibited $P2X_1$ (IC_{50}, 43 nM) and $P2X_3$ (IC_{50}, 84 nM) receptors, whereas its cyclized form (pyridoxine $\alpha^{4,5}$-monophosphate 6-azophenyl 2′,5′-disulfonate; MRS 2220) showed a marked decrease in potency at $P2X_1$ (by 250-fold) and $P2X_3$ (by 700-fold) receptors (Jacobson *et al.*, 1998). The recently synthesised compound PPNDS (pyridoxal-5′-phosphate 6-(2′-naphthylazo-6′-nitro) 4′,8′-disulfonate), showed a 6-fold increase in potency over PPADS at $P2X_1$ receptors (pIC_{50}, 7.84) and P2X receptors in rat vas deferens (pK_B, 7.43) (Lambrecht *et al.*, 2000).

Over the last several years, we have gradually refined the structure of PPADS derivatives to improve their selectivity for P2X receptors (Jacobson *et al.*, 1998, 1999; Kim *et al.*, 1998, 2001). Some 30 PPADS analogues have been synthesised and tested on a wide range of nucleotide receptors ($P2X_{1,2,3,4,7}$ and $P2Y_{1,2,4,6}$), initially to identify compounds selective for P2X over P2Y receptors (Kim *et al.*, 1998) and, then, to identify compounds with a higher potency at $P2X_1$ and $P2X_3$ receptors than other P2X subtypes (Kim *et al.*, 2001). Many of these improved PPADS analogues were still found to inhibit both ecto-ATPase and ecto-apyrase, but only when used at high micromolar concentrations (Hoffman *et al.*, 2000; Ziganshin *et al.*, 2000). Thus, the present study investigated the antagonistic properties of a short series (7 of 30) of PPADS analogues that showed high selectivity for the Group I P2X receptor subtypes and low activity as inhibitors of ectoenzymes.

7.3 Methods

Materials

All common salts were AnalaR grade (Aldrich Chemicals; Gillingham, UK). ATP (disodium salt) was purchased from Boehringer (Mannheim, Germany). Tricaine (3-amino-benzoic acid ethyl ester) and P5P were purchased from Sigma Chemical Co. (Poole, UK), PPADS from RBI (Natick Mass., USA), isoPPADS from Tocris Cookson (St. Albans, UK) and 2′,3′-*O*-trinitrophenyl-ATP (TNP-ATP) from Molecular Probes (Cambridge, UK). Diinosine pentaphosphate (Ip_5I) was a gift from Dr. Jesus Pintor (Madrid, Spain). The synthesis and purification of PPADS derivatives have been described elsewhere (Kim *et al.*, 1998, 2001). The preparation of carbon-bridged analogues has also been described previously (Kim and Jacobson, 2000). Solutions of agonists and antagonists were prepared daily from stock solutions (10 or 100 mM, stored frozen) made up in extracellular bathing solution.

Oocyte preparation and P2X Receptor expression

Xenopus laevis were anaesthetised with Tricaine (0.2%, $w.v^{-1}$) and killed by decapitation (in accordance with Institution regulations). The dissection and removal of ovaries, as well as the preparation of defolliculated *Xenopus* oocytes, have been described in detail elsewhere (King *et al.*, 1997). Defolliculated oocytes do not possess native P1 or P2 receptors that could otherwise complicate the analysis of agonist activity (King *et al.*, 1996c,d). Also defolliculated oocytes are largely devoid of ecto-ATPase activity, so avoiding the complicating issue of ecto-enzyme inhibition by P2 receptor antagonists (Ziganshin *et al.*, 1995). Mature oocytes (stages V and VI) were injected (40 nl) cytosolically with capped ribonucleic acid (cRNA, 1 $mg.ml^{-1}$) encoding either rat $P2X_1$ or

rat $P2X_3$ receptor subunits. Injected oocytes were incubated at 18° C in a bathing solution (pH 7.5) containing (mM): NaCl 110, KCl 1, $NaHCO_3$ 2.4, Tris-HCl 7.5, $Ca(NO_3)_2$ 0.33, $CaCl_2$ 0.41, $MgSO_4$ 0.82, supplemented with gentamycin sulphate 50 $\mu g.l^{-1}$ for 48 h to allow full receptor expression, then stored at 4° C for up to 12 days.

Electrophysiology

Nucleotide-evoked membrane currents were recorded from cRNA-injected oocytes studied under voltage-clamp conditions using a twin-electrode amplifier (Axoclamp 2B; Foster City, CA). Intracellular microelectrodes had a resistance of 1-2 MΩ when filled with KCl (3M). Oocytes were perfused constantly (at 5 ml min^{-1}) with an extracellular solution containing (mM): NaCl 110, KCl 2.5, HEPES 5, $BaCl_2$ 1.8, pH 7.4-7.5. All recordings were made at room temperature (18° C) at a holding potential between –60 and –90 mV. Electro-physiological data were filtered initially at 3 kHz, captured at a rate of 20 Hz on a computer connected to an MP100WSW interface (Biopac Systems, Inc) and displayed using commercial software (Acqknowledge III, Biopac).

Drugs solutions

Solutions were delivered by gravity flow from independent reservoirs placed above the recording chamber (volume, 0.5 ml). ATP was applied for 30 s and, thereafter, washed off for a period of 20 min with extracellular bathing solution. P2 receptor antagonists were applied 20 min prior to, and during, agonist application. Only one antagonist was tested in each experiment. IC_{50} values were determined from Hill plots, using the transform log (I/I_{max}-I) where I was the current evoked by the agonist (used at the EC_{70}

concentration). For agonist concentration-response (C/R) curves, data were normalised to the maximum agonist response (I_{max}) to ATP (300 μM) at pH 7.5. Two C/R curves were produced in each experiment, before and after the addition of one concentration of antagonist. EC_{50} and EC_{70} values were determined from Hill plots, using the transform log (I/I_{max}-I) where I was the current evoked by each concentration of agonist. The Hill coefficient was taken from the slope of Hill plots. Concentration-response curves and inhibition curves were fitted by non-linear regression analysis using commercial software (Prism v2.0, GraphPad; San Diego, CA).

Statistics

Data are presented as mean ± S.E.M. of 4 sets of data from different batches of oocytes. Student's unpaired t test was used and p values ≤ 0.05 were considered significant.

7.4 Results

The Group I P2X receptor-selective antagonists used in this study were PPADS analogues possessing pyridoxal-5′-phosphate or pyridoxal-5′-phosphonate moieties, the structures of which are shown in Figure 7.1. The codename, formula, molecular weight and chemical name of seven key analogues are listed in Table 7.1. The blocking activity of PPADS, isoPPADS, the related analogues and two known standards (Ip_5I and TNP-ATP) were tested over a wide range of concentrations (0.01-30,000 nM) against ATP-evoked inward currents at either $P2X_1$ or $P2X_3$ receptors. The resultant inhibition curves for a total of eleven P2 receptor antagonists acting at each P2X subtype are shown in Figure 7.2. Mean IC_{50} values, 95% confidence intervals and Hill co-efficients are given in Table 7.2. The most potent PPADS analogue was MRS 2257 at $P2X_1$ receptors (mean IC_{50}, 5nM) and $P2X_3$ receptors (mean IC_{50}, 22 nM). The route to its discovery is described in the below.

SAR of PPADS analogues

PPADS was an effective antagonist at submicromolar concentrations and about 3-fold more potent at $P2X_1$ than $P2X_3$ receptors (mean IC_{50}, 68 *vs* 214 nM). The isoform, isoPPADS, was also a potent antagonist at $P2X_1$ and $P2X_3$ receptors (mean IC_{50}, 35 *vs* 79 nM). Thus, isoPPADS was 2- or 3-fold more potent than PPADS at the Group I P2X receptors. MRS 2191, a 5′-phosphonate derivative of isoPPADS, was even more potent than isoPPADS at $P2X_1$ receptors (mean IC_{50}, 9 *vs* 35 nM), although markedly less potent than isoPPADS at $P2X_3$ receptors (mean IC_{50}, 229 *vs* 79 nM).

MRS 2143, a 5′-phosphate derivative lacking sulfonate groups on the azophenyl moiety, was 2-fold more potent than PPADS at $P2X_1$ receptors (mean IC_{50}, 32 *vs* 68 nM) and slightly less potent than PPADS at $P2X_3$ receptors (mean IC_{50}, 345 *vs* 214 nM).

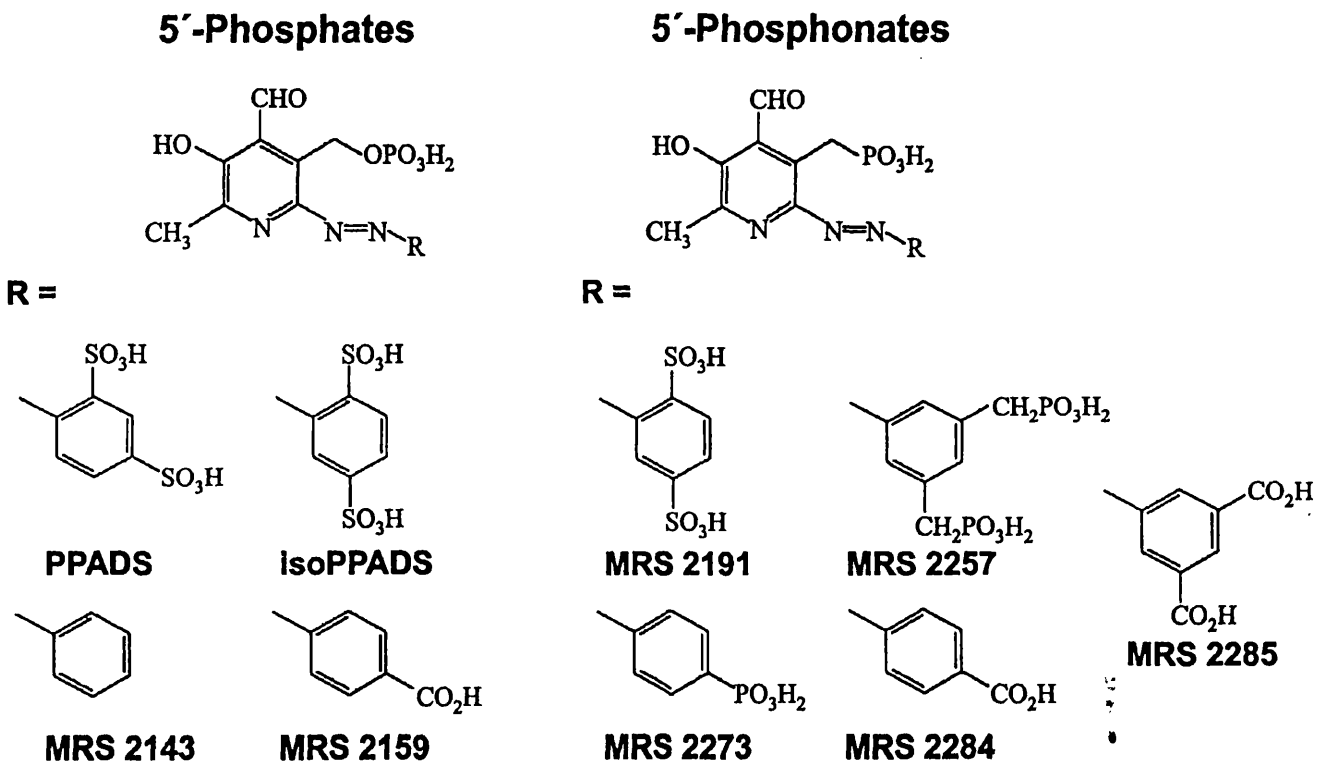


Figure 7.1. *Chemical structure of PPADS and related analogues*

The analogues used in this study were based on pyridoxal-5′-phosphates (left) and pyridoxal-5′-phosphonates (right). Structurally related derivatives of each group were synthesised and tested for pharmacological activity at $P2X_1$ and $P2X_3$ receptors.

Table 7.1. PPADS and related derivatives

Compound	Formula	Mol. Wt.	Name
PPADS	$C_{14}H_{10}N_3O_{12}PS_2Na_4$	599.3	pyridoxal-5′-phosphate-6-azophenyl-2′,4′-disulphonate
isoPPADS	$C_{14}H_{10}N_3O_{12}PS_2Na_4$	599.3	pyridoxal-5′-phosphate-6-azophenyl-2′,5′-disulphonate
MRS 2143	$C_{14}H_{12}N_30_6PNa_2$	395.2	pyridoxal-5′-phosphate-6-azophenyl
MRS 2159	$C_{15}H_{13}N_3O_8PNa$	417.2	pyridoxal-5′-phosphate-6-azophenyl-4′-carboxylate
MRS 2191	$C_{14}H_{14}N_3O_{11}PS_2Na_3$	561.3	pyridoxal-5′-phosphonate-6-azophenyl-2′,5′-disulphonate
MRS 2257	$C_{16}H_{18}N_3O_{11}P_3Na_3$	590.2	pyridoxal-5′-phosphonate-6-azophenyl-3′,5′-bismethylphosphonate
MRS 2273	$C_{14}H_{13}N_3O_8P_2Na_2$	459.2	pyridoxal-5′-phosphonate-6-azophenyl-4′-phosphonate
MRS 2284	$C_{15}H_{14}N_3O_7PNa_1$	402.2	pyridoxal-5′-phosphonate-6-azophenyl-4′-carboxylate
MRS 2285	$C_{16}H_{14}N_3O_9PNa_3$	443.3	pyridoxal-5′-phosphonate-6-azophenyl-3′,5′-dicarboxylate

However, this compound revealed to us that effective antagonism did not require highly polar sulfonate groups on the azophenyl moiety *per se*, an advantage in drug design.

A carboxylate group was added at various positions on the azophenyl moiety of the MRS 2143 template, and the most interesting compound was MRS 2159. This compound increased antagonist potency at both P2X receptor subtypes and, consequently, MRS 2159 was 7-fold more potent than PPADS at $P2X_1$ receptors (mean IC_{50}, 9 *vs* 68 nM) and 2-fold more potent than PPADS at $P2X_3$ receptors (mean IC_{50}, 118 *vs* 214 nM). Conversion of MRS 2159 into a 5′-phosphonate compound (MRS 2284) retained antagonist potency at $P2X_1$ and $P2X_3$ receptors (mean IC_{50}, 7 and 139 nM). However, the addition of a second carboxylate group to MRS 2284, to produce MRS 2285, had a deleterious effect and reduced antagonist potency at $P2X_1$ and, more so, $P2X_3$ receptors (mean IC_{50}, 22 and 260 nM).

Concentrating on pyridoxal-5′-phosphonate analogues, the effects of different substitutions at the azophenyl moiety were explored by replacing sulfonate and carboxylate groups with either phosphonate or methylphosphonate groups. MRS 2273 (phosphonate added) and MRS 2257 (methylene-phosphonates added) showed a marked increase in potency, particularly at $P2X_1$ receptors at which mean IC_{50} values of 11 nM and 5 nM were seen for these two compounds. MRS 2273 was 6-fold, and MRS 2257 14-fold, more potent than PPADS at $P2X_1$ receptors. These derivatives were also effective antagonists at $P2X_3$ receptors (mean IC_{50}, 33 and 22 nM), being 6-fold and 10-fold more potent than PPADS at $P2X_3$ receptors.

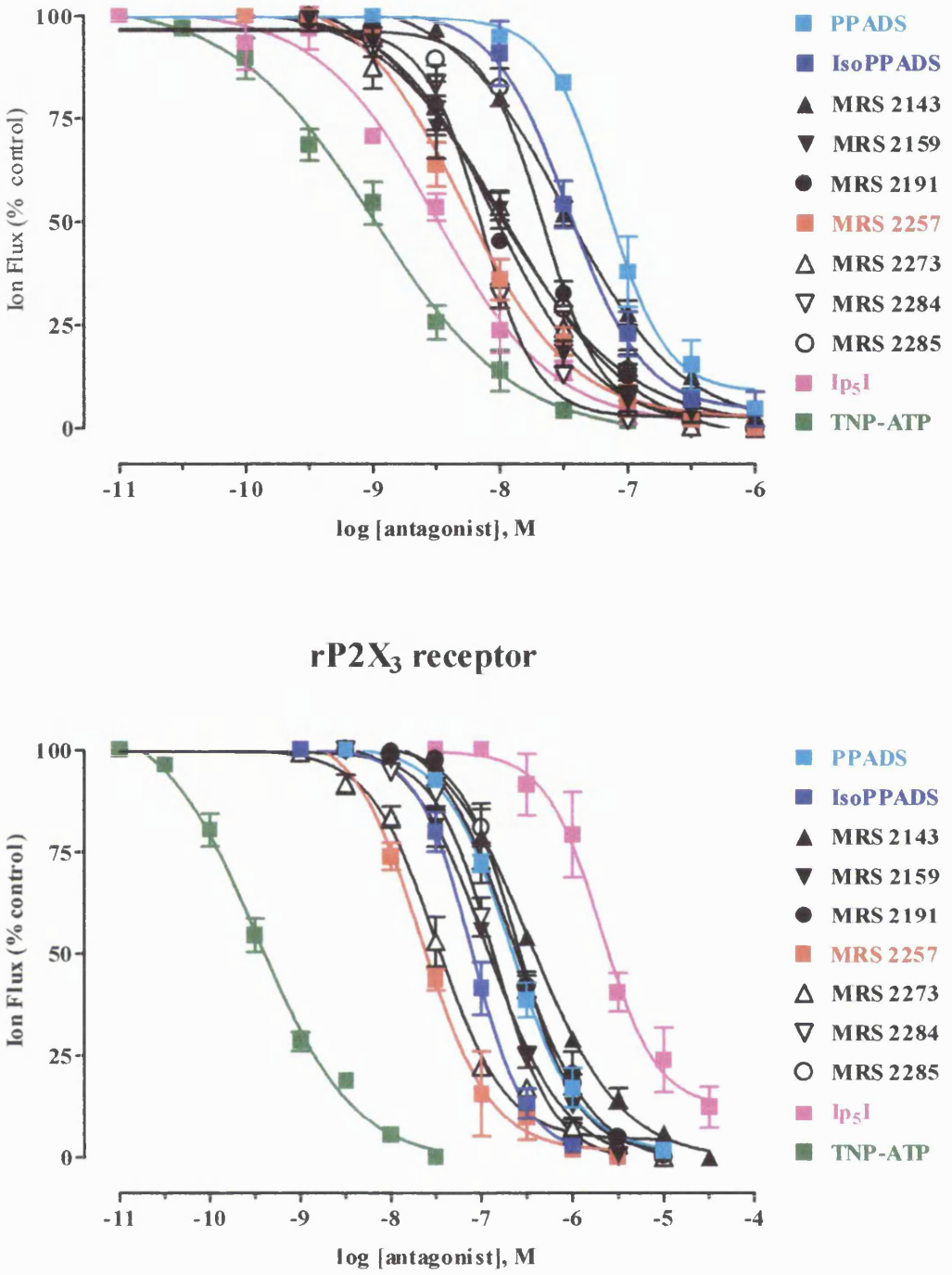


Figure 7.2. *Blocking activity of PPADS and related compounds*

Inhibition curves for PPADS, isoPPADS, seven related PPADS-like compounds and two standards (Ip_5I and TNP-ATP) at $P2X_1$ receptors (A) and $P2X_3$ receptors (B). P2X receptors were activated by ATP at approximately the EC_{70} concentration ($P2X_1$, 1 μM; $P2X_3$, 3 μM). Data represent mean ± SEM for 4 determinations from separate batches of *Xenopus* oocytes.

Table 7.2. Antagonist potency at Group 1 P2X receptors

Compound	rP2X$_1$			rP2X$_3$		
	IC$_{50}$ (nM)	*CI (nM)*	n_H	*IC$_{50}$ (nM)*	*CI (nM)*	n_H
PPADS	**68.5**	**45.9-102.2**	**-1.87**	**213.6**	**173.0-263.7**	**-1.17**
isoPPADS	**34.5**	**25.3-47.1**	**-1.48**	**78.8**	**57.4-108.0**	**-1.46**
MRS 2143	**32.4**	**26.5-40.4**	**-0.97**	**345.3**	**240.6-495.4**	**-0.88**
MRS 2159	**9.4**	**6.6-13.4**	**-1.08**	**117.7**	**93.4-148.3**	**-1.02**
MRS 2191	**8.9**	**5.9-13.6**	**-0.84**	**228.6**	**171.0-305.5**	**-1.01**
MRS 2257	**5.1**	**3.6-7.3**	**-1.02**	**21.8**	**13.8-34.5**	**-1.13**
MRS 2273	**11.3**	**7.5-17.1**	**-0.86**	**32.9**	**25.8-41.9**	**-1.15**
MRS 2284	**7.1**	**6.0-8.4**	**-1.98**	**139.0**	**114.1-169.3**	**-1.33**
MRS 2285	**21.9**	**17.4-27.5**	**-1.9**	**259.8**	**222.3-303.8**	**-1.34**
Ip$_5$I	**3.13**	**2.09-4.69**	**-0.94**	**2059.**	**1252.-3388.**	**-1.43**
TNP-ATP	**1.01**	**0.69-1.47**	**-0.78**	**0.34**	**0.25-0.46**	**-0.82**

Each compound was tested against ATP-responses (rP2X$_1$, 1 μM; rP2X$_3$, 3 μM) and antagonist activity expressed as mean IC$_{50}$ value (nM, for 4 determinations) with confidence intervals for the mean at the 95% level (CI, nM). The mean Hill coefficient (n_H) is also given for each set of inhibition curves.

The azo (-N=N-) linkage

PPADS and isoPPADS slowly decompose when exposed to white light in aqueous solution at room temperature because the azo (-N=N-) bridge connecting the pyridoxal and phenyl moieties is chemically unstable (G. Semple; personal communication). Therefore, three pilot studies of new PPADS analogues were initiated to test the effects of changing the azo-linkage in the sulfonate-free analogue MRS 2143 (Figures 7.3A,B and Table 7.3). In the first pilot study, MRS 2143 was converted into a pyridoxal bisphosphate analogue (MRS 2260) and, thereafter, the azo-linkage replaced by an ethene (-C=C-) bridge in MRS 2259. The antagonist activity of MRS 2260 was significantly reduced (~53% blockade at 10 μM) at $P2X_1$ receptors, and activity of MRS 2259 not appreciably higher (~40% blockade at 10 μM). In a second pilot study, substitution of the azo bridge in MRS 2143 with a methylene (CH_2) bridge maintained the inhibitory activity of MRS 2335 (mean IC_{50}, 37 nM) which was equipotent with MRS 2143 and 2-fold more potent than PPADS. A comparison of the pyridoxal-5′-phosphate compound (MRS 2335) with its pyridoxal-5′-phosphonate derivative (MRS 2305) revealed a reduction in blocking activity (mean IC_{50}, 214 nM), MRS 2305 being markedly less potent than either MRS 2143 (by 6-fold) or PPADS (by 3-fold). In the third pilot study, the azo bridge of MRS 2143 was replaced by an ethene bridge with the derivative (MRS 2379) retaining blocking activity (IC_{50}, 41 nM) at $P2X_1$ receptors.

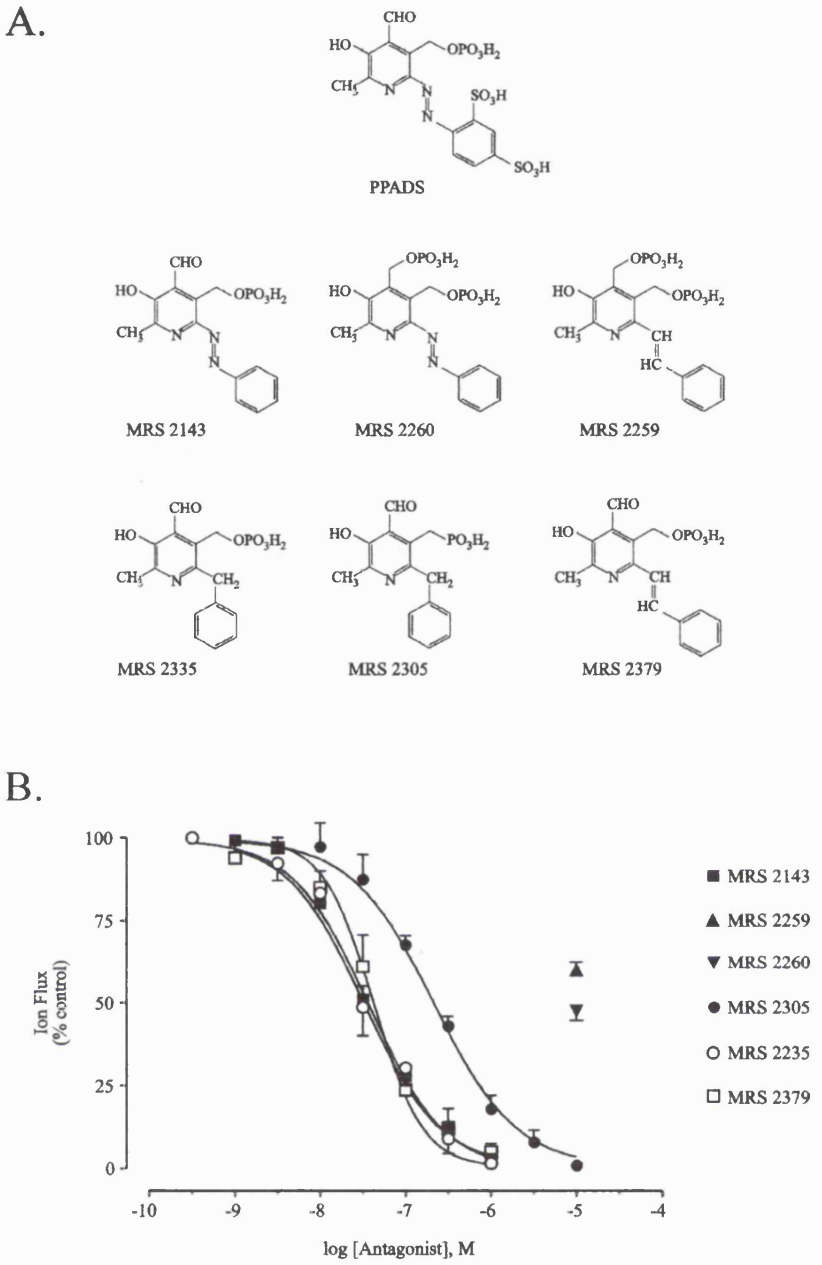


Figure 7.3. *Effect of azo-linkage on antagonist activity.*

In A, chemical structure of compounds related to a nonsulfonated derivative of PPADS (MRS 2143) showing variations in the azo-phenyl linkage. In B, inhibition curves for MRS 2143, MRS 2335, MRS2379 and MRS 2305 at $P2X_1$ receptors activated by ATP (1 μM). MRS 2259 and MRS 2260 were tested at a single concentration (10 μM). Data represent mean ± SEM for 4 determinations from separate batches of *Xenopus* oocytes.

Table 7.3. Antagonist activity at $P2X_1$ receptor

Compound	IC_{50} (nM) or % inhibition	CI (nM)	n_H
PPADS	**68.5**	**45.9-102.2**	**-1.87**
MRS 2143	**32.4**	**26.5-40.4**	**-0.97**
MRS 2335	**36.9**	**22.7-60.1**	**-0.98**
MRS 2305	**213.7**	**127.9-357.2**	**-0.90**
MRS 2379	**40.9**	**27.4-61.2**	**-1.42**
MRS 2259 (10 μM)	**40±3%**	**-**	**nd**
MRS 2260 (10 μM)	**53±3%**	**-**	**nd**

Each compound was tested against $P2X_1$ receptors activated by ATP (1 μM). Antagonist activity is expressed as mean IC_{50} value (nM, for 4 determinations) with confidence intervals for the mean at the 95% level (CI, nM) or, where tested at a single concentration (10 μM), as a percentage inhibition of ATP responses (for 4 determinations). The mean Hill coefficient (n_H) is also given for each set of inhibition curves.

Antagonism by TNP-ATP and Ip_5I

Two recently identified antagonists, with high potency at $P2X_1$ and $P2X_3$ receptors, were also tested as standards to provide a comparative index of antagonism. Nanomolar concentrations of trinitrophenyl-ATP (TNP-ATP) blocked both $P2X_1$ and $P2X_3$ receptors (mean IC_{50}, 1 and 0.34 nM, figure 7.2), this compound showing 3-fold higher potency at $P2X_3$ receptors. Diinosine pentaphosphate (Ip_5I) was found to be highly selective for $P2X_1$ receptors (mean IC_{50}, 3 nM, figure 7.2), and 700-fold less potent at $P2X_3$ receptors (mean IC_{50}, 2059 nM, figure 7.2). Against the most potent PPADS analogue, these two standards were approximately equipotent with MRS 2257 at $P2X_1$ receptors whereas TNP-ATP was 64-fold more potent than MRS 2257 at $P2X_3$ receptors.

Reversibility of P2X receptor antagonism

The ability of $P2X_1$ and $P2X_3$ receptors to recover from blockade was assessed 20 minutes after washout of a maximally effective blocking concentration of each PPADS analogue. Full recovery of agonist responses was observed after isoPPADS washout ($P2X_1$, 101±4%; $P2X_3$, 105±5%; mean±SEM, n=3), and partial recovery observed following PPADS washout ($P2X_1$, 31±10%; $P2X_3$, 44±7%). MRS 2143, the PPADS analogue lacking sulfonate groups, also showed a reasonable level of recovery ($P2X_1$, 74±5%; $P2X_3$, 79±6%). Additionally, MRS 2257 showed a good level of recovery ($P2X_1$, 82±5%; $P2X_3$, 85±4%). The carboxylated 5′-phosphonate compounds (MRS 2284 and MRS 2285) each caused a near irreversible blockade (<25 % recovery).

Nature of antagonism

The nature of antagonism caused by PPADS-like derivatives was assessed from concentration/response (C/R) curves for ATP, in the absence and presence of each compound. PPADS and isoPPADS antagonism were first assessed at $P2X_1$ receptors (Figure 7.4) and $P2X_3$ receptors (Figure 7.5). Thereafter, two further compounds investigated - MRS 2257 (a potent and slowly reversible antagonist) and MRS 2284 (a potent and irreversible antagonist). Each analogue exerted a non-surmountable antagonism at $P2X_1$ and $P2X_3$ receptors (Figures 7.4, 7.5). There was no significant change in the EC_{50} values for ATP in the presence of each compound (data not shown), although the maximum agonist response was progressively decreased in a concentration-dependent manner.

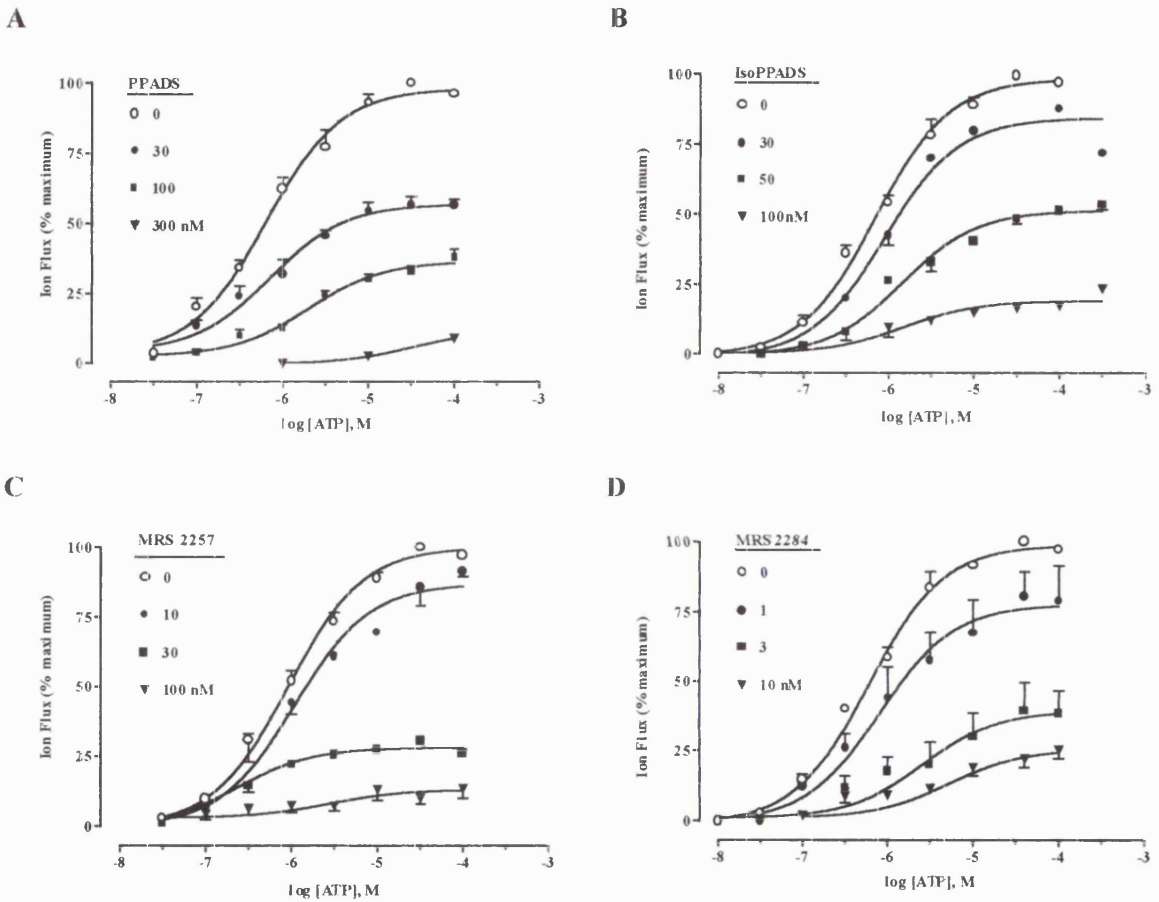


Figure 7.4. Concentration/response (C/R) relationship for ATP at $P2X_1$ receptors, in absence and presence of the $P2X_1$ receptor antagonists. Paired C/R curves (control and test) were obtained for each concentration of antagonist studied. Data (mean ± SEM, n = 4) are expressed as a percentage of the maximum ATP-response (at pH 7.5) prior to the addition of antagonist. Where missing, error bars are smaller than symbol size.

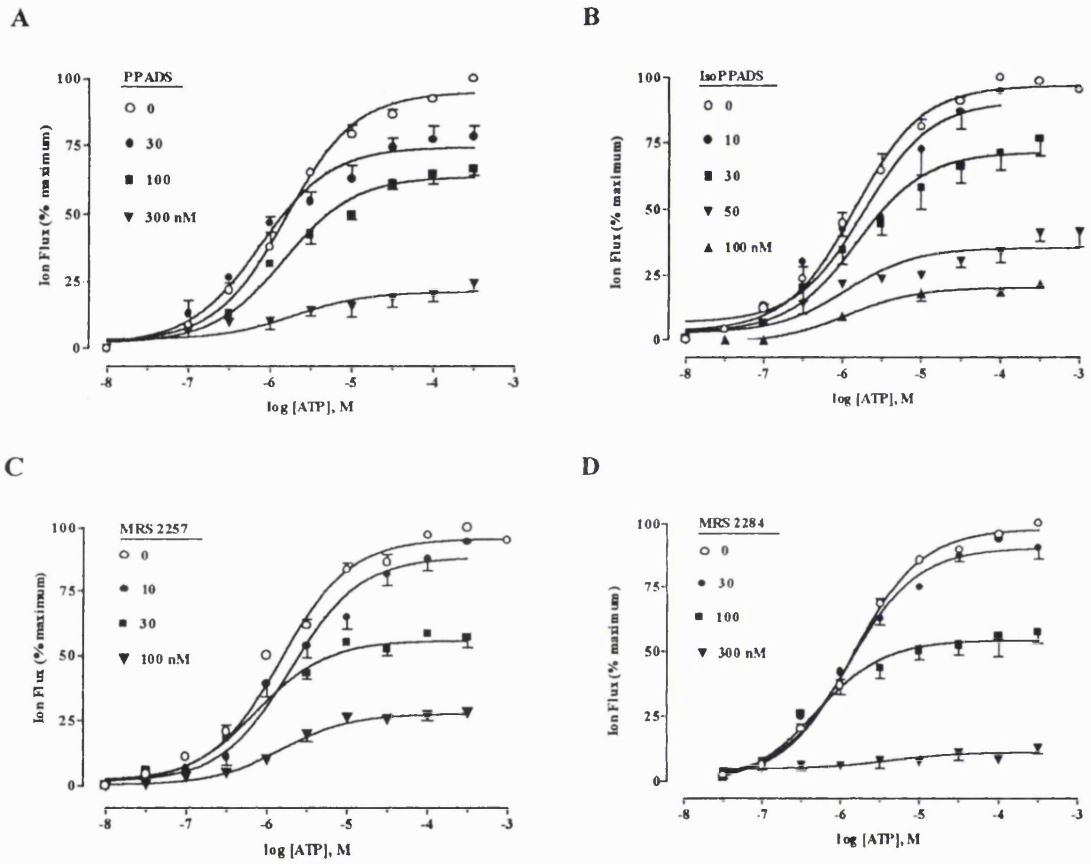


Figure 7.5. Concentration/response (C/R) relationship for ATP at $P2X_3$ receptors, in absence and presence of the $P2X_3$ receptor antagonists. Paired C/R curves (control and test) were obtained for each concentration of antagonist studied. Data (mean ± SEM, n = 4) are expressed as a percentage of the maximum ATP-response (at pH 7.5) prior to the addition of antagonist. Where missing, error bars are smaller than symbol size.

7.5 Discussion

In the present study, we investigated the blocking actions of a short series of PPADS analogues at $P2X_1$ and $P2X_3$ receptors – the Group I P2X receptor subtypes. Seven analogues were selected from a library of 30 PPADS derivatives on the grounds that, except for $P2X_1$ and $P2X_3$ receptors, these compounds did not show significantly different blocking activity to PPADS at other P2X and P2Y receptor subtypes (Kim *et al.*, 2001). Also, these seven compounds showed low inhibitory activity at surface enzymes capable of breaking down ATP (Hoffman *et al.*, 2000; Ziganshin *et al.*, 2000). All seven analogues were shown here to be effective inhibitors of ATP- mediated inward currents at $P2X_1$ and $P2X_3$ receptors. At $P2X_1$, IC_{50} values ranged from 5 to 22 nM and the best compound, MRS 2257, was 14-fold more potent than PPADS. At $P2X_3$, IC_{50} values ranged from 22 to 345 nM and the best compound, again MRS 2257, was 10-fold more potent than PPADS. The high potency of the methylene-phosphonate analogue, MRS 2257, at $P2X_1$ and $P2X_3$ receptors may be related to the avidity of Group I P2X receptors for the methylene-phosphonate based agonists, α,β-meATP and β,γ-meATP.

One drawback to the use of PPADS as an antagonist at P2 receptors is the apparent irreversibility of blockade. In the present study, some of the tested analogues were found to reverse slowly and, as pointed out by Lambrecht and colleagues (1992), the term *pseudo-irreversible blockade* is probably more appropriate. Blockade by the most potent analogue, MRS 2257, was relaxed by approximately 82-85% after 20 minutes washout and this compared favourably against 31-44% relaxation after PPADS. Blockade by isoPPADS was fully relaxed after 20 minutes washout. However, analysis of the C/R curves for ATP showed that blockade by MRS 2257, PPADS and isoPPADS was nonsurmountable in each case. This observation suggests that the off-rate of these

antagonists is significantly slower than either the on-rate for the agonist or the desensitisation rate at these rapidly inactivating P2X receptors.

We also compared the blocking activity of MRS 2257 against two known standards, Ip_5I and TNP-ATP, which currently represent the most potent antagonists at Group I P2X receptors (Virginio *et al.*, 1998; King *et al.*, 1999). By statistical analysis, MRS 2257 was as potent as Ip_5I and TNP-ATP at $P2X_1$ receptors whereas MRS 2257 was 64-fold less potent than TNP-ATP at $P2X_3$ receptors. The activity of Ip_5I and TNP-ATP is about 300- to 1000-fold lower at native P2X receptors *in vivo*, since each of these nucleotidic compounds is broken down by tissue enzymes (Hoyle *et al.*, 1997; Lewis *et al.*, 1998). We have not yet examined the blocking activity of MRS 2257 in whole tissues, because of production problems in generating sufficient quantities of this compound.

The observed blocking activity of the seven tested analogues, PPADS and isoPPADS has helped us to understand the structural requirements for potent antagonists at Group I P2X receptors. In this study, we found that azo-linked analogues with pyridoxal-5′-phosphonate moieties were more potent than their pyridoxal 5′-phosphate counterparts. It was also clear that the pyridoxal bis-4′,5′-phosphate derivatives MRS 2259/2260 (shown in Figure 7.3) were markedly less potent than PPADS and related analogues at Group I P2X receptors. These present results complement our earlier findings where the cyclic pyridoxine-$\alpha^{4,5}$-phosphate derivatives of P5P (MRS 2219) and isoPPADS (MRS 2200) showed diminished or no antagonist activity at Group I P2X receptors (Jacobson *et al.*, 1998). Others have shown that 5′-dephosphorylated pyridoxine hydrochloride is less potent than pyridoxal 5′-phosphate at P2X receptors in rat vas deferens (Trezise *et al.*, 1994c).

When modifications to the azophenyl moiety were analysed, it was clear that PPADS analogues could either dispense with disulfonate groups (*e.g.* MRS 2143) or accommodate other substitutions and still retain antagonist activity at $P2X_1$ receptors. Our present findings showed that azophenyl moieties with carboxylate (MRS 2159, 2284, 2285), phosphate (MRS 2273) and methylene-phosphonate (MRS 2257) substitutions yielded highly potent antagonists for the $P2X_1$ receptor. Closer inspection of inhibition data showed that desulfonated MRS 2143 and carboxylated MRS 2285 were less potent than PPADS at $P2X_3$ receptors, in contrast to their actions at $P2X_1$ receptors. This observation suggests that separate lines of chemistry are possible for $P2X_1$ and $P2X_3$ receptor-selective antagonists, although we have some way to go to improve the selectivity window or specificity for one P2X subtype over the other. Apart from the above modifications, others have shown that the azophenyl moiety can be replaced by naphthylazo groups (*e.g.* PPNDS) and improve antagonist potency at $P2X_1$ receptors (Lambrecht *et al.*, 2000).

PPADS will break down in solution, at room temperature and under white light, due the chemical reactivity of the azo (-N=N-) linkage (G. Semple: personal communication). Accordingly, we have begun to explore ways of dealing with this instability by replacing the azo bridge with other linkages. The conversion of the azo bridge, in MRS 2143, to a methylene bridge, in MRS 2235, caused no significant change in the blocking activity at $P2X_1$ receptors. This observation has prompted us to produce a new series of derivatives based on MRS 2235, with various additions to the benzyl moiety, to improve activity and selectivity at the Group I P2X receptors. Our results with ethene-bridged compounds related to MRS 2379 (see Figure 7.3) were also encouraging and we are exploring synthesis routes for like analogues. We have yet to test either of these new series on a full range of P2X and P2Y receptor subtypes.

In summary, the present results have shown that the PPADS template remains amenable to chemical modification and can yield highly potent antagonists for Group I P2X receptors. Some analogues were more potent at $P2X_1$ receptors and, to this extent, we are starting to see the beginnings of selectivity for this receptor subtype. The scope for modification is not yet exhausted and we hope to improve on the current indices of drug activity by altering the molecule in three positions (pyridoxal and azophenyl moieties and azo linkage). However, the current series of antagonists represent a significant improvement on PPADS - the best compound, MRS 2257, being at least 10-fold more potent at Group 1 P2X receptors and its blocking actions reversible with washout.

CHAPTER 8

HETEROMULTIMERIC $P2X_{1/2}$ RECEPTORS SHOW A NOVEL SENSITIVITY TO EXTRACELLULAR pH

8.1 Summary

1. Rat $P2X_1$ and $P2X_2$ subunits were co-expressed in defolliculated *Xenopus* oocytes and resultant P2X receptors studied under voltage-clamp conditions. Extracellular ATP elicited a biphasic inward current that involved an initial rapidly inactivating component (I_1 or $P2X_1$-like) followed by a second slowly inactivating component (I_2 or $P2X_2$-like). I_1 responses were potentiated by lowering extracellular pH (from 7.5 to 6.5) in a subpopulation of co-injected oocytes, whereas responses at homomeric $rP2X_1$ receptors are inhibited under acidic conditions.
2. The concentration/response (C/R) curve for ATP extended over 5 log-units of concentration (1nM to 300 μM), with an apparent EC_{50} value of 0.56±0.09 μM, in cells exhibiting this pH-enhanced I_1 response. However, the ATP curve for the I_1 response was better described as biphasic and clearly distinct from the monophasic ATP C/R curve for homomeric $rP2X_1$ receptors.
3. Under acidic (pH 5.5, 6.5) and alkaline (pH 8.5) conditions, resultant ATP C/R curves for the I_1 response extended again over a wide concentration range (1nM to 300 μM) and, in each case, revealed increases in either agonist potency and/or maximum response (I_{max}) compared to data obtained at pH 7.5. This was not true for homomeric $rP2X_1$ receptors. Thus, the I_1 response possessed the kinetics of homomeric $P2X_1$ receptors and, to an extent, the acid sensitivity of homomeric $P2X_2$ receptors.
4. Taken together, the pH sensitivity and peculiar pharmacological properties of the I_1 response (in combination with a previous biochemical study showing $rP2X_1$ and $rP2X_2$ co-immunoprecipitate) indicate that these two P2X subunits co-assemble to form novel heteromeric $P2X_{1/2}$ ion-channels.

8.2 Introduction

Adenosine 5′-triphosphate (ATP) acts as a fast excitatory transmitter in the central, peripheral and enteric nervous systems (Ralevic and Burnstock, 1998). Extracellular ATP exerts its effects through two main classes of P2 receptors, the P2X and P2Y families (Burnstock & King, 1996). For the P2X receptor class, seven subunits ($P2X_{1-7}$) have been cloned thus far. P2X subunits have two membrane-spanning domains connected by a large cysteine-rich extracellular loop – with three, or possibly four, subunits assembling to form ligand-gated cation channels selective for ATP (Brake *et al.*, 1994; Valera *et al.*, 1994; Bo *et al.*, 1995; Chen *et al.*, 1995; Collo *et al.*, 1996; Surprenant *et al.*, 1996; Kim *et al.*, 1997; Nicke *et al.*, 1998).

Transcripts for all P2X subunits, except $P2X_7$, have been found in sensory, sympathetic and auditory nerves (Collo *et al.*, 1996; Xiang *et al.*, 1999). It has been suggested that this overlap allows for the co-assembly of P2X receptor subunits into new heteromeric complexes with distinct pharmacology. Indeed, co-expression of $P2X_2$ and $P2X_3$ subunits results in the formation of a heteromer which shows pharmacological properties distinct from homomeric $P2X_2$ and $P2X_3$ receptors (Lewis *et al.*, 1995; Liu *et al.*, 2001). The formation of heteromeric $P2X_{2/3}$ receptors has, in part, helped explain the pharmacological properties of some P2X receptors in sensory and sympathetic nerves (Lewis *et. al.*, 1995; Khakh *et al.*, 1995a; Radford *et al.*, 1997; Zhong *et al.*, 2000). Similar work has been conducted with $P2X_4$ and $P2X_6$ subunits, transcripts for which show an overlapping expression in regions of adult rat brain, and these subunit proteins also generate a novel heteromeric P2X ion-channel (Lê *et al.*, 1998b). $P2X_1$ and $P2X_5$ transcripts have an overlapping expression in the ventral horn of

the spinal cord (Collo *et al.*, 1996) and the co-expression of these subunits results in yet another novel P2X receptor phenotype (Torres *et al.*, 1998b). $P2X_2$ and $P2X_6$ transcripts co-exist in respiratory centers in the rat brainstem and these two subunits form heteromeric $P2X_{2/6}$ receptors with a distinct pharmacology and unique sensitivity to extracellular pH (King *et al.*, 2000).

Biochemical evidence, using co-immunoprecipitation techniques, has highlighted the possibility of heteropolymerisation between various combinations of known P2X subunits (Torres *et al.*, 1999b). Torres and co-workers described the co-precipitation of $P2X_1$ and $P2X_2$ subunits that pointed to a natural occurrence of heteromeric $P2X_{1/2}$ receptors. In common with previous findings where other P2X receptor subunits form heteromeric assemblies, overlapping transcripts and immunoreactivity for $P2X_1$ and $P2X_2$ have been found in neurons. Overlapping expression of $P2X_1$ and $P2X_2$ transcripts was seen in sensory and auditory nerves and in regions of the developing rat brain (Kidd *et al.*, 1995, Xiang *et al.*, 1998, 1999). Furthermore, positive immunoreactivity was seen for $P2X_1$ and $P2X_2$ subunits in the dorsal horn of the spinal cord and in selected regions of the adult rat brain (Kanjhan *et al.*, 1996; Vulchanova *et al.*, 1996; Loesch and Burnstock, 1998).

In the present study, the possibility of heteromeric assemblies of $P2X_1$ and $P2X_2$ subunits was examined by comparing the pharmacological and kinetic profiles of the recombinant P2X receptors formed by subunit co-expression in defolliculated *Xenopus* oocytes. The results indicate the presence of a novel pH-sensitive P2X receptor phenotype and highlight the increased complexity in ATP-mediated excitatory transmission through heteropolymerisation of P2X subunits.

8.3 Methods

8.3.1 Preparation and injection of oocytes with cRNA.

Xenopus laevis were anesthetized with Tricaine (0.2%, wv^{-1}) and killed by decapitation. *Xenopus* oocytes were harvested and prepared for injection as described in detail previously (King *et. al.*, 1997b). Defolliculated oocytes do not possess native P1 or P2 receptors that might otherwise complicate the analysis of agonist activity (King *et al.*, 1996b,d). Defolliculated oocytes were injected cytosolically with a 40 nl mixture of capped RNA (cRNA). The cRNA mixture consisted of 20 nl of cRNA encoding rat $P2X_1$ (1μg/μl; Valera *et al.*, 1994) and 20 nl of cRNA encoding rat $P2X_2$ (0.002μg/μl; Brake *et al.*, 1994). Some batches of oocytes were injected with 40 nl of cRNA for either rat $P2X_1$ or rat $P2X_2$ alone. Injected oocytes were incubated at 18°C in Barth's solution (pH 7.5) containing (mM): NaCl 110, KCl 1, $NaHCO_3$ 2.4, Tris-HCl 7.5, $Ca(NO_3)_2$ 0.33, $CaCl_2$ 0.41, $MgSO_4$ 0.82, supplemented with gentamycin sulfate 50μg l^{-1} for 24 hr and then stored at 4° C for up to 10 days.

8.3.2 Electrophysiology.

Membrane currents were recorded under voltage-clamp conditions using a twin-electrode amplifier (Axoclamp 2B). Intracellular microelectrodes filled with 3 M KCl and showed 1-2 MΩ resistance. Oocytes were placed in a perspex recording chamber and perfused at a constant rate of 5 $ml.min^{-1}$ with Ringer's solution containing (mM): NaCl 110, KCl 2.5, HEPES 5, $BaCl_2$ 1.8 pH 7.5. The pH level of all drugs and solutions stated in the text was

adjusted by adding either 1N HCl or 1N NaOH. Solutions were delivered by a gravity flow system from separate reservoirs placed above the preparation. All drugs were prepared in nominally Ca^{2+}-free Ringer's solution at the concentrations stated in the text. Agonists were perfused for 30s or until the evoked current reached a maximum. Applications of agonists were separated by intervals of 20 minutes. All recordings were made at room temperature (18°C) and at a holding potential of between –60 and –90 mV. Electrophysiological data were recorded using the software package *Acqknowledge III* (Biopac Systems).

8.3.3 Data analysis.

EC_{50} values for agonists were taken from Hill plots using the transformation log (I/I_{max}-I), where I is the current evoked by each concentration of agonist. Hill co-efficients were also taken from the slope of these plots. Concentration/response (C/R) curves were fitted by non-linear regression analysis using commercial software (*Prism v2.0*, GraphPad). Data are presented as mean ± SEM of four or more determinations.

8.4 Results

8.4.1 Use of acidic conditions to distinguish types of P2X receptors.

Co-expression of two P2X subunits, that individually are capable of forming homomeric P2X receptors, has been shown to generate a mixed population of homomeric and heteromeric assemblies (Liu *et al.*, 2001). For $P2X_1/P2X_2$ cRNA co-injected oocytes, ATP (100 μM) evoked biphasic inward currents that comprised an initial rapidly inactivating component (I_1 or $P2X_1$-like) followed by a second slowly inactivating component (I_2 or $P2X_2$-like) (Figure 8.1). Thus, it was necessary to find a way to separate the agonist responses of homomeric $rP2X_1$ and $rP2X_2$ receptors from those mediated by heteromeric $P2X_{1/2}$ receptors.

It has been shown that low extracellular pH (pH_e) influences ATP responses in opposite ways at homomeric $rP2X_1$ and $rP2X_2$ receptors (Stoop *et al.*, 1997; Wildman *et al.*, 1999). Thus, co-injected oocytes were tested at two pH levels (7.5 and 6.5) in the hope of revealing differences between ATP responses at homomeric and heteromeric P2X assemblies (Figure 8.1A,B). In most cells tested (from an initial sample of 73 of 87 co-injected oocytes), the I_1 response was inhibited at pH 6.5 and I_2 response unaffected (Figure 8.1A). These findings were consistent with the involvement of homomeric $P2X_1$ receptors in the I_1 response and homomeric $P2X_2$ receptors in the I_2 responses (*see below and Discussion*). In a smaller subset of tested cells (14 or 87 co-injected oocytes; 16%), the rapidly inactivating I_1 response was significantly potentiated at pH 6.5 and this finding indicated a novel P2X receptor might be involved (Figure 8.1B). Further batches of $P2X_1/P2X_2$ cRNA co-injected oocytes were prepared and surveyed, with the view to exploring the properties of rapidly inactivating I_1 responses potentiated under acidic conditions.

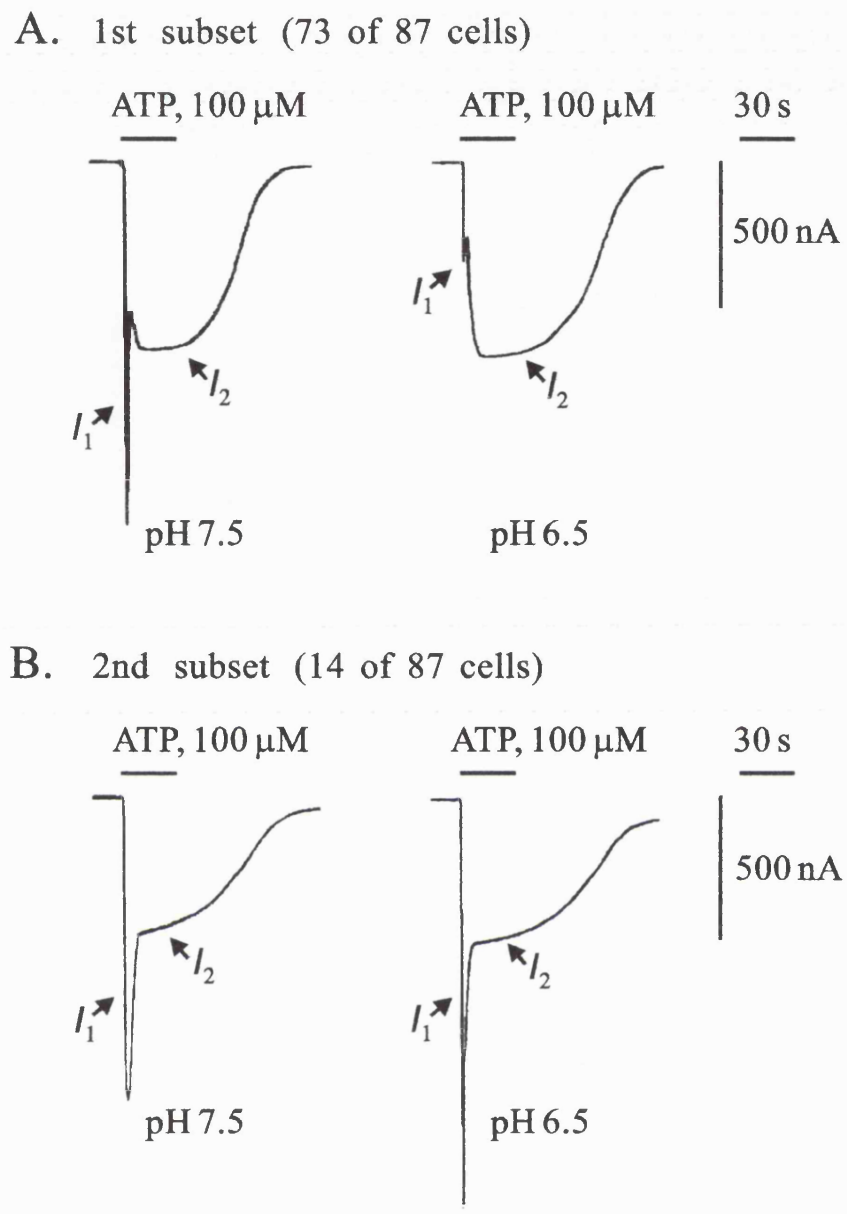


Figure 8.1. Effect of extracellular pH on evoked biphasic responses.

In A, example of a subset of $rP2X_1/rP2X_2$ cRNA co-injected oocytes (73 of 87 cells sampled) that responded to extracellular ATP (100 μM) with biphasic inward currents (I_1 and I_2 responses) and initial $P2X_1$-like response was inhibited by lowering extracellular pH from 7.5 to 6.5. Records in A from the same cell (V_h = -60 mV). In B, example of another subset of co-injected oocytes (14 of 87 cells sampled) where the I_1 response was potentiated by lowering extracellular pH from 7.5 to 6.5. Records in B from another cell, but same batch as oocyte A (V_h = -60 mV).

8.4.2 Potency of agonists mediating pH-sensitive inward (I_1) currents.

Recombinant P2X receptors in co-injected oocytes reacted to low concentrations of ATP, with a threshold below 10 nM, and were activated maximally at high ATP concentrations (100-300 μM). The amplitude of rapidly inactivating inward currents (I_1 response) grew incrementally over this extended concentration range (10 nM to 300 μM) (Figure 8.2A), while the slower I_2 response was evident only over a limited concentration range (approximately 3-300 μM). The concentration-response (C/R) curve for the ATP-mediated I_1 response is shown in Figure 8.3A. The apparent EC_{50} value (and Hill co-efficient) was 0.56 ± 0.09 μM (n_H, 0.37) (n = 9), but the C/R curve was shallow and appeared to be biphasic. Resolving for each phase, mean EC_{50} values were 54 nM (n_H, 1.05) and 3.28 μM (n_H, 0.82). This first EC_{50} value matched the determination for ATP potency at homomeric $hP2X_1$ receptors (mean EC_{50}, 56 nM; Bianchi *et al.*, 1999), but was lower than the determination for homomeric $rP2X_1$ receptors (mean EC_{50}, 98 nM; n_H, 0.80) (Figure 8.3B). Ap_6A and αβ-meATP evoked rapidly inactivating inward (I_1) currents in $P2X_1/P2X_2$ cRNA co-injected oocytes (Figure 8.2B). Ap_6A is inactive at homomeric $rP2X_2$ receptors (Jacobson *et al.*, 2000a) and, accordingly, failed to evoke slow inward (I_2) currents in these experiments (Figure 8.2B). α,β-meATP is a weak partial agonist at homomeric $P2X_2$ receptors (Jiang *et al.*, 2001) and only evoked very small I_2 responses (Figure 8.2B). The resultant agonist C/R curves for the I_1 response were shallow and extended over a wide concentration range (3 nM to 100 μM) (Figure 8.3A) to give EC_{50} values of: Ap_6A, 0.74 ± 0.10 μM (n_H, 0.50) (n = 6); α,β-meATP, 0.43 ± 0.05 μM (n_H, 0.89) (n = 8). The C/R curve for Ap_6A appeared to be biphasic, with mean EC_{50} values of 49 nM (n_H, 1.37) and 2.02 μM (n_H, 1.25). Agonist potency data are summarized in Table 8.1.

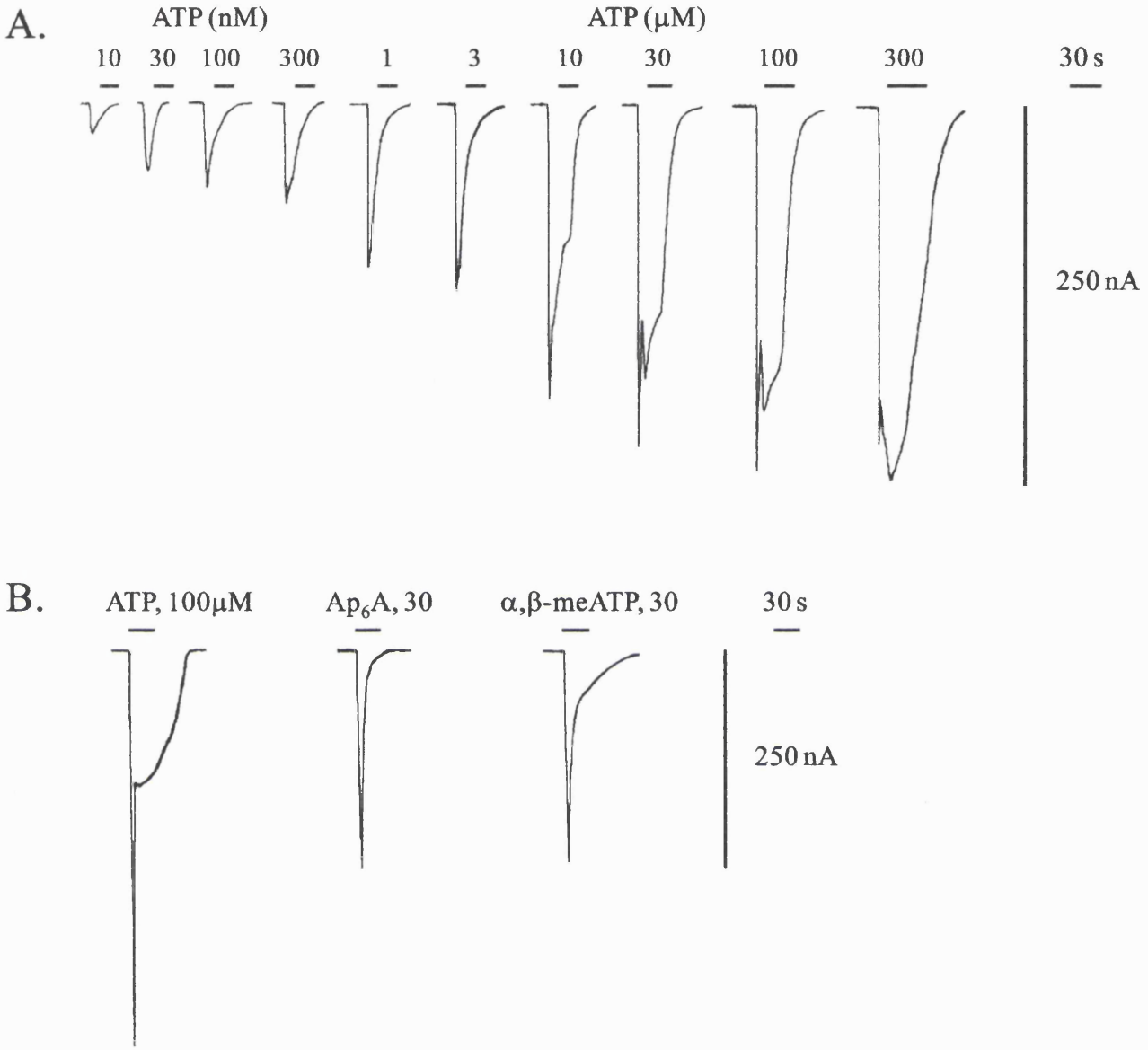


Figure 8.2. Agonist responses in oocytes expressing pH-sensitive P2X assemblies.

In A, concentration-dependent inward currents to ATP (10 nM to 300 μM; for 30 s, and 20 min apart) recorded from a single $rP2X_1/rP2X_2$ cRNA co-injected oocyte (V_h = -60 mV). Monophasic I_1 responses were evoked by low ATP concentrations (<3 μM), and biphasic (I_1 and I_2) responses by higher ATP concentrations. In B, inward currents evoked by saturating concentrations of ATP (100 μM) and the homomeric $rP2X_1$ receptor agonists Ap_6A (30 μM) and α,β-meATP (30 μM). Records in B from the same cell (V_h = -60 mV).

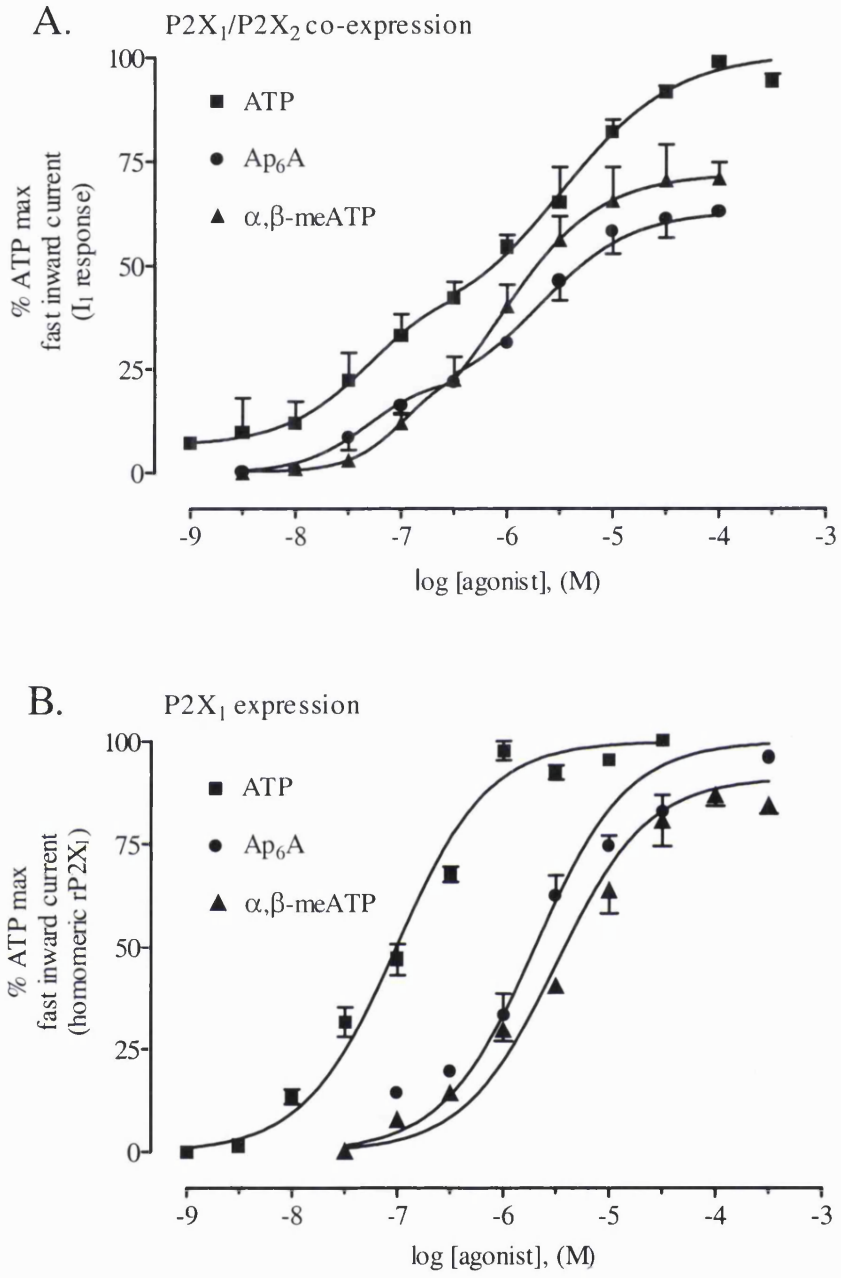


Figure 8.3. Agonist potency at P2X subunit assemblies.

In A, concentration/response (C/R) curves for rapidly inactivating I_1 responses evoked by ATP, Ap_6A and α,β-meATP in oocytes co-expressing $rP2X_1$ and $rP2X_2$ subunits. Data given as mean ± SEM (6-9 sets of observations). In B, C/R curves for ATP, Ap_6A and α,β-meATP in oocytes expressing homomeric $P2X_1$ receptors (n = 4-8). Where missing, error bars are occluded by symbols. Curves fitted to the Hill equation, using Prism v2.0 (GraphPad).

Table 8.1. Agonist potency at P2X receptor assemblies.

Receptor	ATP		Ap_6A		α,β-meATP	
	EC_{50} (nM)	*n_H*	*EC_{50} (nM)*	*n_H*	*EC_{50} (nM)*	*n_H*
homomeric $rP2X_1$	98±11	0.80	2598±721	0.74	3301±492	0.77
I_1 response: *1st phase*	54±19	1.05	49±10	1.37	*not determined*	
2nd phase	3276±309	0.82	2020±163	1.25	*not determined*	
full range	555±84	0.37	741±98	0.50	427±50	0.89
homomeric $P2X_2$	5623±488	1.21	*inactive*		*inactive*	
I_2 response	7726±560	1*	*inactive*		*inactive*	

Determinations of EC_{50} values and Hill co-efficients (n_H) for ATP, Ap_6A and α,β-meATP at homomeric $rP2X_1$ and $rP2X_2$ receptors and P2X receptors responsible for the I_1 and I_2 responses. Data for I_1 response was analyzed for each phase of biphasic C/R curves and re-analyzed for full range of data points. The Hill co-efficient for the I_2 response (see *) was constrained to a value of 1, because of limited C/R data at low agonist concentrations (<10 μM).

8.4.3 Effects of extracellular pH on agonist efficacy at I_1 response.

The C/R relationship for rapidly inactivating I_1 responses to ATP was re-examined at four different levels of extracellular pH (pH_e) (Figure 8.4A-D). At the four levels tested (pH 8.5-5.5), the resultant C/R curves extended over 5 $\log_{10}$ units of agonist concentration (1nM to 100 μM) and, accordingly, their slopes were shallow ($n_H \leq 0.5$). It was difficult to dissect some ATP C/R curves into first and second phases, particularly at pH 5.5, where agonist sensitivity was heightened. However, it was clear that the amplitude of I_1 responses was consistently greater under acidic conditions (pH 6.5 and 5.5) at all ATP concentrations tested (Figure 8.4C,D). Compared to control data at pH 7.5, the relative amplitude of the maximum response (I_{max}) was 134 ± 8% (at pH 6.5; n = 6) and 284 ± 18% (at pH 5.5; n = 4). Thus, one effect of acidic pH_e was an increase in agonist efficacy.

Under alkaline conditions (pH_e = 8.5), the amplitude of I_1 responses at low concentrations (ATP, 1-100 nM) was not significantly different from I_1 responses obtained at pH 7.5 (Figure 8.4B). At higher ATP concentrations (300 nM to 100 μM), the amplitude of I_1 responses was greater at pH 8.5 than pH 7.5 (Figure 8.4B). Compared to control data, the relative amplitude of the maximum response (I_{max}) was 154 ± 16% at pH 8.5 (n = 5). Thus, the amplitude of I_1 responses and agonist efficacy were enhanced under both alkaline (pH 8.5) and acidic (pH 6.5 and 5.5) conditions. It is not unusual for alkaline and acidic conditions to exert the same effect, rather than opposing effects, at P2X receptors. At heteromeric $P2X_{1/5}$ receptors, for example, agonist efficacy and potency are reduced by both acidic and alkaline bathing solutions (Surprenant *et al.*, 2000).

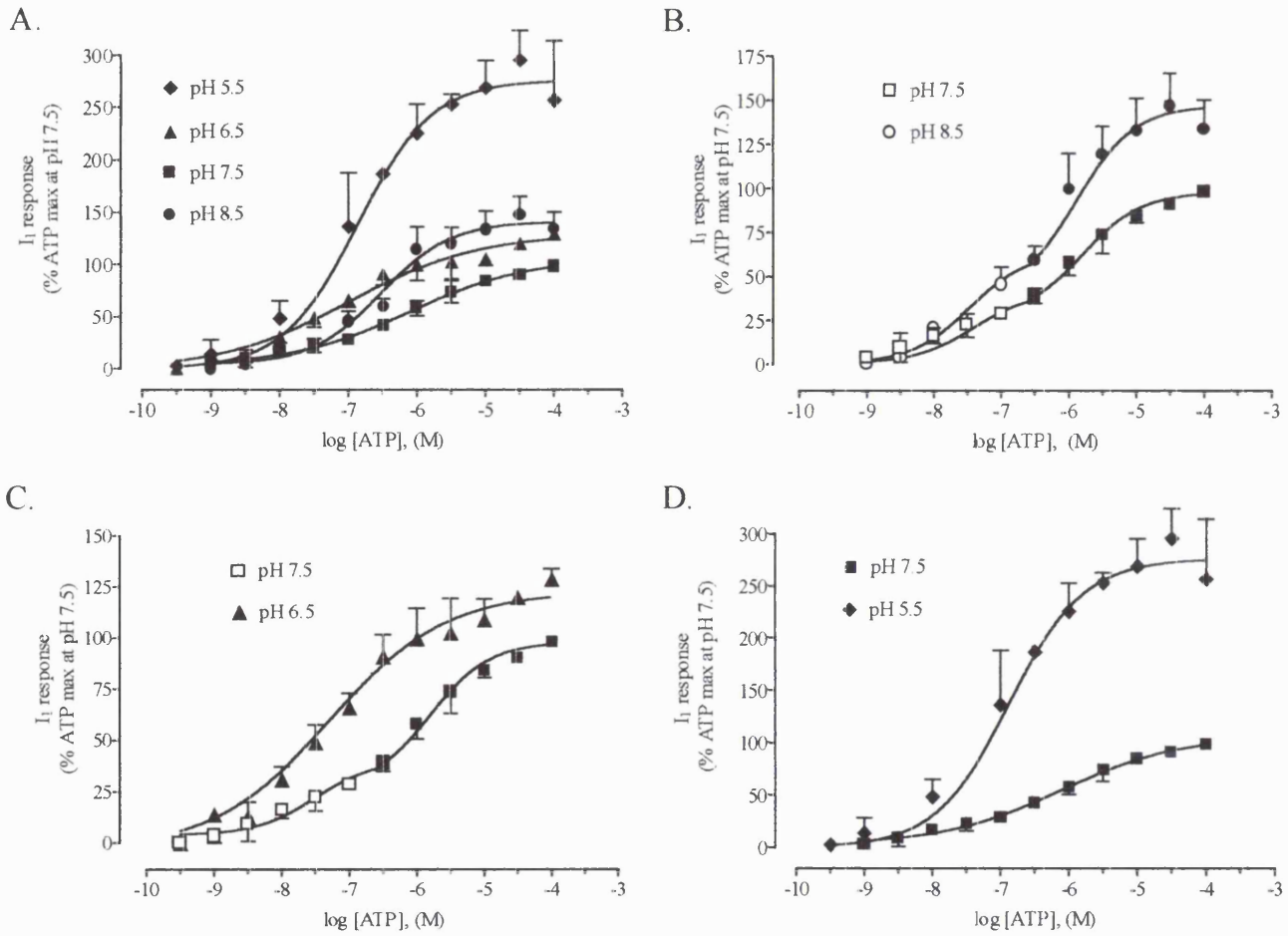


Figure 8.4. Effects of extracellular pH of ATP activity at I_1 responses.

In A, concentration/response (C/R) curves for rapidly inactivating I_1 responses evoked by ATP at four levels of extracellular pH (8.7, 7.5, 6.5 and 5.5) Data given as mean ± SEM (4-9 sets of observations). In B-D, C/R curves are redrawn to compare the effects of test pH levels (B, pH 8.5; C, pH 6.5; D, pH 5.5) against control data (at pH 7.5). Curves fitted to the Hill equation, using Prism V2.0 (GraphPad).

8.4.5 Effects of extracellular pH on agonist potency at I_1 response.

The effects of extracellular pH on agonist potency were assessed in one of two ways. Where there was no clear boundary between two phases of the C/R curve (particularly at pH 6.5 and 5.5), agonist potency was assessed as the EC_{50} value over the full concentration range (1 nM to 100 μM) (see Figure 8.4). Where possible, C/R curves were analyzed over first and second phases of the curve and changes in EC_{50} values noted. This was only possible for C/R curves defined at pH 7.5 and pH 8.5 (see Figure 8.4).

Where C/R curves were analyzed over the full concentration range, apparent EC_{50} values were as follows for the I_1 response: *pH 8.5*, 0.39 ± 0.13 μM (n =5); *pH 7.5*, 0.55 ± 0.09 μM (n = 9); *pH 6.5*, 0.08 ± 0.02 μM (n = 6); *pH 5.5*, 0.12 ± 0.05 μM (n = 4). Thus, ATP potency was not significantly different at pH 8.5 and 7.5, yet was enhanced by 5-7 fold at pH 6.5 and 5.5. These results contrast with data for homomeric $rP2X_1$ receptors where it is known that ATP potency is reduced under acidic conditions (Stoop *et al.*, 1997; Wildman *et al.*, 1999). The effects of extracellular pH on I_1 responses and homomeric $rP2X_1$ receptors are summarized in Table 8.2.

Data were re-analyzed to take into account the biphasic nature of C/R curves defined at pH 7.5 and pH 8.5 (Figure 8.4B). For the first phase, mean EC_{50} values were 48 nM (pH 7.5) and 45 nM (pH 8.5) and, for the second phase, 1.59 μM and 1.42 μM respectively. Thus, ATP potency was not affected under alkaline conditions at either phase of these complex C/R relationships.

Table 8.2. Effect of extracellular pH at P2X receptor assemblies.

Agonist	homomeric $rP2X_1$		heteromeric $rP2X_{1/2}$	
			I_1 response	
	EC_{50} value	*I_{max}*	*EC_{50} value*	*I_{max}*
ATP @ pH 8.5	[a]0.31±0.02 μM	[a]98±2%	0.39±0.13 μM	154±16%
ATP @ pH 7.5	0.10±0.01 μM	100%	0.56±0.09 μM	100%
ATP @ pH 6.5	[a]0.60±0.05 μM	[a]99±1%	0.08±0.02 μM	134±8%
ATP @ pH 5.5	[a]1.70±0.32 μM	[a]96±4%	0.12±0.05 μM	284±18%

Determinations of EC_{50} values and maximum response (I_{max}) for ATP, at different extracellular pH levels, at homomeric $rP2X_1$ receptors and P2X receptors responsible for the I_1 response. The given I_{max} values were normalized to the maximum response to ATP at pH 7.5. [a]Data taken from Wildman *et al.* (1999).

8.4.6 Potency of agonists mediating slow inward (I_2) currents.

Slowly inactivating inward currents (I_2 response) were evoked at ATP concentrations in excess of 3 μM and maximal at high concentrations (300 μM) (see Figure 8.2A). It was not possible to determine the threshold concentration to elicit these slow responses, since of the initial I_1 response showed deactivating tail currents that obscured the smallest of I_2 responses. The C/R curve for the ATP-mediated I_2 response is shown in Figure 8.5. At pH 7.5, the apparent EC_{50} value (and Hill co-efficient) was 7.7 ± 0.6 μM (n_H, 1.00) for the I_2 response, which was not significantly different from the determination for homomeric $rP2X_2$ receptors (5.6 ± 0.5 μM; n_H, 1.21) (Figure 8.5). The EC_{50} value for the I_2 response was 0.71 ± 0.06 μM (n_H, 1.95) at pH 6.5, similar to the determination for homomeric $rP2X_2$ receptors (1.09 ± 0.12 μM; n_H, 1.81) (Figure 8.5). The ATP C/R curves for the I_2 response and homomeric $rP2X_2$ receptors appeared to be monophasic at both pH levels tested. Also, the maximum amplitude for the I_2 response, as for $rP2X_2$ receptors (King *et al.*, 1996a), was not significantly different at the pH levels tested (Figure 8.5B). Furthermore, Ap_6A and α,β-meATP (both 30 μM) were ineffective at eliciting I_2 responses (Figure 8.2B) and activating homomeric $rP2X_2$ receptors (Jacobson *et al.*, 2000a).

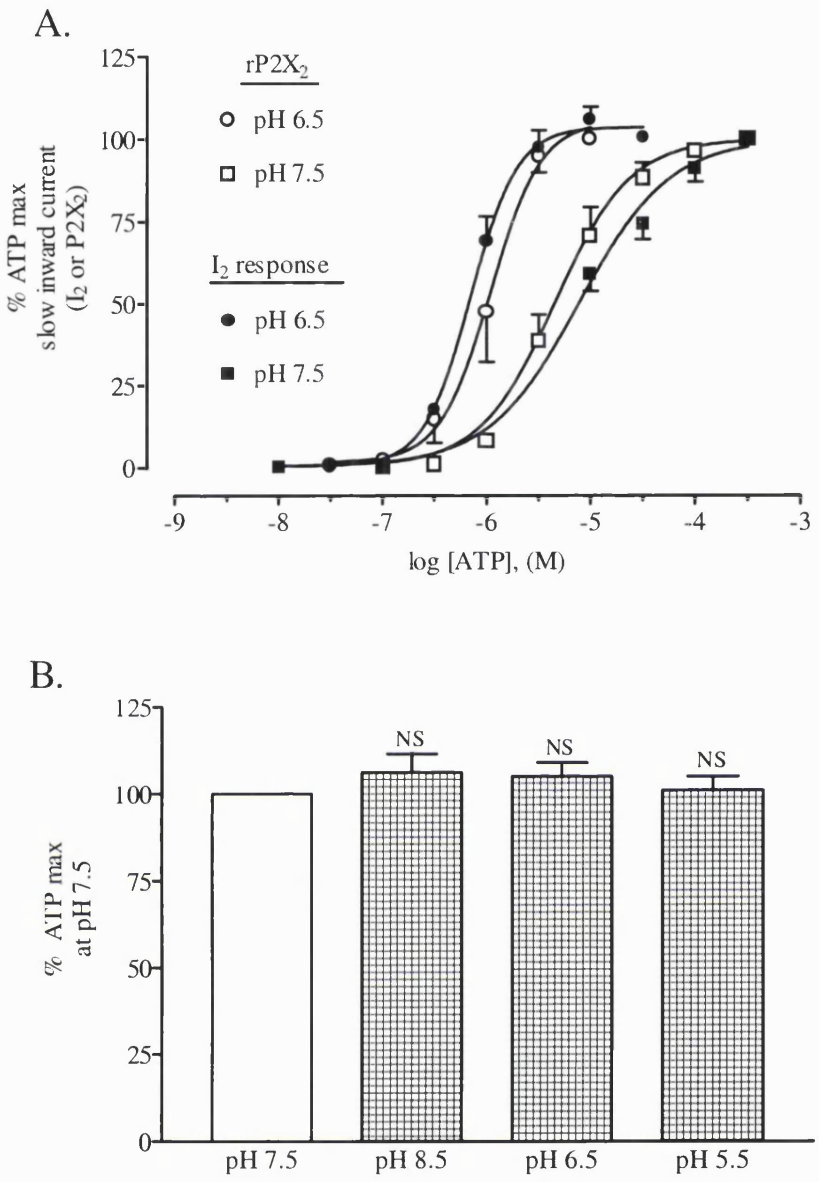


Figure 8.5. Effects of extracellular pH of ATP activity at I_2 responses.

In A, concentration/response (C/R) curves for slowly inactivating inward currents evoked by ATP in oocytes either co-expressing $P2X_1$ and $P2X_2$ subunits or expressing homomeric $rP2X_2$ receptors. ATP activity was assessed at pH 7.5 and again at pH 6.5. Curves fitted to the Hill equation, using Prism v2.0 (GraphPad). In B, relative amplitude of maximum I_2 responses to ATP (100 μM), at three test pH levels (8.5, 6.5 and 5.5) and compared to control data (at pH 7.5), in oocytes co-expressing $P2X_1$ and $P2X_2$ subunits. Data given as mean ± SEM (4-9 sets of observations).

8.5 Discussion

In the present study, heterologous co-expression of $P2X_1$ and $P2X_2$ subunits in defolliculated *Xenopus* oocytes resulted in the formation of a complex population of P2X receptors. Activation of these P2X receptors with extracellular ATP resulted in biphasic inwards currents that involved rapidly and slowly inactivating components and, at first glance, could be explained by the successive activation of homomeric $P2X_1$ and $P2X_2$ receptors. Such a conclusion already has been stated in the earliest report on co-expression of $P2X_1$ and $P2X_2$ subunits (Lewis *et al.*, 1995). In the intervening time, however, much more has been learned about the operational profiles of homomeric $rP2X_1$ and $rP2X_2$ receptors - not least, the influence of extracellular pH on agonist activity (King *et al.*, 1996a, 1997b, 2000; Stoop *et al.*, 1997; Stoop & Quayle, 1998; Wildman *et al.*, 1997, 1999; Ding & Sachs, 1999). Also, recent biochemical evidence has suggested that $P2X_1$ and $P2X_2$ subunits should heteropolymerize (Torres *et al.*, 1999b).

By lowering extracellular pH (from pH 7.5 to 6.5), it was noted that a relatively small sample (14 of 87 cells) of $P2X_1/P2X_2$ cRNA co-injected oocytes responded to ATP with rapidly inactivating inward currents (I_1 or $P2X_1$-like responses) that were potentiated under acidic conditions. This behaviour was atypical of fast inward currents carried by homomeric $rP2X_1$ receptors, which are inhibited under acidic conditions by a mechanism that decreases ATP potency (Stoop *et al.*, 1997; Wildman *et al.*, 1999). The outcome of lowering extracellular pH was more in keeping with homomeric $P2X_2$ receptors, at which ATP potency is enhanced under acidic conditions although $P2X_2$-evoked inward currents are normally slowly inactivating (King *et al.*, 1996a, 1997b, 2000; Stoop *et al.*, 1997; Ding & Sachs, 1999). Thus, the I_1 response possessed the kinetics of homomeric $P2X_1$ receptors and, to an extent,

the acid sensitivity of homomeric $P2X_2$ receptors. It is possible that a significant part of the I_1 response was mediated by heteromeric $P2X_{1/2}$ receptors sharing some of the properties of their constituent P2X subunits.

Alternatively it could be argued that, in those cells showing a potentiation of I_1 responses under acidic conditions, this effect was no more than the relaxation of receptor desensitisation for a significant proportion of the available homomeric $P2X_1$ receptor population. However, several lines of evidence show this was not the case. First, the amplitude of the I_1 response under control conditions was constant for successive agonist applications, consistently potentiated under acidic conditions, and returned to control values when pH levels were restored (Figure 8.6). Second, the potentiation of I_1 responses under acidic conditions was due to an increase in ATP potency - an effect unrelated to the number of P2X receptors available for activation. Third, the I_1 response could be evoked by very low agonist concentrations – not only ATP, but also Ap_6A and α,β-meATP – in contrast to parallel experiments where homomeric $P2X_1$ receptors were studied separately. None of these observations would suggest the influence of desensitised homomeric $P2X_1$ receptors on results.

Only one in every five $P2X_1/P2X_2$ cRNA co-injected oocytes showed a pH-potentiated I_1 response and, accordingly, it was difficult to carry out extensive pharmacological investigations. Different RNA concentrations were injected in an attempt to increase the expression of heteromeric P2X assemblies, as carried out in experiments on heteromeric $P2X_{2/3}$ receptors by Liu and colleagues (2001), but this procedure only resulted in cells with P2X receptors showing the expected properties of homomeric $P2X_1$ and $P2X_2$ receptors (data not shown). This outcome may explain why a previous attempt to co-express $P2X_1$ and $P2X_2$

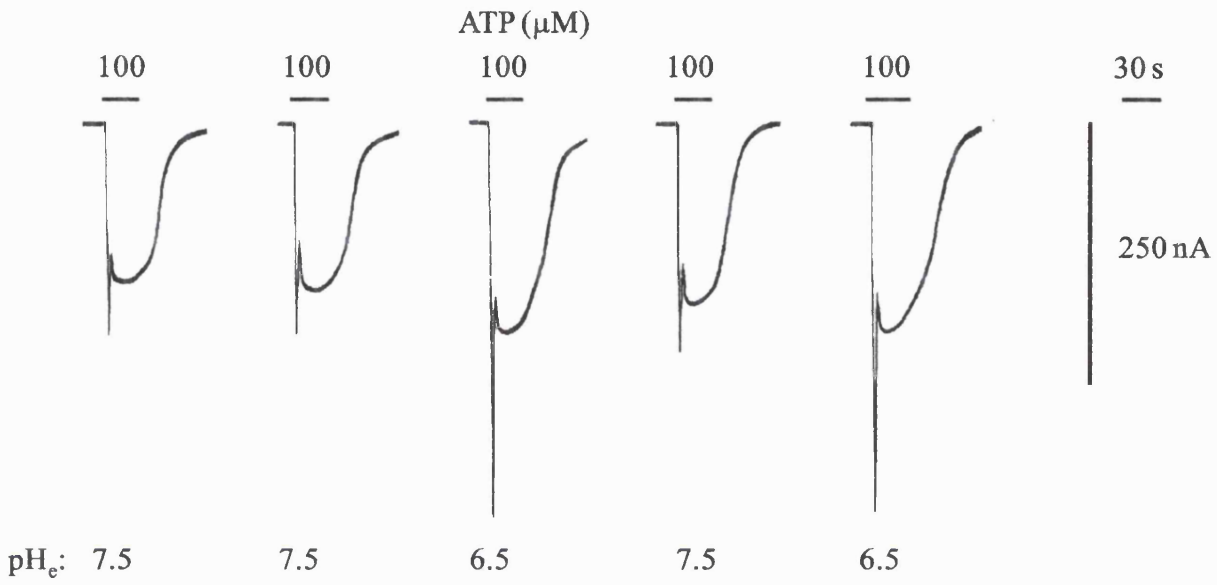


Figure 8.6. Reproducibility of I_1 and I_2 responses.

ATP (100 μM; for 30 s, and 20 min apart) evoked biphasic inward currents (I_1 and I_2 responses) of consistent amplitude, at either pH 7.5 or pH 6.5, in oocytes co-expressing $rP2X_1$ and $rP2X_2$ subunits. The reproducibility of I_1 responses at each pH_e level supported the viewpoint that pH-potentiation was not due to a relaxation of $rP2X_1$ receptor desensitisation. Also, the maximum amplitude of I_2 responses was largely unaffected by extracellular pH.

subunits failed to reveal the presence of heteromeric $P2X_{1/2}$ assemblies (Lewis *et al.*, 1995). There was no evidence in this study to say there was a problem with $P2X_{1/2}$ receptor assembly, only a problem of finding a way to pharmacologically dissect this heteromeric P2X receptor from the homomeric P2X receptors present.

Where $P2X_1/P2X_2$ cRNA co-injected cells were studied carefully, agonist activation of the mixed P2X receptor population resulted in complex concentration/response (C/R) curves for the pH-potentiated I_1 response. C/R curves extended over a large concentration range and, at pH 7.5, were clearly biphasic for ATP and Ap_6A. It seemed likely that biphasic C/R curves resulted from the separate activation of different populations of P2X assemblies capable of generating rapidly inactivating I_1 responses. It is less likely the second phase of complex C/R curves was caused by a simultaneous activation of homomeric $P2X_1$ and $P2X_2$ ion-channels and the addition of inward currents, particularly in the case of Ap_6A which is inert at $rP2X_2$ receptors (see Table 8.1). Also, elevation of the second phase of the ATP C/R curve at pH 8.5 (Figure 8.4B) was inconsistent with the actions of alkaline bathing solutions at homomeric $rP2X_2$ receptors (King *et al.*, 1997b). Additionally, the elevation of ATP C/R curves for the I_1 response under acidic conditions (Figure 8.4C,D) was incompatible with the involvement of homomeric $rP2X_2$ receptors (King *et al.*, 1997b; and see below).

EC_{50} values for ATP and Ap_6A fell in the region of 50 nM for the first phase of C/R curves for the I_1 response - different from agonist EC values at homomeric $rP2X_1$ receptors (Figure 8.3, Table 8.1). It has been reported that the rapidly inactivating inward currents elicited by heteromeric $P2X_{1/5}$ receptors are also very sensitive to ATP (mean EC_{50}, 55 nM) (Surprenant *et al.*, 2000). Thus, a trend is emerging that P2X heteromers comprising $P2X_1$ subunits are unusually sensitive to ATP and, conceivably, this represents a useful adaptation to enhance

purinergic signalling where homomeric $P2X_1$ receptors are also utilized. EC_{50} values for the second phase were in the low micromolar (~2-3 μM) concentration range and these values were unrelated to EC_{50} values for homomeric $P2X_1$ or $P2X_2$ receptors (see Table 8.1). Where EC_{50} values were determined over the full range of data points for each C/R curve, the agonist potency order was α,β-meATP > ATP > Ap_6A which, again, was unrelated to data for homomeric $rP2X_1$ receptors (ATP > Ap_6A > α,β-meATP) and homomeric $rP2X_2$ receptors (ATP active, Ap_6A and α,β-meATP inactive) (see Table 8.1).

A thorough study of ATP potency and efficacy at different extracellular pH levels provided further evidence that the I_1 response was mediated by a novel pH-sensitive heteromeric P2X receptor. Here, it was found that lowering pH_e caused an increase the maximum ATP response and displaced the C/R curve in a leftwards manner. In contrast, acidic conditions decrease ATP potency without altering the maximum response at homomeric $rP2X_1$ receptors (Stoop *et al.*, 1997; Wildman *et al.*, 1999) (see Table 8.2), or increase ATP potency with altering the maximum response at homomeric $P2X_2$ receptors (King *et al.*, 1996a, 1997b; Stoop *et al.*, 1997) (see Figure 8.5). Furthermore, raising pH_e increased the maximum ATP response without altering agonist potency for the I_1 current. In contrast, alkaline conditions have no effect on ATP responses at homomeric $P2X_1$ receptors (Wildman *et al.*, 1999), or decrease ATP potency without changing the maximum response at homomeric $P2X_2$ receptor (King *et al.*, 1996a, 1997b).

It seemed unlikely that the I_1 response could be mediated by homomeric $P2X_2$ receptors, for a number of reasons. The I_1 responses were rapidly inactivating, evoked by Ap_6A and α,β-meATP and their maximal amplitude potentiated by both acidic and alkaline conditions. None of these features match the profile of homomeric $rP2X_2$ receptors (King *et al.*, 1997b;

Jacobson *et al.*, 2000a). Instead, there appears to be a major role for homomeric $rP2X_2$ receptors in the later I_2 response that showed the appropriate sensitivity to ATP at all pH levels tested.

Therefore, it is reasonable to conclude that heteromeric assemblies of $rP2X_1$ and $rP2X_2$ subunits would best explain the unique pH sensitivity and peculiar pharmacological activity of agonists at the I_1 response. P2X receptors are now viewed as either trimeric or tetrameric assemblies (Kim *et al.*, 1997; Nicke *et al.*, 1998) and, so, expression of heteromeric $P2X_{1/2}$ receptors could involve from one to three $P2X_1$ subunits. Perhaps such differences in subunit composition of heteromeric $P2X_{1/2}$ receptors can help explain the complex C/R curves observed in this study, but that is a matter of conjecture. As yet, there are no reports of native P2X receptors in neural systems that are similar to the findings in this study – although, of note, is a report on $P2X_1$-like responses in guinea-pig vas deferens that are potentiated under acidic conditions (Nakanishi *et al.*, 1999). However, nothing is known about guinea-pig $P2X_1$ as a recombinant receptor and, furthermore, species orthologues of $P2X_1$ are known to undergo alternative splicing that generates phenotypically altered P2X receptors (Greco *et al.*, 2001). It is envisaged that naturally occurring $P2X_{1/2}$ receptors would be activated by very low concentrations of released ATP and purinergic transmission would be facilitated under the acidic environment associated with exocytosis of neurotransmitters and with inflammation (as discussed in: King *et al.*, 1997b).

CHAPTER 9

GENERAL DISCUSSION

9.1 Selectivity of novel P2X agonists

In this thesis it has been shown that diadenosine polyphosphates have a wide range of pharmacological profiles at recombinant $rP2X_{1-4}$ receptors expressed in *xenopus* oocytes (chapter 4). As such, they have potential as pharmacological tools to dissect excitatory ATP-mediated responses in tissues containing heterogeneous populations of P2X receptors. It is of particular interest that there are a number of clear differences in potency and efficacy values of Ap_3A, Ap_4A and Ap_5A between homomeric $P2X_1$ and $P2X_3$ receptors, which have been traditionally difficult to separate on agonist activity alone (see table 1.2). However, comparison of my work with other recent studies using diadenosine polyphosphates has revealed potential limitations (see section 9.5). As several of the diadenosine polyphosphates are potent agonists it may be interesting to see if it is possible to convert these molecules into antagonists as was done with ATP to generate TNP-ATP (see figure 1.2 and appendix 1 for structures of TNP-ATP and ATP respectively).

It is interesting to see how chemical manipulation of endogenous nucleotides can generate compounds with new pharmacology. For example, in chapter 5, I reported that the potency of ATP was increased at $rP2X_1$ and $rP2X_3$ receptors upon addition of a 4-aminophenylethylthio group to the adenine moiety, thus generating PAPET. Previous modification of the adenine moiety in the same position, but adding a methythio group to generate 2-meSATP, preserved or slightly increased the potency of ATP at $rP2X_1$ and $rP2X_3$ receptors (see table 1.2). Clearly, however, PAPET is the most significant advance in terms of agonist potency increase reported recently.

Remarkably, AMP, which is inactive at recombinant $rP2X_{1-4}$ receptors, is converted into a potent, but partial, agonist at $rP2X_1$ and $rP2X_3$ receptors when it too has its adenine moiety modified by the addition of a thio-containing group to generate HT-AMP (chapter 5). Chemical manipulation of this type is limited as previous attempts to elongate the chain, added at the 2′ position of the adenine moiety of ATP, has resulted in compounds that are inactive at P2X receptors (Jacobson *et al.*, 1997). As with 2-meSATP, PAPET and HT-AMP potently activate $P2Y_1$ receptors with nanomolar affinity, therefore, in whole tissue preparations containing heterogeneous populations of $P2X_1$ and $P2Y_1$ receptors, selective activation of either subtype, using these compounds alone, is unlikely to prove possible. Nevertheless, agents that show potency higher than ATP and have increased resistance to nucleotidases (Zimmet *et al.*, 1993) are a welcome addition to the pharmacological armoury used in the study of P2X receptors.

9.2 Selectivity of novel P2X antagonists

In this thesis it has been demonstrated that chemical manipulation of PPADS can produce potent antagonists with a reasonably high degree of selectivity for $P2X_1$ and $P2X_3$ receptors. As described, potent agonists with preferential activity at group 1 P2X receptors can be synthesised through linkage of thio-groups to adenine moiety of ATP. As, the new agonists developed are also highly potent against P2Y receptors, the greatest success was achieved in developing antagonists that are not only selective for group 1 receptors but also have low activity at P2Y receptors, since the precursor parent molecule PPADS shows little discrimination between the two P2 receptor subtypes.

Another consideration that must be addressed when determining the selectivity of P2X-receptor antagonists is their effect on enzymes that degrade extracellular ATP. A family of ectonucleotidase enzymes has been cloned and are collectively called, *ectonucleoside triphosphate diphosphohydrolases* (E-NTPases) (Zimmermann, 1999). Their protein topology resembles that of a P2X receptor subunit, with two transmembrane domains, intracellular N- and C- terminals and a large extracellular loop. As well as being membrane anchored enzymes, it has been demonstrated that soluble ectonucleotidase enzymes can be released from sympathetic nerves of the vas deferens (Todorov *et al.*, 1997; Westfall *et al.*, 2000). A recent study used two members of this family, CD39 and CD39L1, to examine the ability of PPADS and selected derivatives (all at 100μM) to inhibit ectonucleotidase activity. PPADS was a poor inhibitor of both enzymes, inhibiting ATP production by approximately 20% in each case (Hoffmann *et al.*, 2000). This makes an interesting comparison to the results seen when investigating the effect of PPADS on the activity of the soluble ectonucleotidase released from the vas deferens. Here, PPADS (100μM) inhibited enzyme activity by approximately 70%, indicating that the soluble enzyme is, as postulated, distinct from CD39 and CD39L1 class of enzymes (Westfall *et al.*, 2000).

Interestingly, isoPPADS, an isomer of PPADS where a sulphonate group is placed in the 5´ position instead of the 4´ position on the azophenyl moiety (See Chapter 6), shows similar inhibitory activity to PPADS against CD39 but is inactive at CD39L1 (Hoffmann *et al.*, 2000). Of the novel PPADS derivatives tested, MRS 2143 and MRS 2191 (chapter 7), have the least effect on enzyme activity. MRS 2143 inhibits both enzymes by about 20% and MRS 2191 is inactive at CD39, with an approximate 20% inhibition of enzyme activity also seen at CD39L1 (Hoffmann *et al.*, 2000). Therefore, this observation improves the pharmacological

profile of MRS 2191 further as it is a potent $P2X_1$ antagonist with a 28-fold selectivity for $P2X_1$ over $P2X_3$ (chapter 7). Low blocking activity at ectonucleotidase enzymes is a desirable quality for antagonists as it has been demonstrated, using antagonists lacking this quality, that their potency is reduced through a self-cancellation effect by prolonging the time that the agonist (ATP) is available to compete with the antagonist and activate the receptors (Crack *et al.*, 1994, Bültmann *et al.*, 1999). As noted in chapter 7, MRS 2257, is a potent, non-surmountable antagonist at $P2X_1$ receptors. It inhibits CD39L1 by approximately 20% but has a considerable higher activity at CD39, over 50% inhibition of ATP degradation at 100μM. MRS 2159 is a very potent $P2X_1$ antagonist and shows 15-fold selectivity for $P2X_1$ over $P2X_3$ receptors (Chapter 7). However, it also inhibits CD39 activity by over 50% but is inactive at CD39L1 (Hoffmann *et al.*, 2000). The selective blocking activity between CD39 and CD39L1 seen in particular with isoPPADS and MRS 2159, although an undesirable characteristic for a P2X receptor antagonist, is a useful observation and may lead to the development of agents selective purely for particular isoforms of these enzymes and will allow the facilitation of purinergic transmission.

The development of novel pharmacological agents for P2X receptors has largely been through chemical modification of previously effective antagonists (e.g. suramin and PPADS, van Rhee *et al.*, 1994; Damer *et al.*, 1998; Klapperstück *et al.*, 2000). Success has been achieved in attempting to model P2Y receptors and therefore identify residues that are important in ligand recognition (Jacobson *et al.*, 1999). A recent study has addressed similar issues at the $P2X_1$ receptor (Ennion *et al.*, 2000). Here, it was found that 4 lysine residues are involved in ATP binding and activation of the receptor. Interestingly, the authors noted that mutations of these residues considerably reduced the potency of ATP but had no effect

on the potency of the antagonist suramin, thereby indicating that these residues are not important for the binding of suramin.

9.3 Heterogeneity of P2X receptors

Seven P2X receptors have been cloned and characterised to date (King, 1998; North and Surprenant, 2000). Analysis of their pharmacological and kinetic properties in isolation in expression systems has allowed comparison of these responses with ATP-mediated responses in tissues. This approach, in combination with molecular and immunohistochemical techniques, has permitted an attempt to identify the P2X receptors that account for ATP-mediated fast excitatory responses in tissues. Many P2X responses appear to be firmly established in some of the more intensively studied preparations. For example, $P2X_1$-like responses have been identified in the smooth muscle of blood vessels, vas deferens and urinary bladder (Ralevic and Burnstock, 1998; Mulryan *et al.*, 2000; Sneddon, 2000). Yet it is clear that other ATP-sensitive receptors can mediate responses in some of these tissues (Ziganshin *et al.*, 1993; Lewis and Evans, 2000a; Surprenant *et al.*, 2000; Chapter 3).

Early in the history of cloning of P2X receptors it was noted that there was overlapping transcript expression in sensory neurones (Lewis *et al.*, 1995). Co-expression of $P2X_2$ and $P2X_3$ resulted in a phenotype distinct from the individual receptor subtypes (Lewis *et al.*, 1995). These results helped explain *in vitro* recordings from sensory and sympathetic neurones (Khakh *et al.*, 1995a; Zhong, *et al.*, 2000). Perhaps remarkably, the initial differentiation of receptor subpopulations in these neurones relied primarily on the pharmacological activity of αβ-meATP and the kinetic profile of the receptors activated by this agonist. Early investigations on recombinant receptors demonstrated that $P2X_3$ was

sensitive to αβ-meATP (Chen *et al.*, 1995) whereas $P2X_2$ was not (Brake *et al.*, 1994). The heteromeric receptor $P2X_{2/3}$ was also sensitive to αβ-meATP but fortunately the response was slowly inactivating in comparison to $P2X_3$, therefore indicating a distinct receptor population (Lewis *et al.*, 1995). As similar responses to αβ-meATP were seen in sensory neurones (Khakh *et al.*, 1995a) it was suspected that heteromeric $P2X_{2/3}$ receptors may account for these responses (Lewis *et al.*, 1995). This was later confirmed using the novel antagonist TNP-ATP (Thomas *et al.*, 1998). Two studies using recombinant receptors showed just how close the pharmacological phenotypes of $P2X_3$ and $P2X_{2/3}$ are (Bianchi *et al.*, 1999; Liu *et al.*, 2001), highlighting the fortune that their kinetic profiles differ so noticeably. This is yet another example demonstrating the need to develop selective agents to pharmacologically isolate specific P2X receptor populations in a tissue containing a heterogeneous population. Clearly therefore, the potential exists for individual P2X receptors to co-assemble to form an ATP-sensitive receptor distinct from the homomeric receptors.

Biochemically, there are 11 possible combinations for heteropolymerisation of 2 different P2X subunits (Torres *et al.*, 1999b). However, only a few of these combinations have been demonstrated functionally and once again, this is by virtue of studying these receptors in isolation in expression systems and demonstrating that the distinctions made between the properties of heteromeric and homomeric P2X receptors can be subtle ones. For example, heteromeric $P2X_{1/5}$ receptors show similarities to homomeric $P2X_1$ receptors in their sensitivity to agonists but have conflicting reports on the sensitivity of the heteromeric $P2X_{1/5}$ receptors to TNP-ATP (Haines *et al.*, 1999; Lê *et al.*, 1999; Surprenant *et al.*, 2000). The clearest difference is the resistance to run-down of responses at the heteromeric $P2X_{1/5}$ receptors following repeated applications of agonist at short intervals (Lê *et al.*, 1999).

Equally, the results obtained with co-expression of $P2X_4$ and $P2X_6$ receptors support the formation of a heteromer receptor (Lê *et al.*, 1998b) but, even in the recombinant system, the differences between homomeric $P2X_4$ and $P2X_{4/6}$ are very subtle. Therefore, to isolate this receptor conclusively, if it exists, in tissues is currently impossible with the tools available. The demonstration of heteromeric $P2X_{2/6}$ receptors was slightly more clear cut, despite the same agonist potency order as homomeric $P2X_2$ receptors (King *et al.*, 2000). Heteromeric $P2X_{2/6}$ receptors showed a biphasic response to ATP that was accentuated in the presence of Zn^{2+}. Also there was a unique pH effect where ATP-responses potentiated at pH 6.5 but reduced at pH 5.5 (King *et al.*, 2000).

Regarding my studies, the identification of $rP2X_{1/2}$ receptors relied primarily on demonstrating fast responses to ATP that were sensitive to changes in extracellular pH. Ideally, it would have been prudent to test a wider range of agonists, including those reported in chapters 4 and 5, along with the new antagonists I have studied, in conjunction with the more traditional compounds used, in order to attempt to distinguish further characteristics of $P2X_{1/2}$ receptors.

Only when armed with the information concerning these P2X receptor complexes obtained from recombinant studies is it possible to return to results obtained from tissues and attempt to label ATP-mediated responses, based on overlapping transcript expression aswell as pharmacological and kinetic data (Lê *et al.*, 1999; Surprenant *et al.*, 2000). Clearly, this is not ideal, as such an approach is often speculative (Lê *et al.*, 1998b; 1999) leaving questions unanswered (for example, see Thomas *et al.*, 1999, Thomas & Spyer, 2000; King *et al.*, 2000). Moreover, due to the subtleties in P2X receptor properties and the overlapping expression of transcripts for multiple P2X receptors in, for example, sensory and sympathetic

neurones (Xiang *et al.*, 1998) it is possible that subpopulations of P2X receptors exist together, apart from those that have been positively identified.

This highlights an interesting area that remains to be investigated: the mechanisms that determine functional expression of P2X receptors in biological membranes. An example of the controlled expression of endogenous P2X receptors was demonstrated when examining the effects of ivermectin on ATP mediated currents on hippocampal CA1 neurones (Khakh *et al.*, 1999). Khakh and co-workers showed that ivermectin could potentiate ATP-mediated currents at recombinant $P2X_4$, and $P2X_{4/6}$, receptors when expressed in *Xenopus* oocytes. In CA1 pyramidal neurones, known to contain high levels of $P2X_4$ and $P2X_6$ mRNA, the averaged data showed that ivermectin had no significant potentiating effect on ATP-mediated currents, although there was suggestion of potentiation in a subpopulation of neurones tested (Khakh *et al.*, 1999). Therefore, it was demonstrated that although there is abundant expression of P2X receptor message there are post-translational control mechanisms that seem to limit the production and/or incorporation of a functional P2X receptor protein into the membrane.

Similarly, positive immunoreactivity and transcript expression for $P2X_{1\text{-}6}$ have been found in DRG cells (Collo *et al.*, 1996; Xiang *et al.*, 1998). However, ATP-responses in DRG cells appear to be mediated largely through homomeric $P2X_2$ and $P2X_3$ or heteromeric $P2X_{2/3}$ receptors, with further fine control exhibited through cell type-specific ATP-responses in subsets of DRG neurones (Lewis *et al.*, 1995; Robertson *et al.*, 1996; Rae *et al.*, 1998; Burgard *et al.*, 1999; Ueno *et al.*, 1999; Burnstock 2000). As previously mentioned in chapter 8, in the *Xenopus* oocytes receptor expression system, control over receptor populations appears to be much more crude and can only be manipulated through alteration in the

concentration ratio of P2X subtypes. Heteromeric receptor expression occurred over a narrow range for $P2X_{1/2}$ receptors and showed a high variability within this range. This contrasts with coexpression of $P2X_2$ and $P2X_3$ in *Xenopus* oocytes yields ATP-responses that are mediated through 3 populations of P2X receptors: $P2X_2$, $P2X_3$ and $P2X_{2/3}$ over a comparatively wide range of mRNA ratios (Liu *et al.*, 2001). A possibility waiting to be explored is that more than two P2X subtypes can contribute to a function channel that has distinct pharmacological and/or kinetic profiles.

9.4 P2X receptors and pathogenesis of disease

Pharmacological analysis in conjunction with molecular and immunohistochemical techniques identify $P2X_1$-like receptors as being predominant in smooth muscle of the vasculature (Valera *et al.*, 1994; Nori *et al.*, 1998; Lewis *et al.*,1998; Ralevic and Burnstock, 1998; Hansen *et al.*, 1999; Lewis *et al.*, 2000), vas deferens (Valera *et al.*, 1994; Kidd *et al.*, 1995; Valera and Burnstock, 1998; Barden *et al.*, 1999; Lee *et al.*, 2000b) and urinary bladder (Valera *et al.*, 1994; Hansen *et al.*, 1998; Ralevic and Burnstock, 1998). *In vivo*, it has been demonstrated that purinergic neurotransmission plays a major role in the parasympathetic innervation of the urinary bladder of pithed rats (Hegde *et al.*, 1998). In humans, the role of purinergic transmission in the urinary bladder has remained more controversial with results obtained against atropine-resistant resistant responses in the detrusor muscle from healthy subjects (Sjögren *et al.*, 1982; Kinder and Mundy, 1985; Chen *et al.*, 1994). However, in disease states a significant purinergic component may be present (Sjögren *et al.*, 1982; Hoyle *et al.*, 1989; Palea *et al.*, 1993). In the $P2X_1$-receptor knockout

mouse, bladder function appeared normal (Mulryan *et al.*, 2000). Several explanations are possible to explain these findings. Firstly, $P2X_1$-like receptors may not play an important role in mouse bladder function. Or perhaps, the cholinergic component adapts during development in order to ensure near-normal function. Another reason maybe that the non-$P2X_1$-like response to ATP in the urinary bladder (Ziganshin *et al.*, 1993; Hashimoto and Kokobun, 1995) is present in the mouse and remains following $P2X_1$ knockout. The presence of these residual purinergic and non-purinergic receptors then ensures adequate bladder function. In terms of drug development for treatment of conditions where a purinergic component may play a significant role in the pathogenesis of bladder problems, it is imperative to identify which ATP-sensitive receptors are present and their relative contribution to the neurogenic response of the bladder. Interestingly, a novel splice variant for $P2X_1$ has been identified in the human bladder and its functional role, if any, remains to be investigated (Hardy *et al.*, 2000). From the study using the $P2X_1$ knock-out mice (Mulryan *et al.*, 2000), it was clear that $P2X_1$-receptors are essential for mediating the neurogenic response of the vas deferens as homozygous knock out male mice were infertile, yet their sperm remained functional. Furthermore, the vasa deferentia were unresponsive to $P2X_1$ receptor agonists (Mulryan *et al.*, 2000). Also in that study, it was noted that the mean systolic blood pressure increased slightly (Mulryan *et al.*, 2000). A meaningful interpretation of this blood pressure effect is difficult due to the possible central effects of a loss of $P2X_1$ function along with adaptations that may have occurred during development.

Whole animal studies have demonstrated a role for P2X receptors in pressor activity. Administration (i.v. and i.a.) of $\alpha\beta$-meATP resulted in vasopressor activity (Bulloch and McGrath, 1988; Schlicker *et al.*, 1989; Urbanek *et al.*, 1990). Furthermore, these responses

could be attenuated by treatment with suramin and related compounds (Schlicker *et al.*, 1989; Urbanek *et al.*, 1990). The results are complicated through the fact that αβ-meATP may not be $P2X_1$-specific *in vivo* (Schlicker *et al.*, 1990) and that there is species variation between the purinergic component of sympathetic nerves supplying the vasculature (Bulloch and McGrath, 1988). However, vascular P2X receptors still remain a potential target in the treatment of hypertension (Burnstock, 1998), especially considering that *in vitro* vascular smooth muscle preparations, from spontaneously hypertensive rats, may have an enhanced purinergic component (Vidal *et al.*, 1986; Brock and Van Helden, 1995).

Transcripts coding for multiple P2X isoforms have been detected in vascular smooth muscle indicating that the population of P2X receptors may be heterogeneous (Nori *et al.*, 1998; Lewis and Evans, 2000a). Moreover, recent functional evidence has been forthcoming that further supports the idea of P2X receptor heterogeneity in different vascular smooth muscle preparations (Lewis and Evans, 2000a; Surprenant *et al.*, 2000). The exact phenotypes remain undetermined and highlight the need for novel pharmacological agents that can distinguish between homo- and heteromeric receptors. The heterogeneous population of P2X receptors, in combination with P2Y-mediated vascular activity (Boarder and Hourani, 1998; Ralevic and Burnstock, 1998; Lewis *et al.*, 2000a), provides the potential for selective treatment for problems in specific areas of the vasculature.

P2X-like and P2Y-like responses were identified in human platelets (Hallam and Rink, 1985; MacKenzie *et al.*, 1996; Sage *et al.*, 1997) prior to the cloning of $P2X_1$ receptors from these blood cells (Clifford *et al.*, 1998; Scase *et al.*, 1998; Sun *et al.*, 1998). The exact role of $P2X_1$ remains controversial, with claims that this receptor is not needed for platelet aggregation *in vitro* (Takano *et al.*, 1999). However, the *in vivo* role for $P2X_1$ receptors may be more subtle,

as demonstrated in patients with a dominant platelet $P2X_1$ receptor mutation in the second transmembrane region, in which the receptor fails to be activated by ATP. These patients have a severe bleeding disorder in which they have reduced platelet aggregation in response to ADP (Oury *et al.*, 2000). Furthermore, the recent cloning of a shortened version of $P2X_1$ ($P2X_{1del}$) from human platelets complicates the argument of the role of $P2X_1$ receptors in platelets (Greco *et al.*, 2001). This new form has a marked reduction in sensitivity to ATP but is potently activated by ADP. It remains to be determined if and how this new form assembles into the membrane of platelets in relation to homo- or heteromeric assemblies with $P2X_{1wt}$ and any influence on the pharmacology and physiology of platelet function.

Over 20 years ago ATP was identified as an algogenic substance that could be released from damaged cells (Bleehen *et al.*, 1976; Bleehen and Keele, 1977). Following the cloning and identification of P2X subtypes it was discovered that one of these subtypes, $P2X_3$, was apparently exclusively expressed in a subpopulation of small diameter sensory neurones (Chen *et al.*, 1995). Subsequently it was proposed that ATP maybe involved in mediating pain through activation of purinoceptors on afferent nerve terminals (Burnstock, 1996). Functional evidence was forthcoming to prove that ATP excites sensory neurones *in vitro* (Robertson *et al*, 1996; Rae *et al.*, 1998; Grubb and Evans, 1999). Direct excitation of primary afferent neurones has been demonstrated (Dowd *et al.*, 1998; Hamilton *et al.*, 1999a; Kirkup *et al.*, 1999) as well as behavioural studies highlighting the algogenic nature of ATP (Bland-Ward and Humphrey, 1997; Sawynok and Reid, 1997; Hamilton *et al.*, 1999b). Along with functional studies, immunolocalisation experiments lend weight to the idea that $P2X_3$ receptors may mediate the excitatory effects of ATP in small diameter nociceptive neurones that are capsaicin-sensitive (Vulchanova *et al.*, 1997; Bradbury *et al.*, 1998; Bo *et al.*, 1999).

Electrophysiological studies also identified a non-desensitising ATP-mediated current in sensory neurones (Bean *et al.*, 1990; Khakh *et al.*, 1995a; Li *et al.*, 1999; Ueno *et al.*, 1999) that is likely to be mediated through heteromeric $P2X_{2/3}$ receptors (Lewis *et al.*, 1995; Thomas *et al.*, 1998). Interestingly, it has been proposed that the heteromeric $P2X_{2/3}$ receptor may be involved in mediating the pain response seen in response to a mechanical stimulus (Tsuda *et al.*, 2000). It was proposed that ATP may act on $P2X_{2/3}$ receptors present on the peripheral terminals of capsaicin-insensitive afferent fibres and manifest in mechanical allodynic response. However, it was noted that due to a lack of pharmacological agents that can distinguish between $P2X_{2/3}$ and $P2X_3$ receptors that it was not possible to rule out the involvement of homomeric $P2X_3$ receptors (Tsuda *et al.*, 2000). Nevertheless, if the mechanical allodynia is, as proposed, mediated through medium sized capscaicin-insensitive neurones then this is an important finding as it demonstrates distinct pathways for the transduction of ATP-mediated pain stimuli (Tsuda *et al.*, 2000).

The nociceptive $P2X_3$-positive neurones project to lamina II_{inner} of the dorsal horn of the spinal cord where $P2X_3$ protein appears to be transported to the central terminals from the nerve cell bodies (Bradbury *et al.*, 1998), indicating a possible central role for P2X receptors in nociceptive transmission. Furthermore, it has been demonstrated that ATP can excite postsynaptic neurones in the spinal cord (Jahr and Jessell, 1983; Li and Pearl, 1995) but with large differences in estimation of ATP-sensitive neurones (Bardoni *et al.*, 1997; Gu *et al.*, 1998). GABAergic interneurones have been proposed as the source of ATP and that changes in output from these interneurones may manifest in painful conditions (Jo and Schlicter, 1999).

In contrast, ATP, acting via P2X receptors, appears to have a modulatory role on presynaptic function through facilitation of glutamate release from afferent fibres (Li and Pearl, 1995; Gu and MacDermott, 1997; Li *et al.*, 1998). The P2X receptors responsible for mediating the actions of ATP in the dorsal horn have not been conclusively identified. However, postsynaptic responses are PPADS and suramin sensitive and αβ-meATP insensitive (Bardoni *et al.*, 1997). There is strong immunoreactivity for $P2X_2$, $P2X_4$ and $P2X_6$ (Collo *et al.*, 1996), therefore raising the possibility of multiple populations of heteromeric and/or homomeric receptors. Equally, the $P2X_3$ protein reported to be transported to the terminals of nociceptive primary sensory neurones (Bradbury *et al.*, 1998; Yiangou *et al.*, 2000) and upregulated following neuropathic injury (Novakovic *et al.*, 1999) may have functional significance. Indeed, it has been proposed that presynaptic P2X receptors (probably $P2X_3$) present on capscaisin-sensitive primary afferent neurones can be activated by ATP to facilitate neurotransmitter release from the primary afferent fibres in response to plantar injection of both formalin and capsaicin (Tsuda *et al.*, 1999a).

As well as being involved in the development of neurogenic pain, ATP appears to participate in mediating the development of the more persistent, formalin-induced inflammatory pain at the level of the spinal cord. PPADS can suppress the development of the second phase, an effect independent of the first phase (Tsuda *et al.*, 1999a). Due to the lack of available tools, the ATP-sensitive receptors involved in the second phase of inflammatory pain in this model could not be conclusively identified (Tsuda *et al.*, 1999a). However, two recent studies using $P2X_3$-receptor deficient mice has shed light on the role that $P2X_3$ receptors play in sensory processing. It was noted that in the $P2X_3$ null mutant mice behaviour indicative of pain was reduced in both phases of the formalin-induced inflammation (Cockayne *et al.*, 2000;

Souslova *et al.*, 2000). Furthermore, peripheral injection of ATP into the hindpaw of wild type mice resulted in nociceptive behaviour that was significantly reduced in the null mutant mice. This behaviour could be further reduced through treatment with PPADS (Cockayne *et al.*, 2000). $P2X_3^{-/-}$ dorsal root ganglion neurones lacked rapidly desensitizing responses to ATP or αβ-meATP, although a small proportion of neurones exhibited a slowly desensitizing response to ATP, presumably via $P2X_2$ receptors (Cockayne *et al.*, 2000; Souslova *et al.*, 2000). Therefore the reduction in nociceptive behaviour in null mutant mice appears to be due largely to a loss of expression of functional $P2X_3$ receptors in dorsal root ganglion neurones. In contrast, it was found in both studies that pain related behaviour to noxious mechanical and thermal stimuli were the same in both, wild type and mutant, groups (Cockayne *et al.*, 2000; Souslova *et al.*, 2000). Yet, $P2X_3$ maybe present on neurones responsive to heat (Hamilton *et al.*, 1999a; Tsuda *et al.*, 1999a). Interestingly, although the threshold level in dorsal horn neurones for noxious heat was approximately the same in both wild type and null mutant, the electrical activity in wild type neurones exhibited a graded response to increasing temperature that was absent in the neurones from the null mutant mice (Souslova *et al.*, 2000). Therefore P2X receptors, particularly $P2X_3$, represent a target for analgesics in the treatment of chronic pain and migraine (Burnstock and Wood; 1996), inflammation, visceral and vascular pain (Burnstock, 1996; Ferguson *et al.*, 1997; Burnstock, 2000). Studies investigating the possibility of analgesia following P2X receptor block have been hampered due to the unsuitable pharmacological profiles of the tools that are currently available. However, in electrophysiological and behavioural tests, treatment with P2 antagonists appears to reduce activity that maybe associated with pain (Dowd *et al.*, 1998; Tsuda *et al.*, 1999a,b; Stanfa *et al.*, 2000).

In my investigations I have demonstrated that enhanced potency of synthetic compounds at $rP2X_1$ and $rP2X_3$ is possible while maintaining a degree of selectivity between the two receptors. This would be desirable in treatment of disease conditions such as those of the urinary bladder, where a reduction in mechanical activity may be required whilst maintaining the sensory output via P2X receptors (presumably including $P2X_3$) present on the afferent nerves (Namasivayam *et al.*, 1999). Conversely, results obtained using the $P2X_3$ knock out mice have showed that block of $P2X_3$ in sensory neurones innervating the urinary bladder may provide a new route for the treatment of bladder disorders. Null mutant mice had reduced voiding frequency and increased bladder capacity but no increase in bladder pressures at any volume (Cockayne *et al.*, 2000). It is proposed that ATP can be released from the epithelium of the bladder as it is stretched during filling. The ATP then activates $P2X_3$ receptors on sensory neurone nerve terminals to excite afferent neurones and thus mediate mechanosensation (Cook and McCleskey, 2000). Therefore, agents that block $P2X_3$ receptors to impair this afferent activity from the bladder may serve to treat storage disorders (Cockayne *et al.*, 2000). Currently, however, agents that are selective for $P2X_3$ are limited and restricted only to agonists.

$P2X_3$ mRNA is not localised exclusively to sensory neurones (Garcia-Guzman *et al.*,1997) and agents designed to treat pain through blocking $P2X_3$ receptors are perhaps unlikely to be without side effects. Indeed, loss of $P2X_3$ receptors resulted in an increased thermal hyperalgesia to chronic inflammation (Souslova *et al.*, 2000), although this may be a consequence of the model used (Cook and McCleskey, 2000).

Elucidation of definitive roles for $P2X_2$ and $P2X_4$ receptors in physiological and pathophysiological states has been hampered by the lack of selective agonists or antagonists

for these P2X subtypes. In our investigations into the antagonistic ability of PPADS derivatives, ATP-mediated responses at $rP2X_2$ receptors could be effectively abolished by many of the compounds (see chapter 6) albeit with low potency in comparison to their activity at $P2X_1$ and $P2X_3$ receptors. However, $P2X_4$ receptors are considerably more resistant to antagonism by the current tools available, including the novel PPADS derivatives (see table 1.4, chapter 6). Transcripts for $P2X_2$, $P2X_4$ and $P2X_6$ receptors have a widespread distribution in the brain (Collo *et al.*, 1996; Lê *et al.*, 1998a; Kanjahn *et al.,* 1999) and functional studies have demonstrated a fast excitatory role for ATP in the CNS (Edwards *et al.*, 1992; Nieber *et al.*, 1997; Inoue, 1998; Mateo *et al.*, 1998; Pankratov *et al.*, 1998; Ross *et al.*, 1998; Garcia-Lecea *et al.*,1999; Khakh *et al.*, 1999; Spyer and Thomas, 2000). In certain instances, homomeric populations appear to dominate the ATP-response (Mateo *et* al., 1998; Garcia-Lecea *et al.*, 1999). Furthermore, it is evident that heteromeric assemblies may also contribute to the heterogeneous population of P2X receptors in the CNS (Lê *et al.*, 1998b; King *et al.*, 2000). However, it is apparent that positive identification of specific functional subtypes in the CNS, and therefore elucidation of their physiological and pathophysiological roles, will not occur until selective pharmacological tools are developed. A collaboration involving our laboratory is currently undertaking the task of developing and testing agents that are selective for $P2X_2$ receptors (Jacobson *et al.*, 2000b).

9.5 Factors affecting the pharmacological phenotype of P2X receptors

As discussed, the pharmacological characterisation of P2X receptors has relied largely upon the relative sensitivity of these receptors to ATP, αβ-meATP and antagonists such as suramin and PPADS. However, as a wider range of tools becomes available it is becoming

apparent that the properties of these receptors not only depend upon species orthologue but also, the expression system that the recombinant receptors are studied in. This is demonstrated by comparing several investigations, including my own, examining the pharmacological properties of diadenosine polyphosphates at recombinant receptors. Comparing the rat $P2X_1$ ($rP2X_1$) and human $P2X_1$ ($hP2X_1$) receptors, the results indicate a difference in agonist potency order and efficacy of the diadenosine molecules between ortholgues (table 9.1). However, these receptors are expressed in two different expression systems, namely oocytes ($rP2X_1$) and human astrocytoma cells ($hP2X_1$). This may be significant as it can seen by comparing these results with $hP2X_1$, expressed in astrocytoma cells, with those obtained using $hP2X_1$ in oocytes and HEK293 cells that receptor expression system appears to influences the pharmacological properties of the receptor as the efficacy of Ap_5A is different in each case (table 9.1). Therefore, it may be possible that these cells slightly alter the tertiary structure of the receptor through different post-translational modification processes such that the receptor properties are altered slightly. Interestingly, a species-specific difference in post-translational modification for $P2X_3$ receptors has already been reported (Yiangou *et al.*, 2000).

It has been demonstrated that the kinetics of $P2X_1$ channel activation and inactivation changes over several days in culture following expression in HEK293 cells (Parker, 1998). The kinetics of the channel changes from an extremely rapid activation/inactivation to a phenotype that resembles endogenous $P2X_1$-like responses. The channel could be returned to its rapid activation/inactivation phenotype through disruption of the actin cytoskeleton (Parker, 1998). These findings contrast with results obtained in oocytes (Werner *et al.*, 1996), indicating a possible difference between assembly and/or anchorage of the receptor into the

membrane between oocytes and HEK293 cells. Similarly, there is a discrepancy between the reported time needed for recovery from desensitization of the $P2X_4$ receptor when expressed in oocytes and HEK293 cells. In HEK293 cells desensitization has been reported to be minimal (Buell *et al.*, 1996), but in oocytes, desensitization was more pronounced and required longer periods of wash out to permit full recovery (Bo *et al.*, 1995). Interestingly, in single HEK 293 cells, $rP2X_4$ receptors were subject to an application-dependent run down of ATP responses that did not occur with $rP2X_2$. However, in cell rafts, where groups of cells are electrically coupled, ATP responses showed no run down and were reproducible every 5 minutes (Miller *et al.*, 1998). Therefore, it appears that in HEK293 cells an intracellular mediator may assist in determining the biophysical properties of $rP2X_4$ receptors that is not necessary, or is needed in a smaller concentration, for $rP2X_2$ receptors.

It is possible that the endogenous ATP release, that has been demonstrated from oocytes (Bodas *et al.*, 2000) and HEK cells (Surprenant *et al.*, 2000), alters the apparent activity of the diadenosine polyphosphates, presuming that such a phenomenon does not occur with the astrocytoma cells. Indeed, the measured EC_{50} value for ATP at $hP2X_1$ receptors (Bianchi *et al.*, 1999) is in the region of 14-fold more potent than at the same receptors expressed in oocytes (Evans *et al.*, 1995, Appendix 2), indicating that the receptors in the astrocytoma cell line appear to be in a more favourable state for activation. However, partial receptor desensitization occurring as a result of endogenously released ATP is unlikely to account for the significant difference in activities of some of the diadenosine compounds seen at $P2X_2$, $P2X_3$ and $P2X_4$ receptors. The results point to the fact that the receptor expression system influences the pharmacological properties of the receptor. Ap_4A is the only diadenosine molecule to show activity at $rP2X_2$ when expressed in oocytes, where it acts as a full agonist

(Pintor *et al.*, 1996). However, when the same receptor is expressed in astrocytoma cells, Ap_4A is inactive (Bianchi *et al.*, 1999). Similarly, activity of Ap_3A is absent at $rP2X_3$ receptors when expressed in the human cell line and diadenosine agonist potency orders are altered (table 9.1). Ap_5A is inactive at $rP2X_4$ receptors but is a partial agonist at the human orthologue, however sequence differences, aswell as a potential influence of expression system on the pharmacological properties, may determine the activity of Ap_5A at $P2X_4$ (table 9.1). Clearly, there are some pharmacological differences between P2X receptor orthologues (see also table 1.4 for antagonist data). Therefore, it would seem prudent that this is addressed early on when examining the potential of drugs with activity at P2X receptors to influence disease states where ATP is implicated in the pathogenesis. It may be possible that the phenotype of a particular endogenous P2X subtype can be subtly varied depending on where it is expressed, although this is speculative at this stage. Diadenosine polyphosphates may prove to be useful investigative tools when examining this hypothesis.

It should be noted that diadenosine polyphosphates seem to be the most noticeable case of significant differences in pharmacological profiles between expression systems and that generally data obtained between expression systems is comparable. Nevertheless, to accurately characterise a receptor and to represent and compare results with endogenous receptors closely, the possibility of anomalous receptor properties arising through its study in different expression systems must be taken into account. Therefore currently, diadenosine polyphosphates represent another tool for investigating P2X receptors but are not a definitive test as to the phenotype of a P2X receptor population.

The environment that they exist in may also alter the phenotype of native P2X receptors. For example, protons and Zn^{2+} are released during neurotransmission (Krishtal *et al.*, 1987; Assaf

and Chung, 1984; Rose and Deitmer, 1995), and are known to facilitate ATP-mediated responses at recombinant (King *et al.*, 1996a; Stoop *et al.*, 1997; Miller *et al.*, 1998; Wildman *et al.*, 1998, 1999; Liu *et al.*, 2000) and native P2X receptors (Cloues *et al.*, 1993; Koizumi *et al.*, 1995; Li *et al.*, 1996; Garcia-Lecea *et al.*, 1999). However, only endogenous receptors are consistently exposed to these mediators *in vivo*. Potentiation of ATP-mediated responses has also been reported at other P2X receptors with various agents and raises the intriguing possibility of mediators existing *in vivo* that enhance purinergic transmission (for example Ap_2A and Ap_3A, Chapter 4). Allosteric modulation (that is, the binding of a modulator to the receptor at a site distinct from the agonist binding site, so as to induce a conformational change with a resultant, in these examples, enhancement of agonist-induced activation of the receptor (Kenakin, 1997; Colquhoun, 1998)) at P2X receptors is reported with physiological relevant, as well as pharmacological, substances. Allosteric modulation at P2X receptors has been proposed to explain the potentiation of ATP responses by antagonists seen in tissue preparations (Michel *et al.*, 1997). These authors proposed that the radioligand [^{35}S]ATPγS preferentially binds to a desensitized state of the $rP2X_4$ receptor and that the antagonist cibacron blue binds to the receptor promoting a switch from the desensitized state (Michel *et al.*, 1997). If this were to occur with other antagonists in functional studies then a greater number of receptors would be in a state that favours activation, thereby producing an apparent increase in response in the presence of the antagonist from control responses. Functional evidence demonstrated that cibacron blue could potentiate ATP-mediated responses at recombinant $rP2X_4$ receptors (Miller *et al.*, 1998). Cibacron blue also modulated responses at human and rat $P2X_3$ receptors but in a manner that differed from its actions at $rP2X_4$. At $P2X_3$, cibacron blue shifted the ATP-dose response curve to the left, increased the

maximum response and, interestingly, in relation to the hypothesis of Michel *et al.*, 1998, cibacron blue increased the recovery time from desensitization (Alexander *et al.*, 1999). Potentiation of ATP-responses through a decrease in refractory period following desensitization at $P2X_3$ has also been reported for high concentrations of Ca^{2+} (Cook *et al.*, 1998). Much remains to be discovered regarding the mechanisms of modulation as there is evidence that some of these effects are independent of each other (Alexander *et al.*, 1999). Furthermore, it will be interesting to discover the receptor sites involved in modulation aswell as the mechanisms of desensitization. It is of note that at the single channel level, the rate of activation of $P2X_2$ receptors is increased upon a reduction of pH (Ding and Sachs, 1999). In relation to mechanisms of activation, it was also noted that the binding of ATP was cooperative and that the channel has at least 3 binding sites (Ding and Sachs, 1999).

9.6 Future experiments

Now that the actions of agonists and antagonists reported in chapters 5, 6 and 7 have been closely examined at homomeric recombinant rP2X receptors there is scope for a large number of studies. Ideally, I envisage immediate investigations could begin on in vitro smooth muscle preparations, examining the effect of these agents in the more traditional models e.g. vas deferens, urinary bladder, rat tail artery. To complete the pharmacological profiles of the diadenosine polyphosphates and the new compounds described in this thesis at homomeric P2X receptors, it would necessary to study their effects at $P2X_5$, $P2X_6$ and $P2X_7$ in expression systems, such as HEK293 or CHO cells, where their expression has previously been successful.

Another line of study could continue in oocytes looking at the effects of these compounds on heteromeric receptors, including, as mentioned above, at $rP2X_{1/2}$ receptors. Results from such studies on $P2X_{2/3}$ receptors, combined with the present data of their activity at $rP2X_3$ receptors, could compliment investigations on these agents at homomeric and heteromeric P2X receptors present in sensory neurones.

With regards to co-expression of P2X subunits, there is still a number of combinations that still remain to be studied and reported in expression systems (see table 1.3). The only combination using $P2X_1$ that has not been reported thus far is an attempt to co-express $P2X_1$ with $P2X_6$. These experiments are currently at a preliminary stage in our laboratory.

Recently, it has been demonstrated that $P2X_1$ receptors in rat vas deferens and HEK293 cells are internalised upon exposure to agonists (Dutton *et al*, 2000; Ennion & Evans, 2001). These receptors are subsequently rapidly recycled back to the cell surface. It will be fascinating to undercover the mechanisms involved in this dynamic process along with the effects, if any, on other populations of receptors. For example, there have been a number of recent reports on the occurrence of cross-talk between P2X receptors and other receptor types e.g. $\alpha 3\beta 4$ nicotinic, GABA (Khakh *et al*, 2000; Sokalova *et al.*, 2001). For cross-talk to occur it is likely that the receptor type have to be in contact with each other (Khakh *et al.*, 2000 and references therein). Therefore, if cross-talk can occur with $P2X_1$ and other receptor types or if the P2X receptors currently reported to be involved in cross-talk are internalised following activation then receptor number of other receptor populations may decrease also without themselves having been activated.

In itself, cross-talk between P2 receptors holds huge potential as there are many cases of P2X receptors co-localising with P2Y receptors, not least in platelets where $P2X_1$ receptors are

present along with $P2Y_1$ and $P2Y_{12}$ receptors (Nicholas, 2001; see section 9.3). Due to their widespread tissue distribution, the possibility exists for P2X and P2Y receptors to interact with numerous other receptors.

A. $P2X_1$

Relative potency orders

$rP2X_1$ (chapter 4)	$Ap_4A > ATP > Ap_6A \geq Ap_5A > Ap_3A$
$hP2X_1$ (Bianchi *et al.*, 1999)	$ATP > Ap_4A \geq Ap_5A > Ap_6A$
$hP2X_1$ (Evans *et al.*, 1995)	$ATP > Ap_5A$

Efficacy values (%ATP maximum)

	Ap_3A	Ap_4A	Ap_5A	Ap_6A
$rP2X_1$ (Chapter 4)	15	35	45	90
$hP2X_1$ (Bianchi *et al.*, 1999, 1321N1 astrocytoma cells)		100	100	55
$hP2X_1$ (Evans *et al.*, 1995, oocytes)			60	
$hP2X_1$ (Evans *et al.*, 1995, HEK293 cells)			80	

B. $P2X_3$

Relative potency orders

$rP2X_3$ (Chapter 4)	$Ap_4A > Ap_3A > Ap_5A > Ap_6A > ATP$
$rP2X_3$ (Bianchi *et al.*, 1999)	$ATP > Ap_4A > Ap_5A$
$hP2X_3$ (Bianchi *et al.*, 1999)	$ATP > Ap_4A > Ap_5A = Ap_6A > Ap_3A$

Efficacy values (%ATP maximum)

	Ap_3A	Ap_4A	Ap_5A	Ap_6A
$rP2X_3$ (Chapter 4)	60	100	100	100
$rP2X_3$ (Bianchi *et al.*, 1999)	IA	34	100	ND
$hP2X_3$ (Bianchi *et al.*, 1999)	53	64	81	82

C. $P2X_4$

Relative potency orders

$rP2X_4$ (Chapter 4)	$Ap_4A > ATP >> Ap_6A$
$hP2X_4$ (Bianchi *et al.*, 1999)	$ATP > Ap_4A > Ap_5A > Ap_6A$

Efficacy Values (%ATP maximum)

	Ap_3A	Ap_4A	Ap_5A	Ap_6A
$rP2X_4$ (Chapter 4)	IA	25	IA	10
$hP2X_4$ (Bianchi *et al.*, 1999)	IA	59	55	60

Table 9.1 Summary of pharmacological properties of diadenosine polyphosphates at recombinant P2X orthologues. Two tables of information are given for each diadenosine molecule relating to their activity at each receptor subtype. The relative potency order is based on calculated EC_{50} values. Efficacy values are expressed as a percentage of the maximum ATP response.

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Ziganshin, A.U., Zaganshina, L.E., King, B.F. & Burnstock, G. (1995). Characteristics of ecto-ATPase of *Xenopus* oocytes and the inhibitory actions of suramin on ATP breakdown. *Eur. J. Pharmacol.*, 429, 412-418.

Ziganshin, A.U., Ziganshina, L.E., King, B.F., Pintor, J. & Burnstock, G. (1996). Effects of P2 purinoceptor antagonists on degredation of adenine nucleotides by ecto-nucleotidases in folliculated oocytes of *Xenopus Laevis*. *Biochem. Pharmacol.*, 51, 897-901.

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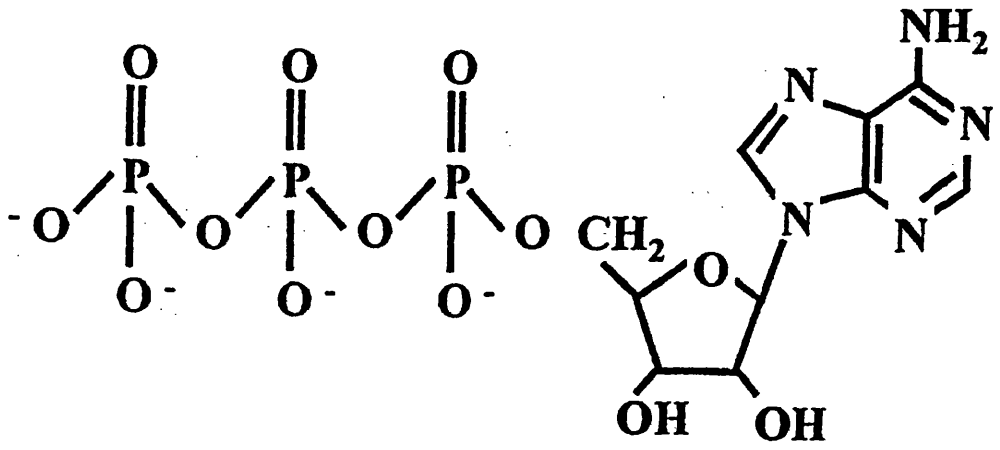
Ziyal, R., Ziganshin, A.U., Nickel, P., Ardanuy, U., Mutschler, E., Lambrecht, G. & Burnstock, G. (1997). Vasocontstrictor responses via P2X-receptors are selectively antagonized by NF023 in rabbit isolated aorta and saphenous artery. *Br. J. Pharmacol.*, 120, 954-960.

APPENDICES

APPENDIX 1

STRUCTURE OF ATP AND GENERAL STRUCTURE OF ADENINE DINUCLEOTIDES

A



B

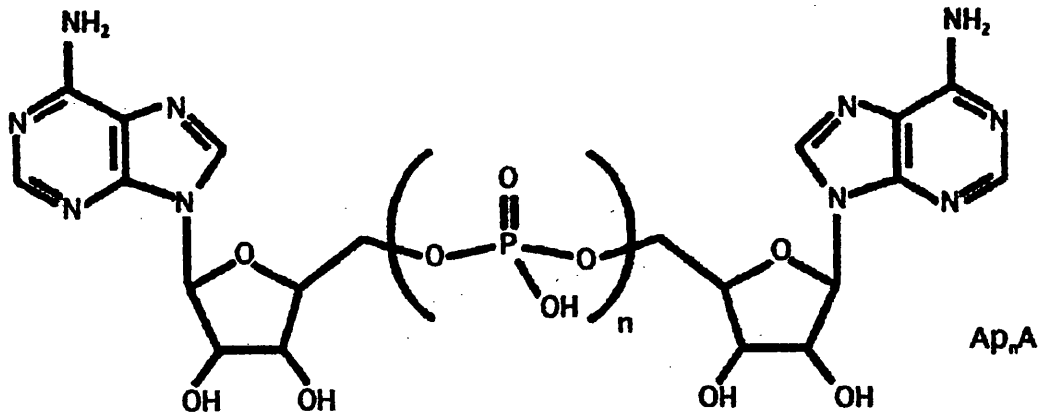


Figure A1.1 Chemical structure of **A**. ATP signified by (from right to left) an adenine structure, a ribose moiety and three phosphate groups. **B**. Diadenosine polyphosphates as denoted by two nucleoside moieties linked by a varying number of phosphate groups (n = 2 – 6).

APPENDIX 2

THE $P2X_1$ RECEPTOR: A SENSITIVE BIOASSAY FOR ATP

A.2.1. SUMMARY

Four commercially available samples of adenosine 5′-triphosphate disodium salt (ATP-Na_2) were tested at $P2X_1$ receptors expressed in *Xenopus* oocytes. This sensitive bioassay system revealed significant differences in the potency of these four ATP samples, with mean EC_{50} values ranging from of 81-636 nM (8-fold difference). ATP samples with the lowest levels of divalent cation contamination were most potent - a *Sigma-RBI* product ATP *SigmaUltra*™ (A7699) and a *Calbiochem* product ATP *Low Metals Grade* (119125) being joint first.

A2.2. INTRODUCTION

It is widely acknowledged ATP directly activates a cation-selective ion channel (*a P2X receptor*) to cause depolarisation and Ca^{2+}-influx in a variety of cell types (Burnstock, 1997). Seven P2X subunits have been identified in mammalian cells (Khakh *et al.*, 2001a,b). These P2X subunits must polymerise with other P2X proteins to form an ion channel and, presently, it is believed a P2X receptor is formed by three subunits (Nicke *et al.*, 1998). Six P2X receptors can be formed as homomeric assemblies (*i.e.* same P2X subunits) and are called $P2X_1$-$P2X_5$ and $P2X_7$ receptors (Khakh *et al.*, 2001a). Biochemical evidence suggests another eleven P2X receptors can be formed as heteromeric assemblies (*i.e.* different P2X subunits) (Torres *et al.*, 1999b) although, so far, only four subtypes - $P2X_{1/5}$, $P2X_{2/3}$, $P2X_{2/6}$ and $P2X_{4/6}$ receptors - have been examined for function (Khakh *et al.*, 2001a). Arguably there may be as many as seventeen P2X receptor subtypes in mammalian cells, although some P2X subtypes appear to be more prevalent than others in specific tissues (Jacobson *et al.*, 2000; Khakh *et al.*, 2001a).

A2.3. RESULTS AND DISCUSSION

A2.3.1. *The $P2X_1$ receptor - a sensitive bioassay for ATP*

Homomeric $P2X_1$ receptors are found in blood platelets, vascular and visceral smooth muscles, cardiac muscle, sensory neurons in spinal ganglia and, to a lesser extent, central neurones in the CNS (in cerebellum, periaqueductal grey, dorsal horn of spinal cord) (Jacobson *et al.*, 2000). Of the known homomeric assemblies, the $P2X_1$ receptor is most sensitive to ATP and gives rise to fast, rapidly–inactivating membrane currents carried by Na^+, K^+ and Ca^{2+} ions (Jacobson *et al.*, 2000). The threshold concentration for ion channel activation by ATP is of the order of 3-30 nM, with maximum activation occurring over a range of 1000-100,000 nM. Previously published determinations of ATP potency fall over a range of 56-3200 nM (mean EC_{50} values) at human and rat $P2X_1$ receptors (Table A2.1). Such small differences in potency can still be important when assessing the activity of ATP against other nucleotide agonists or comparing the pharmacological properties of $P2X_1$ receptors against other P2X receptor subtypes. These differences in potency may be due to subtle changes in experimental conditions. For example, the presence of Ca^{2+} and Mg^{2+} ions in the extracellular solution affected ATP potency at native P2X receptors in rat vagus nerve, in part by altering the degree of ATP breakdown by Ca^{2+}/Mg^{2+}-dependent surface enzymes (Trezise *et al.*, 1994b). However, ATP breakdown cannot adequately explain the observed differences in ATP potency seen at recombinant $P2X_1$ receptors, since common expression systems such as *Xenopus* oocytes are largely devoid of surface enzymes that hydrolyse ATP (Ziganshin *et al.*, 1995). Also, the kinetics of activation and inactivation for $P2X_1$ ion channels are appreciably faster in expression systems than kinetics of ATPases in whole tissues (Ziganshin *et al.*, 1995). Furthermore, the breakdown product ADP is not an agonist at

Table A2.1. ***ATP potency at species orthologues of homomeric $P2X_1$ receptor.***

Species Orthologue	Expression System	EC_{50} value (nM)	Threshold (nM)	Maximum (nM)	Hill Coefficient	Reference
Human $P2X_1$	1321N1 astrocytoma	56	3	1000	nd	A
	Xenopus oocyte	790	10	100,000	nd	B
	Xenopus oocyte	800	30	100,000	nd	C
	HEK 293	900	10	30,000	nd	D
Rat $P2X_1$	*Xenopus* oocyte	300	30	30,000	1.47	E
	Xenopus oocyte	420	30	30,000	0.99	F
	HEK 293	500	10	30,000	nd	G
	Xenopus oocyte	960	30	30,000	1.1	H
	Xenopus oocyte	1200	30	100,000	0.99	I
	Xenopus oocyte	3200	nd	100,000	nd	J

References. [A]Bianchi *et al.* (1999) *Eur. J. Pharmacol.* **376**:127-138. [B]Ennion *et al.* (2000) *J. Biol. Chem.* **275**:29361-29367. [C]Valera *et al.* (1995) *Recept. Chann.* **3**:283-289. [D]Evans *et al.* (1995) *Mol. Pharmacol.* **48**:178-183. [E]Wildman *et al.* (1999) *Br. J. Pharmacol.* **128**:486-492. [F]King *et al.* (1999) *Br. J. Pharmacol.* **128**:981-988. [G]Valera *et al.* (1994) *Nature* **371**:516-519. [H]Rettinger *et al.* (2000) *Neuropharmacol.* 39:2044-2053. [I]Soto *et al.* (1999) *Neuropharmacol.* **38**:141-149. [J]Werner *et al.* (1996) *PNAS* **93**:15485-15490.

rat $P2X_1$ receptors and its presence in ATP solutions does not complicate assays of agonist potency (Mahaut *et al.*, 2000).

A2.3.2. *Assaying $P2X_1$ receptors expressed in Xenopus oocytes*

Two grades of adenosine 5′-triphosphate disodium salt from *Sigma-RBI* (Product No. A-7699, *Sample A*; and A-3377, *Sample A**), as well as samples from *Boehringer Mannheim* (Product No. 519 979, *Samples B and B**) and *Calbiochem* (Product No. 119125, *Sample C*), were tested at rat $P2X_1$ receptors expressed in *Xenopus* oocytes and concentration/response (C/R) curves plotted (Figure A2.1).

Sample A (purity > 99%) and *Sample C* (purity > 95%) were equipotent by statistical comparison (unpaired *t*-test) with mean EC_{50} values in the region of 80-100 nM (Table A2.2). The threshold concentration for activation for both ATP samples was 1-3 nM, with maximal activation at 1000-3000 nM. Both samples were notable for exceptionally low levels of metal cation contamination (*Sample A*: calcium, <0.01%; copper, <0.0005%; iron, 0.005%; magnesium, <0.005%; zinc, <0.0005%; *Sample C*: aluminium, <0.003%; calcium, <0.001%; magnesium, <0.001%). At submicromolar and micromolar concentrations, each of these divalent or trivalent cations is known to affect the probability of ion channel opening at native and recombinant P2X receptors (Cloues, 1995; Ding and Sach, 2000; Xiong *et al.*, 1999) and, therefore, will subtly alter the position of C/R curves for ATP (King *et al.*, 1997b; Wildman *et al.*, 1999; Michel *et al.*, 1994).

*Sample A** (purity >99%) showed a higher threshold for ion channel activation (~10 nM), a higher mean EC_{50} value (~330 nM) and steeper Hill slope (Table A2.2). This ATP sample had slightly higher levels of trace metal elements [aluminium, 0.008%; calcium, 0.07%; magnesium, 0.002%] and such contaminants might possibly explain the skewed nature of the C/R curve.

A fourth sample, *Sample B* (purity >99%), showed interesting pharmacological properties. The threshold concentration for receptor activation was low (1-3 nM), the mean EC_{50} value (268 nM) higher than both *Sample A* and *Sample C*, and the concentration-response curve showed an unusually shallow slope with full activation occurring at high concentrations (~300,000 nM) (Figure A2.1). Where $P2X_1$ receptor assays were repeated extensively (n = 50; *see B* in Figure A2.1*), the C/R curve for this ATP sample now gave a Hill slope similar to determinations for *Sample A* and *Sample C* but higher threshold concentration (~10 nM) and mean EC_{50} value (636 nM). This sample of ATP powder had been used in the laboratory on numerous occasions and lower threshold and EC_{50} values may be due to slow hydrolysis of the agonist by repeated freeze-thaw cycles. Reasonably low levels of trace metals were reported for *Sample B/B** [aluminium, 0.002%; calcium, 0.0005%; iron, 0.001%; magnesium, 0.001%; zinc, 0.005%].

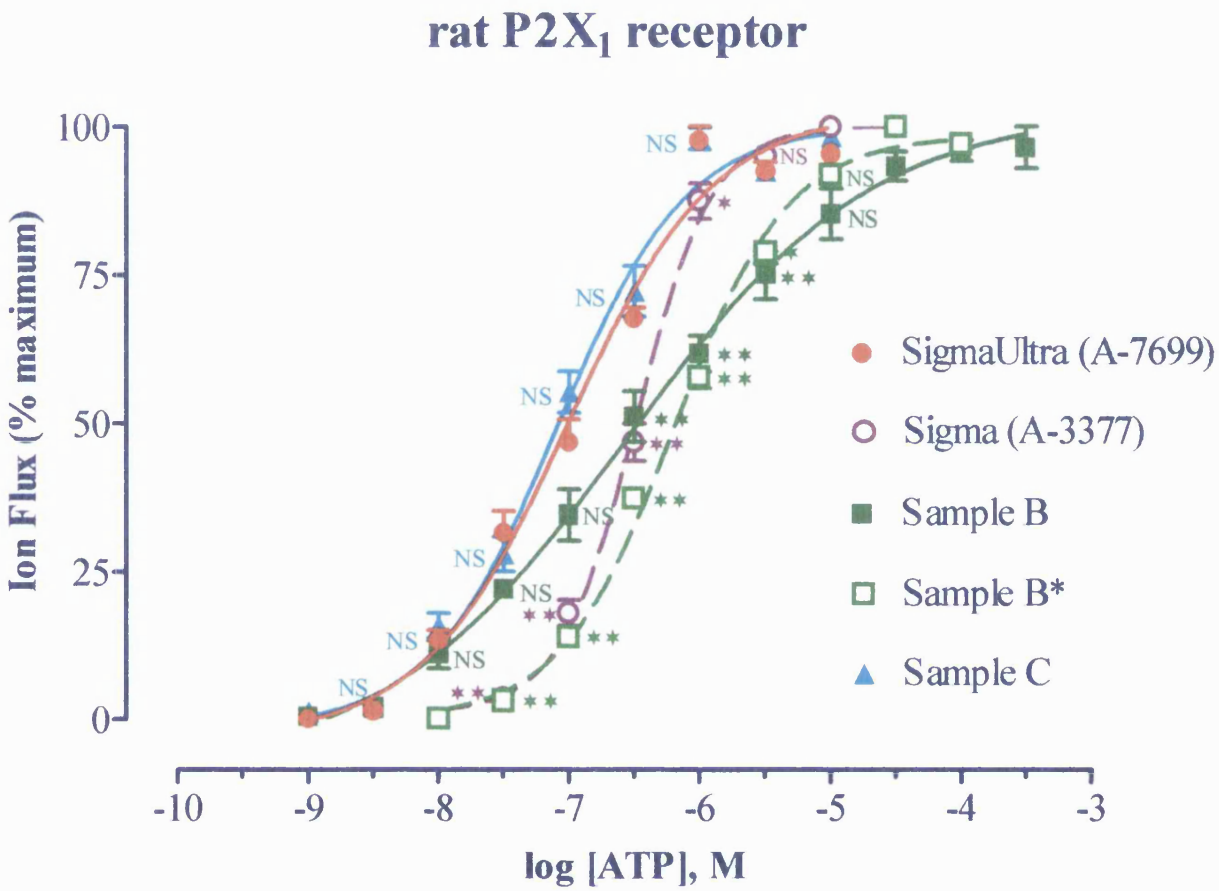


Figure A2.1. ***Concentration-dependent ATP activation of rat P2X$_1$ receptors***

Concentration/response curves for four commercial samples of ATP. Data points are expressed as mean±SEM (n = 5-50) and curves are fitted to the Hill equation using Prism v2.0 (GraphPad). Data for *Sample A* were compared statistically against data for other three ATP samples using Student's unpaired *t*-test (NS, not significant; ✱ <0.05; ✱✱ <0.01). Data for *Sample B** represent accumulated historical data from a number of C/R relationships for ATP carried out over a period of 4 months.

Table A2.2. ***ATP potency at homomeric $P2X_1$ receptor from rat.***

ATP Sample	Threshold (nM)	Maximum (nM)	EC_{50} value (nM)	CI (95%) (nM)	Hill Coefficient	N
Sample A	1	1000	103.6	74.4-144.3	0.77	8
*Sample A**	10	10,000	326.9	285.1-374.9	1.49	5
Sample B	1	300,000	267.8	159.1-450.8	0.46	6
*Sample B**	10	100,000	635.6	570.4-708.3	0.80	50
Sample C	1	10,000	80.9	59.3-110.2	0.88	7

Data from Figure A2.1.

A.2.3.3. *Conclusions*

The single use of fresh stock solutions can avoid the problem of slow hydrolysis of ATP in recycled (freeze/thawed-freeze/thawed) samples. The highest potency values at $P2X_1$ receptors were seen with ATP samples with the lowest reported levels of trace metals. *Sample A* and *Sample C* were equipotent and their EC_{50} values came close to the best seen activity index for ATP in the earliest studies of this recombinant P2X receptor (see Table A2.1). These ATP samples are preferable for future experiments and are currently being evaluating at other ATP receptors, including P2X heteromeric receptors as well as metabotropic P2Y receptor subtypes.

A.2.3.4. *Drug handling issues*

Vials of ATP powder was opened at room temperature and once only, except for *Sample B** which had been reopened on a number of occasions. A stock solution of 10 mM was prepared, aliquoted into sterile Eppendorff tubes and frozen immediately (-20° C). Each aliquot was use once and discarded at the end of each experiment. Stock solutions were kept on ice in a covered icebox. Superfused ATP solutions were made up 30 s prior to application, using a bathing solution devoid of magnesium and calcium ions (although containing (mM): Na^+, 110; K^+, 2.5; Ba^{2+}, 1.8; HEPES, 5; Cl^- 114.3; and pH 7.5). Flow rates were fast (5 $ml.min^{-1}$) and the electrophysiological chamber had a volume of 0.5 ml. The dead-space between the chamber and reservoir was 0.6 ml and it took 7 s for the superfusate to reach the recording chamber and oocyte. ATP-evoked membrane currents were recorded under twin-electrode voltage clamp conditions (see Methods in King *et al.*, 1997b).

APPENDIX 3

P2X RECEPTOR IMMUNOHISTOCHEMICAL STAINING IN RAT VAS DEFERENS

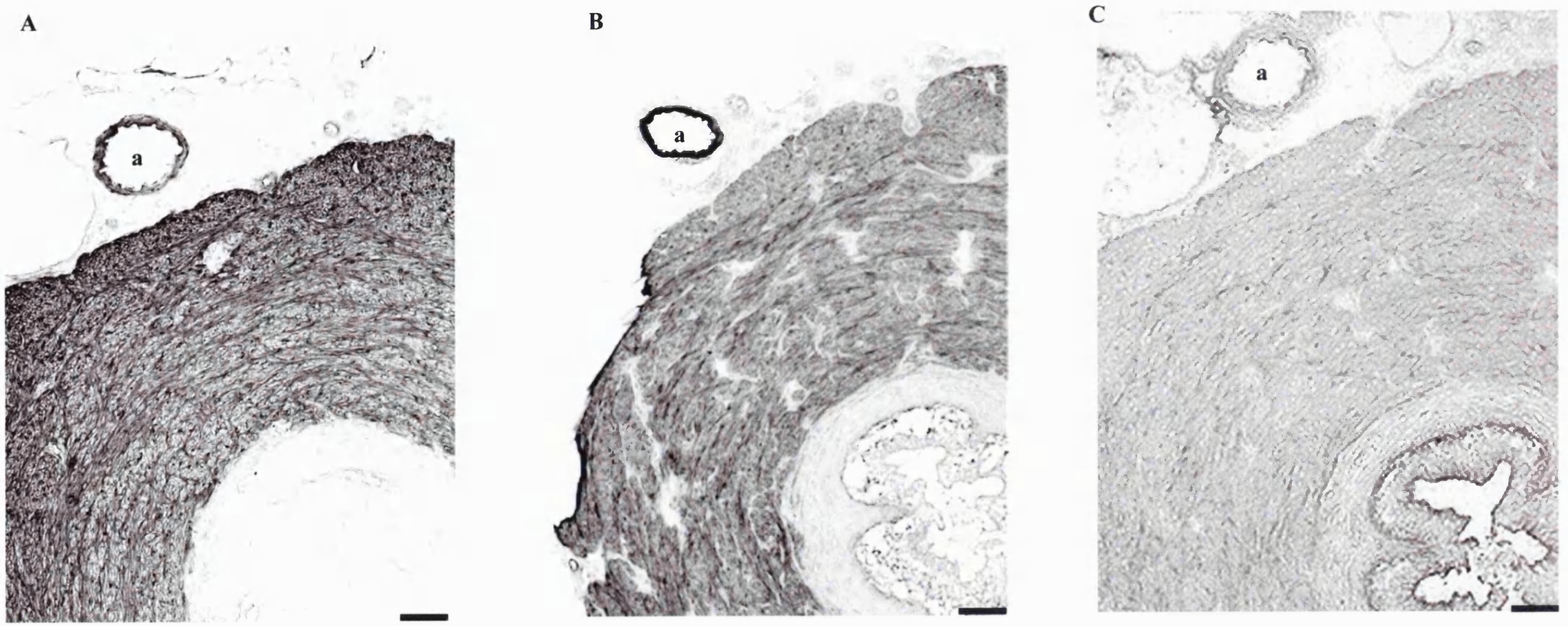


Figure A.3.1 P2X receptor staining in transverse sections of rat vas deferens. **A**. Positive immunoreactivity for P2X1 in the smooth muscle membranes of smooth muscle layers. P2X1 immunostaining also seen in the smooth muscle artery (a). **B**. Similar immunopositive staining seen for P2X2, although not as widespread. **C**. For comparison, no immunostaining detected for P2X6. Results similar to those obtained previously (Lee *et al.*, 2000). *Bars* 100um

APPENDIX 4

PUBLICATIONS ARISING FROM THIS THESIS

Abstracts

Brown, S.G., Wildman, S.S., King, B.F. & Burnstock, G. (1999). Diadenosine polyphosphates as pharmacological tools to identify $P2X_{1,2,3,4}$ subunits. *Brit. J. Pharmacol.,* 126, 24P.

Full Papers

Wildman, S.S., Brown, S.G., King, B.F. & Burnstock, G. (1999). Selectivity of diadenosine polyphosphates for rat P2X receptor subunits. *Eur. J. Pharmacol.,* 367, 119-123.

Jacobson, K.A., Hoffman, C., Kim, Y.C., Camaioni, E., Nandanan, E., Jang, S.Y., Guo, D.P., Ji, X.D., Kugelgen, I.V., Moro, S., Ziganshin, A., King, B.F., Brown, S.G., Wildman, S.S., Burnstock, G., Boyer, J.L. & Harden, T.K. (1999). Molecular modeling in P2 receptors: Ligand development aided by molecular modeling and mutagenesis. *Brain Res.* 120, 119-132.

Kim, Y.C., Brown, S.G., Harden, T.K., Boyer, J.L., Dubyak, G., King, B.F., Burnstock, G. & Jacobson, K.A. (2000) Structure activity relationships of pyridoxal phosphate derivatives as potent and selective antagonists of $P2X_1$ receptors. *J. Med. Chem.*, 44, 340-349.

Brown, S.G., King, B.F., Kim, Y.C., Jang, S.Y., Burnstock, G. & Jacobson, K.A. (2001). Activity of novel adenine nucleotide derivatives as agonists and antagonists at recombinant rat P2X receptors. *Drug Dev. Res.*, 49, 253-259.

Brown, S.G., Jacobson, K.A., Kim, C.Y., Burnstock, G. & King, B.F. (2001). Actions of a series of PPADS analogues at $P2X_1$ and $P2X_3$ receptors. *Drug Dev. Res.*, (In press).

Brown, S.G., Townsend-Nicholson, A., Jacobson, K.A., Burnstock, G. & King, B.F. (2001). Heteromultimeric $P2X_{1/2}$ receptors show a novel sensitivity to extracellular pH. *J. Pharmacol. Expt. Ther.*, (submitted).