Coexpression of recombinant P2X receptors and their relation to ATP-gated channels in sensory neurons

by

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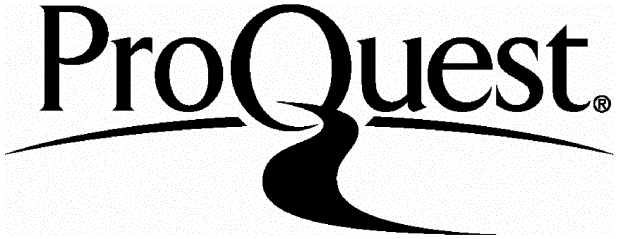

ABSTRACT

Recombinant P2X receptors expressed in *Xenopus* oocytes, which reflect the properties of ATP-gated channels in sensory neurons, were studied individually, or expressed together, or coexpressed with VR1 receptors. Coexpression of $P2X_2$ and $P2X_3$ subunits gave rise to three populations of receptors: homomeric $P2X_2$ and $P2X_3$ receptors, and heteromeric $P2X_{2/3}$ receptors, the proportions of which were changed by altering the ratios of $P2X_2$ and $P2X_3$ cRNA injected. The pharmacological characteristics of ATP responses evoked by this complement of P2X receptors bore a close similarity to the range of ATP responses evoked in sensory neurons. Agonist and antagonist profiles of $P2X_{2/3}$ receptors were closer to those of $P2X_3$ receptors yet showed the desensitization kinetics and sensitivity to extracellular pH and Zn^{2+} of $P2X_2$ receptors.

Activation of $P2X_3$ receptors neither enhanced nor diminished the activation of VR1 receptors, both of which occur on nociceptors. In contrast, the activation of VR1 receptors inhibited the activation of $P2X_3$ receptors, but only when ATP was applied during the peak of capsaicin-activated currents. The degree of one-way modulation was dependent on the degree of activation of VR1 receptors. Capsaicin responses, but not ATP responses, were altered to the same extent by extracellular pH as when these receptors were expressed alone. This one-way modulation of $P2X_3$ receptors by the activation of VR1 receptors may influence nociceptive transmission in sensory neurons.

Diinosine pentaphosphate (Ip_5I) exhibited selectivity for $P2X_1$ receptors over $P2X_3$ receptors, but otherwise failed to inhibit ATP responses at $P2X_2$, $P2X_4$ and $P2X_{2/3}$ receptors. The selective inhibition by Ip_5I for $P2X_3$ over $P2X_{2/3}$ receptors provided a useful pharmacological tool to study the composition of P2X subunits in sensory neurons.

This novel P2X antagonist might fill a long-term need for highly potent and selective antagonists to study P2X receptors and nociception in sensory neurons.

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Finally, I would like to dedicate this thesis to my parents and my son Yuan-Yuan, whom I love very much. I thank you for your continued support, encouragement and love.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 PREFACE

The sensory nervous system detects events in the external world by means of receptors sensitive to physical and chemical stimuli from the environment. A variety of noxious stimuli activate pain-sensing sensory neurons or nociceptors via specific ion channels. Two of the ion channels involved in nociception are the ATP-gated ion channel (P2X receptor) and the vanilloid receptor type 1 (VR1). P2X receptors are a family of ligand-gated cation channels with a distinctive structural motif. To date, seven P2X subtypes have been cloned and characterized at the molecular level (see reviews: King, 1998; North & Surprenant, 2000). ATP acts as a fast excitatory neurotransmitter at P2X receptors in many neuronal preparations (see reviews: Burnstock, 1996b; 1997). Electrophysiological and *in vivo* experiments suggest that P2X receptors may also play an important role in sensory signaling (see reviews: Burnstock, 2000; Bland-Ward & Humphrey, 2000; Hamilton & McMahon, 2000). Six ($P2X_{1-6}$) out of seven cloned P2X receptor transcripts are expressed in sensory neurons and immunoreactivity for $P2X_2$ and $P2X_3$ receptors is present in DRG neurons (Collo *et al.*, 1996; Vulchanova *et al.*, 1997). In this study, coexpression of $P2X_2$ and $P2X_3$ subunits in *Xenopus* oocytes has been investigated and a spectrum of agonist evoked responses has been compared with that seen in sensory neurons. The potential functional interaction between ATP-gated $P2X_3$ receptors and capsaicin-gated VR1 receptors has also been investigated with $P2X_3$ and VR1 receptors expressed together in *Xenopus* oocytes.

It has long been hoped that the study of heterologously expressed P2X receptors would lead to the development of selective and potent antagonists, which are

needed to investigate the physiological role of ATP and explore therapeutic potentials. The antagonist activity of diinosine polyphosphates (Ip_nI, where n = 3, 4 and 5) has been examined at homomeric $P2X_{1-4}$ and heteromeric $P2X_{2/3}$ receptors expressed in *Xenopus* oocytes. In an attempt to search for a model for native $P2X_2$ receptors, ATP-gated ion channels in adrenal chromaffin cells dissociated from adrenal medullae of the rat and guinea-pig have been investigated.

This thesis is organized as follows: Chapter 1 is a general introduction of purinergic transmission and molecular characterization of P2X receptors. The development of agonists and antagonists of P2X receptors has also been included in this chapter. This is followed by a description of general methods (Chapter 2) employed throughout this study. Experimental work in this thesis is divided into two sections. Section A is concerned with coexpression of $P2X_3$ and $P2X_2$ receptor subunits and coexpression of $P2X_3$ and VR1 receptors in *Xenopus* oocytes (Chapter 3 and 4). Section B focuses on the antagonist activity of diinosine polyphosphates at homomeric $P2X_{1-4}$ and heteromeric $P2X_{2/3}$ receptors (Chapter 5 and 6). Results regarding functional expression and pharmacological properties of P2X receptors in chromaffin cells dissociated from rat and guinea-pig adrenal glands are also included in Section B (Chapter 7). This is followed by a general discussion (Chapter 8) concerning the heterogeneity of P2X receptors in sensory neurons and modulation of P2X receptors by H^+ and Zn^{2+}. Several other ion channels involved in nociception and their potential functional interaction with P2X receptors are also considered in the general discussion.

1.2 OVERVIEW

Intracellular ATP is a ubiquitous carrier of chemical energy. ATP and other nucleotides can be released from different cell types through regulated pathways, or through the damaged plasma membrane. Extracellular ATP activates P2 receptors, which have been divided into two families: P2X and P2Y receptors according to their structures and transduction mechanisms (see reviews: Fredholm *et al.*, 1997; Ralevic & Burnstock, 1998). P2X receptors are ligand-gated ion channels possessing two transmembrane domains, whilst P2Y receptors are seven transmembrane domain G-protein-coupled nucleotide receptors. To date, seven P2X receptors ($P2X_{1-7}$) have been cloned from mammals and pharmacological characteristics of homomeric assemblies of $P2X_{1-7}$ receptors have been well documented (see reviews: Buell *et al.*, 1996a; Evans *et al.*, 1998; Humphrey *et al.*, 1998a; King, 1998; North & Surprenant, 2000). Thirteen P2Y-like receptors, ($P2Y_{1-12}$) plus tp2y, have been cloned so far and functional analysis of recombinant P2Y receptors has been reviewed (King *et al.*, 1998; Ralevic & Burnstock, 1998; Hollopeter *et al.*, 2001). Molecular cloning and functional studies of P2X and P2Y receptors in various cells and biological tissues have provided direct evidence for the physiological role of ATP as an extracellular messenger (see reviews: Burnstock, 1986; 1996a; 1996b; 1997; 1999; 2000). Of the many physiological functions of extracellular ATP, purinergic transmission in the nervous system has been extensively studied and is now well established.

1.3 PURINERGIC TRANSMISSION AND MODULATION

1.3.1 Early evidence for purinergic transmission

Classically, the autonomic nervous system (ANS) consists of two components: cholinergic and adrenergic nerves. The first hints for the existence of a third component, the non-adrenergic non-cholinergic (NANC) nerve, can be traced as far back as the end of the 19th century (Langley & Anderson, 1895; Bayliss & Starling, 1900). These studies demonstrated the presence of atropine-resistant responses in the urinary bladder and the large intestine. Drury and Szent-Györgyi (1929) documented the first biological effects of extracellular ATP, including bradycardia and vasodilatation. However, direct evidence supporting the view that nucleotides are capable of acting as neurotransmitters only started to accumulate in the last 30-40 years. For example, electrical stimulation of the guinea-pig taenia coli produced a large hyperpolarization, which was maintained in the presence of atropine (cholinergic receptor antagonist) and guanethidine (presynaptic adrenergic neuron blocker) (Burnstock *et al.*, 1963; 1964; Bennett *et al.*, 1964). These results suggested the presence of NANC nerves, which fundamentally challenged the concept that each nerve makes and releases only one nerve transmitter. Studies on the nature of the neurotransmitter released from inhibitory enteric nerves have led to the suggestion that ATP is the inhibitory neurotransmitter in the gut (Burnstock *et al.*, 1970; 1972). "Purinergic" receptors were first coined and purinergic transmission proposed by Burnstock (see review: Burnstock, 1972). Several years later, it was proposed that purinoceptors could be divided into P1 (adenosine) receptors, which are activated by adenosine, and

P2 receptors, which are activated by ATP or UTP (see reviews: Burnstock, 1978; 1980).

1.3.2 ATP storage and release

ATP is continuously produced in mitochondria and is present in millimolar concentrations in the cytoplasm of all cells. There are various reports showing that mechanical, chemical and electrical stimuli induce ATP release from vascular smooth muscle, endothelial cells, myocardial cells and bladder epithelial cells (Doyle & Forrester, 1985; Sedaa *et al.*, 1990; Bodin *et al.*, 1991; Ferguson *et al.*, 1997). There are three possible pathways for ATP release: exocytotic release from storage vesicles, carrier-mediated release from the cytoplasm and cytolytic release (see review: Sperlágh & Vizi, 1996). Cytolytic release of ATP is of significance only under pathological conditions.

As a neurotransmitter, the important question is whether ATP is stored in the nerve terminal and can be released during nerve depolarization. ATP was first found co-stored and co-released with catecholamines in the adrenal medulla (Blaschko *et al.*, 1956; Douglas *et al.*, 1966), a finding confirmed by other studies (Winkler & Westhead, 1980; Rojas *et al.*, 1985). The presence of ATP in cholinergic nerve terminals was first demonstrated in 1974 (Dowdall *et al.*, 1974). Subsequent studies extended this finding in sympathetic nerve terminals (Westfall *et al.*, 1978; White & MacDonald, 1990) and central synapses (Wieraszko *et al.*, 1989; Fiedler *et al.*, 1992; Sawynok *et al.*, 1993). There is now abundant evidence that ATP is present in all types of synaptic vesicles of nerve terminals and is released during nerve stimulation

(see review: Sperlágh & Vizi, 1996). Once released into the extracellular fluid, ATP is rapidly degraded by ectoenzymes, which remove phosphate groups from ATP and appear to be expressed in most tissues (see reviews: Zimmermann, 1996; Zimmermann *et al.*, 1998).

Several studies have shown that ATP is also stored and released from sensory nerve terminals. Holton and colleague (Holton & Holton, 1953; Holton, 1959) demonstrated for the first time the outflow of various purines from the peripheral terminals of sensory nerves and also suggested that ATP release might also occur at central sensory terminals. It has been shown that dorsal, but not ventral spinal cord synaptosomes release endogenous ATP when exposed to capsaicin (Sweeney *et al.*, 1989). These results suggest that ATP, released at peripheral and central terminals of sensory nerves, may play an important role in sensory signaling.

1.3.3 Purinergic transmission

It is well established that extracellular ATP mediates fast synaptic transmission in the nervous system (see reviews: Brake & Julius, 1996; Burnstock, 1996b). The importance of ATP as a neurotransmitter was first appreciated in neuro-effector junctions in the gut as described in Section **1.3.1**. Subsequently, ATP was identified as a neurotransmitter in sympathetic nerves innervating blood vessels (Cheung, 1982; Sneddon & Burnstock, 1984a; Suzuki, 1985; Bao & Stjärne, 1993) and vas deferens (Fedan *et al.*, 1982; Sneddon & Burnstock, 1984b; Sneddon & Westfall, 1984). In addition, ATP was also recognized as a neurotransmitter in parasympathetic nerves innervating the urinary bladder (Brown *et al.*, 1979; Kasakov & Burnstock, 1982;

Westfall *et al.*, 1983; Hoyle & Burnstock 1985). The purinergic transmission at these neuro-effector junctions was demonstrated by the selective blockade by P2 antagonists and desensitization following application of α,β-meATP. For example, an electrical stimulation of sympathetic nerve evoked a biphasic contraction in the vas deferens. The initial phase of the response was selectively blocked by P2X antagonists including arylazidoaminopropionyl-ATP ($ANAPP_3$) or desensitized by α,β-meATP, whilst the second phase of contraction was selectively blocked by α_1-adrenoceptor antagonists such as prazosin (Fedan *et al.*, 1982; Sneddon & Burnstock, 1984b; Sneddon & Westfall, 1984). Therefore, it was suggested that the first component of this sympathetic response was mediated by ATP, whilst the second component was mediated by noradrenaline.

In addition to its action at neuron-to-effector junctions, ATP mediates fast excitatory potentials at neuron-to-neuron synapses (see reviews: Brake & Julius, 1996; Burnstock, 1996b). In the peripheral nervous system (PNS), a role for ATP in synaptic transmission has been demonstrated using rat coeliac ganglion neurons which form a network in culture (Evans *et al.*, 1992). ATP evoked excitatory synaptic potentials and currents in cultured coeliac ganglion neurons, which were blocked by P2 receptor antagonist suramin and desensitized by α,β-meATP. The first evidence of purinergic transmission in the CNS came from recordings of fast excitatory postsynaptic potentials in the medial habenula (Edwards *et al.*, 1992). A cocktail of drugs that antagonize the actions of all other known fast excitatory and inhibitory transmitters failed to completely block fast synaptic potentials evoked by electrical stimulation. The residual fraction of fast excitatory potentials was strongly inhibited

by P2 antagonists including suramin. This finding suggests a fast transmitter function of extracellular ATP in the CNS, a view supported by several other studies in neuro-neuronal junctions in the brain. For example, glutamate-evoked release of ATP induced an increase in the intracellular Ca^{2+} concentration in hippocampal neurons, which could be blocked by a P2 antagonist (Inoue *et al.*, 1995). A purinergic component in the excitatory synaptic transmission was also found in locus coeruleus and hippocampal synapses (Nieber *et al.*, 1997; Pankratov *et al.*, 1998). However, the study of ATP and P2X receptors in the CNS is still in its infancy.

It is now clear that cotransmission is the general rule rather than the exception in the nervous system. For example, ATP functions as a cotransmitter with noradrenaline in sympathetic nerves innervating the vas deferens and blood vessels (Allcorn *et al.*, 1986; Saville & Burnstock, 1988), whilst ATP acts with acetylcholine in parasympathetic nervous system innervating the urinary bladder (Kasakov & Burnstock, 1982; Hoyle & Burnstock, 1985). In addition to acetylcholine, ATP mediates synaptic transmission in myenteric neurons of the guinea-pig ileum (Zhou & Galligan, 1996). However, the relative contribution of each transmitter varies enormously in the ANS. The contribution made by ATP and noradrenaline in sympathetic vasoconstriction varies considerably from one artery to another (see reviews: Burnstock, 1988; 1990). For example, ATP was the principal transmitter acting postjunctionally in the rabbit jejunal artery, whilst it made little contribution to sympathetic vasoconstriction in the rabbit ear artery (Goncalves & Guimaraes, 1991). This variable importance of purinergic transmission may reflect differences in ratios of neurotransmitters in storage vesicles (see review: Sperlágh & Vizi, 1996), expression

levels and properties of postsynaptic transmitter-gated receptors (Lewis & Evans, 2000).

1.3.4 Presynaptic modulation by P2X receptors

Following the role of P2X receptors in fast postsynaptic transmission, evidence has been presented that P2X receptors may also play a role in presynaptic modulation of neurotransmitter release (see review: Khakh & Henderson, 2000). The modulation of transmitter release by P2X receptors first came from studies using peripheral neurons and neuron-muscle co-cultures (Fu & Poo, 1991; Sun & Stanley, 1996). ATP is co-stored and co-released with acetylcholine at cholinergic synaptic vesicles, though the function of ATP remains largely unclear (Dowdall *et al.*, 1974). In neuron-muscle co-cultures, ATP stimulated acetylcholine release was shown by a marked increase in the frequency of spontaneous synaptic currents at the neuromuscular synapse (Fu & Poo, 1991). Direct evidence for P2X receptors expressed on presynaptic nerve terminals came from the study of chicken ciliary ganglion synaptosomes (Sun & Stanley, 1996). Most recently, evidence suggests that a P2X receptor at presynaptic sites directly mediates sympathetic transmitter release in superior cervical ganglion neurons (Boehm, 1999).

Electrophysiological studies have also demonstrated a modulatory effect of ATP on sensory synaptic transmission at the spinal cord level (Li & Perl, 1995; Gu & MacDermott, 1997; Li *et al.,* 1998). ATP enhanced the excitatory action of glutamate in sensory synaptic transmission in dorsal horn of the spinal cord (Li & Perl, 1995; Li *et al.,* 1998). In DRG and dorsal horn neuron co-cultures, ATP increased the

frequency of spontaneous glutamate release, suggesting that ATP through P2X receptors enhanced sensory signal output between primary afferents and dorsal horn neurons (Gu & MacDermott, 1997). A similar modulatory action of ATP has also been observed in brainstem neurons (Khakh & Henderson, 1998). Both exogenous ATP and endogenous ATP released by electrical stimulation enhanced the frequency of spontaneous fast excitatory postsynaptic currents in trigeminal mesencephalic motor nucleus neurons. It has been suggested that ATP, via presynaptic P2X receptors, enhances glutamate release from the central terminals of trigeminal mesencephalic nucleus neurons. These functional results are compatible with those of immunohistochemical studies (Vulchanova *et al.*, 1997; Bradbury *et al.*, 1998; Lê *et al.*, 1998b). $P2X_{1-4}$ receptor proteins have been found in sensory nerve terminals projecting to the spinal cord and $P2X_4$-like proteins concentrated at afferent synaptic junctions in the brain.

Evidence suggests ATP also inhibits the release of glutamate and noradrenaline from hippocampal neurons, possibly via P2X receptors at presynaptic terminals (Koizumi & Inoue, 1997). ATP seems to have opposing roles on neurotransmitter release via the activation of P2X at presynaptic sites. However, mechanisms other than P2X receptor activation may also contribute to the stimulatory and inhibitory effects of ATP on neurotransmitter release. For example, ATP and ATPγS, a hydrolysis-resistant analogue of ATP, decreased the electrical evoked overflow of noradrenaline in the mouse and rat vas deferens and rat atria, whilst β,γ-meATP had no effect on the neurotransmitter release in rat atria (von Kügelgen *et al.*, 1994; 1995). These findings suggest that endogenously released ATP might activate

presynaptic P2Y receptors to mediate a negative feedback modulation at sympathoeffector junctions. Nevertheless, the functional significance of presynaptic P2X and P2Y receptors in the CNS and PNS is far from clear.

1.3.5 Pharmacological classification of P2 receptors

Following the original classification of purinergic receptors proposed by Burnstock in 1978, the classification of P2 purinoceptor has been continuously updated and modified (Burnstock, 1980; Burnstock & Kennedy, 1985; Abbracchio & Burnstock, 1994; Fredholm *et al.*, 1994). Biochemical, pharmacological and receptor-binding assays have demonstrated the existence of multiple P2 purinoceptors and led to the proposed subclassification of P2 purinoceptors. Five functional subtypes have been defined according to their distinct pharmacological profiles. P_{2X} purinoceptors mediate rapid excitatory responses via intrinsic cation channels with an agonist potency order of α,β-meATP>ATP=2-MeSATP. In contrast, P_{2Y} (2-MeSATP>>ATP≈ADP>α,β-meATP), P_{2U} (ATP≈UTP>>2-MeSATP) and P_{2T} purinoceptors (2-MeSADP>ADP, ATP as an antagonist) are G-protein-coupled receptors, involving the activation of phospholipase C and mobilization of internal Ca^{2+}. P_{2Z} purinoceptors are characterized by channel-to-pore transformation by ATP^{4-} and are permeable to large hydrophilic solutes.

As both purines and pyrimidines can act at some purinoceptor subtypes, the term P_2 purinoceptor has been replaced by P2 receptor. Following the cloning and molecular characterization of P2X and P2Y receptors, the above provisional sub-classification has now been superseded and P2 classification is now based on results of

molecular biology studies (See reviews: Fredholm *et al.*, 1997; King, 1998; Ralevic & Burnstock, 1998). The current classification divides P2 receptors into two classes: P2X receptors, which form intrinsic ionic channels, and P2Y receptors, which are coupled to G-proteins. This classification results in a simplification of P2 receptor nomenclature. P2X receptors are subdivided into seven subtypes, termed $P2X_1$ to $P2X_7$ (see reviews: Buell *et al.*, 1996a; Evans *et al.*, 1998; Humphrey *et al.*, 1998a; King, 1998; Ralevic & Burnstock, 1998). The P2Y receptors are subdivided into at least eleven subtypes, designated $P2Y_1$ to $P2Y_{11}$ (see review: King *et al.*, 1998; Ralevic & Burnstock, 1998).

1.4 CLONING AND MOLECULAR CHARACTERIZATION OF P2X RECEPTORS

1.4.1 General remarks

The first two P2X receptors were cloned and characterized in 1994 (Brake *et al.*, 1994; Valera *et al.*, 1994), and the P2X family rapidly expanded to seven members in the next few years. $P2X_{1-6}$ subtypes share approximately 40% sequence identity distributed fairly evenly over their length, which ranges from 379-472 residues (Brake *et al.*, 1994; Valera *et al.*, 1994; Chen *et al.*, 1995; Bo *et al.*, 1995; Buell *et al.*, 1996b; Collo *et al.*, 1996). The $P2X_7$ receptor, a cytolytic receptor for extracellular ATP, was first cloned from the rat brain (Surprenant *et al.*, 1996). The isolated $P2X_7$ cDNA encodes a 595 amino acid protein. The first 395 amino acids are 35-40% identical to those of the other six P2X receptors. However, the C-terminal domain of the $P2X_7$

receptor is much longer than that of the other P2X subtypes, and shows no sequence similarity with known membrane receptor proteins.

1.4.2 Molecular characterization of P2X receptors

Molecular cloning of ATP receptors has led to the discovery of a family of P2X receptors that has a novel structure for ligand-gated ion channels (see reviews: Brake & Julius, 1996; King, 1998; North & Surprenant, 2000). It has been suggested that both N- and C- termini of $P2X_1$ and $P2X_2$ receptors are located inside the cell with a large extracellular loop (~270 amino acids) containing 10 conserved cysteine residues and consensus sites for N-glycosylation (Brake *et al.*, 1994; Valera *et al.*, 1994). Indeed, all seven ATP-gated channels appear to have only two hydrophobic regions (~20 amino acids each) sufficiently long to span the plasma membrane. These findings define a novel structural motif for ligand-gated ion channels, which is markedly different from that of the other two major ligand-gated channel families: nicotinic acetylcholine and glutamate superfamilies (North, 1996). Each receptor subunit of the nicotinic acetylcholine family possesses a large extracellular ligand-binding domain followed by four transmembrane segments (see review: Lester, 1992), whilst the glutamate receptor subunit is characterized by three transmembrane domains plus a hydrophobic loop that partially traverses the membrane (see review: Gasic & Heinemann, 1991). Interestingly, the proposed topology of the P2X receptor resembles that of amiloride-sensitive sodium channels, with which no similarity of amino acid sequence has been found (North, 1996).

There are several experimental results that are compatible with the predicted topology of the P2X receptor. Firstly, desensitization of the $P2X_1$ receptor can be removed by replacing either of the two hydrophobic segments from the appropriate segment of the $P2X_2$ receptor (Werner *et al.*, 1996). Desensitization can also be introduced into the $P2X_2$ receptor by inserting both proposed transmembrane domains from the $P2X_1$ receptor. These findings also indicate that the hydrophobic domains of the $P2X_1$ receptor are involved in desensitization and possibly contribute to the pore-forming molecule. Secondly, the rat $P2X_4$ receptor is resistant to P2 antagonists including pyridoxal-5'-phosphate-6'-azophenyl-2',4'-disulfonic acid (PPADS) and pyridoxal-5'-phosphate (P5P). Replacing one amino acid within the putative extracellular loop can restore sensitivity to these antagonists (Buell *et al.*, 1996b). Thirdly, the human $P2X_4$ receptor displays a higher sensitivity to P2 antagonists including suramin and PPADS. Sensitivity to suramin and PPADS can be transferred by exchanging a segment within the first half of the proposed extracellular loop between human and rat isoforms (Garcia-Guzman *et al.*, 1997).

The proposed membrane topology of the P2X receptor has been confirmed using N-glycosylation site tagging techniques, which has been successfully used to determine the topology of other membrane proteins (Newbolt *et al.*, 1998; Torres *et al.*, 1998a). The endogenous N-glycosylation sites of the $P2X_2$ receptor were identified and a mutant lacking all three N-glycosylation sites was developed and used as a background construct to introduce artificial glycosylation sites. Subsequent N-glycosylation could only occur at the engineered sites. When engineered sites were inserted at positions within the proposed N- or C-terminal region, no glycosylation

was observed. These results indicate that both N- and C- termini are not accessible to glycosylating enzymes and therefore are located intracellularly (Newbolt *et al.*, 1998; Torres *et al.*, 1998a).

Recently, several studies have attempted to identify the residues contributing to the ligand-binding site or the pore formation (Werner *et al.*, 1996; Rassendren *et al.*, 1997a; Egan *et al.*, 1998). Substituted cysteine accessibility method has been successfully used in identified ion-conducting pore domains of other channel proteins including the acetylcholine receptor (Akabas *et al.*, 1992). Amino acids throughout the second hydrophobic domain of the $P2X_2$ receptor were mutated individually to cysteine and the accessibility of the side-chain in the mutants was investigated with modifying reagents including (2-aminoethyl) methanethiosulfonates (MTSEA) and ionic Ag^+ (Rassendren *et al.*, 1997a; Egan *et al.*, 1998). The guiding principles are that the secondary structure of functional mutants is not altered by the mutation and that a blockade of inward current by MTSEA or Ag^+ indicates that the residue in question is accessible and modified. Data from these experiments suggest that side-chains of certain residues in the second hydrophobic segment of the $P2X_2$ receptor contribute to the ionic pore.

It remains unclear whether one or more mutated P2X subunits is required to incorporate into a channel to achieve complete blockade by MTSET. Based upon the results in substituted cysteine accessibility experiments (Rassendren *et al.*, 1997a; Egan *et al.*, 1998), Stoop and colleagues have attempted to solve the stoichiometry of the P2X receptor. The contribution of the individual subunit to the channel formation was investigated by coexpression of wild-type and mutant $P2X_2$ subunits. It has been

shown that the inhibition by MTSET depends on the number of mutant subunits in the channel. Electrophysiological results on concatenated P2X subunits have revealed that introduction of an extra mutant subunit in a concatenated construct leads to a progressive increase in channel block in the dimeric and trimeric constructs, but not in the tetrameric and hexameric constructs. These data indicate that neither four nor six subunits are the structural basis for the $P2X_2$ receptor, but that three subunits may represent the structural basis to form the $P2X_2$ receptor (Stoop *et al.*, 1999). These results are in general agreement with the findings that $P2X_1$ and $P2X_3$ receptors migrate as trimers both in analysis by gel electrophoresis and after chemical cross-linking (Nicke *et al.*, 1998). Interestingly, the trimeric subunit organization has not been observed for any other ion channels. So far, our understanding of the structure and function of P2X receptors is still very limited.

1.4.3 Pharmacological properties of recombinant P2X receptors

Heterologous expression of P2X receptors allows us to determine the likely subunit composition of the native P2X receptors by comparing the functional activities of expressed receptors with those of native cells. Each of the identified subunit proteins ($P2X_{1-7}$) has been reported to form homomeric receptors when expressed heterologously, though it still remains controversial whether the $P2X_6$ subunit is capable of forming homomeric assemblies (see reviews: Buell *et al.*, 1996a; King, 1998; North & Surprenant, 2000). The $P2X_{1-7}$ receptors are non-selective cation channels with a reversal potential of about 0 mV. They can be broadly subdivided into three groups. Group 1 is almost equally well activated by ATP and α,β-meATP

and includes $P2X_1$ and $P2X_3$ subtypes. Group 2, which is activated by ATP but not α,β-meATP, consists of $P2X_2$, $P2X_4$ and $P2X_5$ subtypes. The only receptor in the Group 3, $P2X_7$ (P2Z) receptor, is distinguished by its unique ion permeability (Surprenant *et al.*, 1996). The conductance of the $P2X_7$ receptor increases by repeated agonist applications in an extracellular solution with a low concentration of divalent cations. It appears that the concentration of extracellular ions have a significant effect on agonist responses at the $P2X_7$ receptor (Surprenant *et al.*, 1996; Rassendren *et al.*, 1997b). Another defining feature is that the $P2X_7$ receptor is more sensitive to 2', 3'-*O*-(4-benzoylbenzoyl) ATP (BzATP) than ATP. However, BzATP is not a selective agonist at $P2X_7$ receptors and also activates other P2X receptor subunits.

Of the seven P2X receptor subtypes, $P2X_5$ and $P2X_6$ have not been systematically tested due to poor expression in heterologous expression systems. Under similar experimental conditions, the amplitude of ATP-activated currents in cells expressing homomeric $P2X_5$ receptors are approximately 100-fold smaller than that observed at recombinant $P2X_{1-4}$ receptors (Collo *et al.*, 1996). $P2X_6$ proteins were found to form functional homomeric receptors when expressed in HEK293 cells (Collo *et al.*, 1996), but not in *Xenopus* oocytes (Soto *et al.*, 1996b; Lê *et al.*, 1998a; King *et al.*, 2000). The lack of assembly detected by co-immunoprecipitation assay also supports the view that the $P2X_6$ subunit is unable to form functional homomeric receptors (Torres *et al.*, 1999).

1.4.4 Heteromeric assemblies of P2X receptors

Almost all known ligand-gated ion channels exist as hetero-oligomeric complexes. For example, the muscle type of nicotinic receptor possesses five separate polypeptide chains in one complex, symbolized $\alpha_2\beta\gamma\delta$. The neuronal nicotinic receptor has subunits that are different from those of muscle type, but again is a pentameric assembly. It has been suggested that heteromeric subunit assembly of ligand-gated channels is of physiological importance in generating functional diversity of responses to naturally occurring agonists. Recently, it has been suggested that all P2X subunits with the exception of $P2X_7$ subunit are capable of forming heteromeric assemblies (Torres *et al.*, 1999). The $P2X_7$ subunit is not able to co-assemble with other subunits to form stable complexes, except with itself. In contrast, the $P2X_6$ subunit can form hetero-oligomeric assemblies with many other subunits, but it is unable to form homomeric complexes.

There is both functional and biochemical evidence for heteromeric assemblies of P2X receptors. Firstly, pharmacological studies have suggested the occurrence of polymeric P2X receptors in sensory neurons (Bean, 1990; Bean *et al.*, 1990; Evans *et al.*, 1995; Khakh *et al.*, 1995; Surprenant *et al.*, 1995). The activation of native P2X receptors in sensory neurons has a Hill coefficient greater than one. Secondly, a P2X receptor with a novel operational profile, pharmacology similar to that of the $P2X_3$ receptor and time course similar to that of the $P2X_2$ receptor, is formed in oocytes expressing $P2X_2$ and $P2X_3$ receptor subunits (Lewis *et al.*, 1995). The novel pharmacology of evoked currents in co-injected oocytes has led to the suggestion of formation of heteromeric $P2X_{2/3}$ receptors, which could account for ATP-activated

currents observed in nodose ganglion neurons. Thirdly, immunohistochemical and *in situ* hybridization studies suggest a close relationship between the distribution of $P2X_2$ and $P2X_3$ receptors in sensory neurons (Collo *et al.*, 1996; Vulchanova *et al.*, 1997; Xiang *et al.*, 1998).

Following the $P2X_{2/3}$ receptor, three other heteromers including $P2X_{1/5}$, $P2X_{4/6}$ and $P2X_{2/6}$ receptors have so far been characterized. α,β-meATP evokes an inward current that completely desensitizes within 1 sec at the $P2X_1$ receptor (Buell *et al.*, 1996c), whereas α,β-ATP is inactive at the $P2X_5$ receptor (Collo *et al.*, 1996). Transcripts of $P2X_1$ and $P2X_5$ receptors have an overlapping distribution in subsets of sensory neurons and spinal motoneurons (Collo *et al.*, 1996). A novel P2X phenotype showing the pharmacology of $P2X_1$ and the kinetics of $P2X_5$ receptor has been demonstrated by coexpression of $P2X_1$ and $P2X_5$ receptor subunits (Torres *et al.*, 1998b; Haines *et al.*, 1999; Lê *et al.*, 1999). This heteromeric assembly has been confirmed by co-immunoprecipitation study (Torres *et al.*, 1998b). Although the $P2X_6$ subunit functions poorly as homomeric assemblies, it has been suggested that the $P2X_6$ subunit can contribute to form functional heteromeric assembles (Lê *et al.*, 1998a; Torres *et al.*, 1999). It has been shown that the distribution of $P2X_4$ and $P2X_6$ mRNAs has a significant overlap in the CNS (Séguéla *et al.*, 1996; Soto *et al.*, 1996a; 1996b). Coexpression of $P2X_4$ and $P2X_6$ subunits gives rise to the formation of a novel pharmacological phenotype of P2X receptors. The putative $P2X_{4/6}$ receptor is activated by low micromolar concentrations of α,β-meATP and blocked by suramin and Reactive blue 2, which are inactive at the homomeric $P2X_4$ receptor (Lê *et al.*, 1998a).

Recently, King and colleagues (2000) reported a heteromeric P2X receptor formed by coexpression of $P2X_2$ and $P2X_6$ receptor subunits. Both $P2X_2$ and $P2X_6$ mRNA and proteins are widely distributed through the CNS (Collo *et al.*, 1996; Kanjhan *et al.*, 1999). The $P2X_{2/6}$ receptor shows some of the characteristics of the $P2X_2$ receptor. However, a closer inspection of pH sensitivity and suramin blockade has revealed a significant difference between $P2X_{2/6}$ and $P2X_2$ receptors. It has been suggested that the $P2X_{2/6}$ receptor may act as a functionally modified $P2X_2$ receptor (King *et al.*, 2000).

1.4.5 Splicing variants

Differences in biophysical and pharmacological properties of P2X receptors suggest the presence of heteromeric assemblies between different P2X subunits and alternatively spliced variants of P2X subunits. So far, at least three P2X receptor subunits, $P2X_2$, $P2X_4$ and $P2X_5$ have been shown to undergo alternative splicing detected by receptor specific reverse transcription-polymerase chain reaction (RT-PCR) (Housley *et al.*, 1995; Brändle *et al.*, 1997; Lê *et al.*, 1997; Dhulipala *et al.*, 1998; Townsend-Nicholson *et al.*, 1999). The first splice variant of the $P2X_2$ receptor was isolated in the rat pituitary and stria vascularis of the cochlea, which included an 85-bp insertion, possibly forming a novel C-terminal end of the second transmembrane domain and part of the cytoplasmic domain (Housley *et al.*, 1995). Subsequently, several truncated forms of the $P2X_2$ receptor have been identified in different tissues (Brändle *et al.*, 1997; Simon *et al.*, 1997; Parker *et al.*, 1998). Heterologous expression of $P2X_{2\text{-}1}$ ($P2X_{2a}$), $P2X_{2\text{-}2}$ ($P2X_{2b}$), and $P2X_{2\text{-}3}$ variants has

generated functional homomeric receptors with different characteristics. Functional comparison of $P2X_{2\text{-}1}$, $P2X_{2\text{-}2}$ and $P2X_{2\text{-}3}$ variants with the wild-type $P2X_2$ receptor has revealed that $P2X_{2\text{-}1}$ and $P2X_{2\text{-}2}$ receptors exhibit marked rapid desensitizing kinetics (Brändle *et al.*, 1997; Chen *et al.*, 2000), whilst $P2X_{2\text{-}3}$ variant shows slow desensitizing kinetics and low efficacy for P2X agonists (Chen *et al.*, 2000). However, $P2X_{4a}$, a splicing variant of the $P2X_4$ receptor isolated from the mouse brain cDNA library is weakly activated by the maximal concentration of ATP and is insensitive to other nucleotides (Townsend-Nicholson *et al.*, 1999). A truncated human $P2X_5$ receptor, missing sequence corresponding to exon 10 of the known P2X genes is also unable to form functional homomeric channels (Lê *et al.*, 1997). One striking difference between rat and human $P2X_5$ receptors is the high expression of human $P2X_5$ transcripts in the brain and immune system, but the distribution of rat $P2X_5$ mRNA is highly restricted (Collo *et al.*, 1996; Lê *et al.*, 1997).

It has been speculated that production of the truncated form of the $P2X_2$ receptor may be due to alternative splicing of nuclear transcripts rather than a unique gene (Housley *et al.*, 1995). Analysis of splice donor and acceptor sequence of the $P2X_2$ receptor gene, consisting of eleven exons and ten introns, strongly supports the existence of multiple splice variants (Brändle *et al.*, 1997). It is now known that $P2X_{1\text{-}7}$ receptor subunit are encoded by different genes. As a single gene is able to give rise to more than one mRNA sequence, the distinct properties of these splice variants may contribute to the differences in the responses to ATP observed in native P2X receptors in variable tissues.

1.4.6 Tissue distribution

The tissue distribution of P2X receptors has been well documented in the nervous system. Six out of seven cloned P2X receptor mRNAs are expressed in sensory neurons (Collo *et al.*, 1996). Immunoreactivity for $P2X_{1-6}$ receptor subunits has been found in varying amounts in DRGs and trigeminal ganglia (Xiang *et al.*, 1998). However, the $P2X_3$ receptor is almost exclusively expressed by small-sized sensory neurons, most of which are nociceptors (Chen *et al.*, 1995). In total, approximately 25-40% of neurons in rat DRG express a high level of $P2X_3$ transcripts shown by *in situ* hybridization. This finding is comparable to that detected by immunohistochemical studies. The latter have shown that $P2X_3$ receptor-like proteins are localized in 35-40% of DRG neurons (Vulchanova *et al.*, 1997; Bradbury *et al.*, 1998). These $P2X_3$-positive neurons are small in diameter and predominantly nonpeptidegeric nociceptors. In addition, $P2X_3$ receptors are approximately equally expressed in afferents projecting to the skin and viscera (Bradbury *et al.*, 1998). In the spinal cord, the $P2X_3$ receptor staining is restricted to a thin band of axon terminals projecting to the inner layer of lamina II of the dorsal horn. This staining can be abolished following dorsal rhizotomy, confirming that $P2X_3$-like proteins are not expressed by intrinsic spinal neurons. It has been suggested that $P2X_3$-like proteins are transported from sensory neuron bodies to their central terminals in the spinal dorsal horn (Bradbury *et al.*, 1998).

All of the seven cloned P2X transcripts are found in the CNS, though the distribution of $P2X_1$, $P2X_3$, $P2X_5$ and $P2X_7$ receptor subtypes is highly restricted (Collo *et al.*, 1996). The strongest labeling for $P2X_1$ mRNA was detected in the

cerebellum and the less intense labeling observed in the striatum, hippocampus, dentate gyrus and cortex of the neonatal rat whole brain (Kidd *et al.*, 1995). $P2X_1$ immunoreactivity was localized in subpopulations of synapses between granule cells and Purkinje cells in the rat cerebellum (Loesch & Burnstock, 1998). In contrast, Vulchanova and colleagues (1996) failed to detect $P2X_1$-like proteins in these restricted regions in the adult rat brain. Early data showed that $P2X_3$ mRNA was undetectable in RNA isolated from the adult rat brain (Chen *et al.*, 1995). Compatible with this finding, $P2X_3$-like immunoreactivity was not found in the adult rat brain, though it was detected in discrete regions of the embryonic and neonatal rat brain (Kidd *et al.*, 1998). There is no evidence for $P2X_5$ transcripts in the CNS, with the exception of trigeminal mesencephalic nucleus and motor nuclei of the ventral spinal cord (Collo *et al.*, 1996). $P2X_7$ mRNA was found strongly expressed in microglial cells, but not neurons in the brain and spinal cord (Surprenant *et al.*, 1996). The $P2X_7$ receptor has been suggested to be involved in the neurotoxic process as a result of excessive calcium influx through nonselective pore.

In contrast, $P2X_2$, $P2X_4$ and $P2X_6$ receptors are widely distributed through the CNS. The $P2X_2$ receptor immunoreactivity was found particularly dense in: olfactory bulb, cerebral cortex, caudate putamen, amygdala, hippocampus, thalamus, hypothalamus, cerebellar cortex and midbrain and medullary nuclei (Kanjhan *et al.*, 1999). These findings are in broad agreement with earlier studies using *in situ* hybridization and Northern blot techniques (Brake *et al.*, 1994; Kidd *et al.*, 1995). Transcripts of $P2X_4$ and $P2X_6$ receptors are expressed abundantly in the CNS (Bo *et al.*, 195; Buell *et al.*, 1996b; Collo *et al.*, 1996; Séguéla *et al.*, 1996; Soto *et al.*, 1996a;

1996b). *In situ* hybridization analysis of rat brain sections showed that the expression pattern of $P2X_6$ mRNA was virtually identical to that of $P2X_4$ mRNA (Collo *et al.*, 1996; Soto *et al.*, 1996b). $P2X_4$-like proteins were observed in widespread areas including the cerebral cortex, hippocampus, thalamus and brainstem, with the strongest staining in the olfactory bulb, lateral septum, cerebellum and spinal cord (Lê *et al.*, 1998b).

The spinal cord expresses all mRNAs except that of the $P2X_3$ subtype, whilst only $P2X_4$ and $P2X_6$ transcripts are found throughout the spinal cord, with stronger signals in laminae II, VII and IX (Collo *et al.*, 1996). In agreement with the studies using *in situ* hybridization techniques, no cell somata were labeled by $P2X_3$ antibodies in the spinal cord, indicating intrinsic spinal neurons do not express $P2X_3$ receptors (Vulchanova *et al.*, 1997; Bradbury *et al.*, 1998). Immunoreactivity of $P2X_{1-4}$ receptors was also found in primary nerve fibers and axon terminals in dorsal horn of the spinal cord (Vulchanova *et al.*, 1996; Bradbury *et al.*, 1998; Lê *et al.*, 1998b). This dense staining for P2X receptor proteins at the level of afferent synaptic junctions suggests an important presynaptic role of P2X receptors in central synapses between primary afferent terminals and second order sensory neurons in the spinal cord. The functional importance of these presynaptic P2X receptors has been discussed in the section **1.3.4**.

1.4.6.1 Differential expression of $P2X_3$ receptors in DRG neurons

Nagy and Hunt (1982) first showed that small sensory neurons could be divided into two major neurochemical subtypes. One group contains neuropeptides such as

calcitonin-gene related neuropeptide (CGRP), somatostatin and substance P (SP), while the other group lacks peptides but contains the fluoride-resistant acid phosphatase activity (FRAP). SP- somatostatin- and CGRP-containing neurons can be identified immunohistochemically, whereas labeling FRAP requires enzyme histochemistry. However, double labeling peptide- and FRAP-containing neurons in single tissue sections has proved to be impossible because these two histochemical approaches are incompatible (Vulchanova *et al.*, 1998). Nevertheless, nearly all FRAP-containing cells bind *Griffonia Simplicifolia* isolectin B4 (IB4). Thus, small dark DRG neurons can be broadly subdivided into neuropeptide-containing neurons and IB4-positive neurons because these two groups show a very limited overlap. These two populations terminate in distinct but overlapping regions of the superficial dorsal horn of the spinal cord. Neuropeptide-containing neurons project to lamina I and the outer part of lamina II, whilst IB4-positive neurons terminate in the inner part of lamina II (Hunt *et al.*, 1992a).

Neuropeptide-containing neurons have been traditionally regarded as neurons associated with nociceptive transmission (Levine *et al.*, 1993). So far, little is known about the function of IB4-positive neurons, the transmitters that they utilize and receptors they express. Nevertheless, there are several lines of evidence supporting the view that these neurons are likely to be nociceptors. Firstly, a tetrodotoxin (TTX)-resistant sodium channel is preferentially expressed in IB4-positive small DRG neurons (Fjell *et al.*, 2000). There is strong evidence showing that TTX-resistant sodium channels contribute to injury-induced increase in nociceptor excitability (see review: McCleskey & Gold, 1999). Secondly, VR1 proteins have been found to be

expressed by 60-80% of IB4-positive DRG neurons (Tominaga *et al.*, 1998). VR1-positive neurons are mainly nociceptors, which are activated by noxious heat and modulated by protons (Caterina *et al.*, 1997; Tominaga *et al.*, 1998). Thirdly, a striking feature of IB4-positive neurons is that many of them selectively express $P2X_3$ receptors (Bradbury *et al.*, 1998; Vulchanova *et al.*, 1998), which has also been implicated in nociceptive transmission (Chen *et al.*, 1995; Burnstock, 1996a; Cook *et al.*, 1997).

1.4.6.2 Colocalization of $P2X_3$ with neurofilament, neuropeptides and IB4

It is well documented that six out of seven P2X receptor subunits ($P2X_{1-6}$) have been found in neurons of DRGs, trigeminal and nodose ganglia (Chen *et al.*, 1995; Collo *et al.*, 1996; Cook *et al.*, 1997). Of these subunits, the $P2X_3$ receptor subunit is almost exclusively localized in sensory neurons (Chen *et al.*, 1995). Combined labeling with IB4 or antisera against neurofilament or neuropeptides allows simultaneous identification of $P2X_3$ receptor immunoreactivity with other sensory neuron markers (Bradbury *et al.*, 1998; Vulchanova *et al.*, 1998). There are several anti-neurofilament antibodies including NF200, which label the classically defined large light sensory neurons. Double labeling studies have showed the very little overlap between $P2X_3$ receptor immunoreactivity and NF200 immunoreactivity. Of the NF200-positive profiles, less than 1% stained for $P2X_3$ receptor (Bradbury *et al.*, 1998). Analysis of double-labeled images has also shown that 3% of the $P2X_3$ receptor-positive neurons were colocalized with substance P immunoreactivity and 7% of them with somatostatin immunoreactivity. In contrast, almost all $P2X_3$ receptor-positive profiles

were also labeled by IB4 (Vulchanova *et al.*, 1998). These results indicate that $P2X_3$ receptor proteins are expressed in neurofilament-negative, non-peptidergic, IB4-positive small DRG neurons.

$P2X_3$ receptors have been identified in nerve fibers supplying a variety of tissues including the tooth pulp, corneal epithelium, tongue, skin and urinary bladder (Cook *et al.*, 1997; Vulchanova *et al.*, 1997; Bo *et al.*, 1999; Cockayne *et al.*, 2000). Central terminals containing $P2X_3$ immunoreactivity are restricted to a narrow band in the inner layer of lamina II (Bradbury *et al.*, 1998; Vulchanova *et al.*, 1998), which is the site of termination of IB4-positive primary afferents (Hunt *et al.*, 1992a). The $P2X_3$ immunoreactivity in the dorsal horn was depleted following dorsal root rhizotomy, indicating that P2X receptor proteins are transported to central terminals in the spinal cord and that $P2X_3$ receptors are not expressed by intrinsic spinal neurons (Bradbury *et al.*, 1998; Vulchanova *et al.*, 1998).

1.4.6.3 Colocalization of $P2X_3$ with VR1 receptors

An ion channel opened by noxious temperature (>42 °C) was found on about 50% of small sensory neurons (Cesare & McNaughton, 1996). The VR1 receptor, which binds capsaicin and is activated by noxious heat, has been cloned and characterized (Caterina *et al.*, 1997). A high proportion of C- and some Aδ- fibers from sensory neurons are sensitive to capsaicin (see review: Holzer, 1991). Mice lacking VR1 receptors show no vanilloid-evoked pain behaviour impaired capacity to detect painful heat and little thermal hypersensitivity following inflammation (Caterina *et al.*, 2000). These results indicate that the VR1 receptor plays an important role in nociceptive

transmission. However, it still remains controversial, that apart from noxious heat, ligands for the VR1 receptor naturally occur in the nervous system.

It has long been speculated that capsaicin-binding receptors are expressed in the neuropeptide-containing afferent fibers because capsaicin induces SP and CGRP release from primary afferent terminals (Yaksh *et al.*, 1979; Micevych *et al.*, 1983). Quantitative analysis has shown that 33% VR1-labelled neurons are SP-positive and 79% are CGRP-positive, indicating a differential localization of VR1 receptor proteins with neuropeptides SP and CGRP (Michael & Priestley, 1999). Evidence has shown that VR1 mRNA at varying levels is concentrated in both neuropeptide-containing neurons and IB4-positive neurons (Guo *et al.*, 1999; Michael & Priestley, 1999). These results are compatible with the distribution of VR1-like immunoreactivity in the spinal dorsal horn. VR1-like proteins were found not only in the lamina I and the outer part of lamina II (site for the termination of neuropeptide-positive neurons) but also in the inner layer of lamina II (the site where IB4-positive nerves project to) (Guo *et al.*, 1999).

It has been found that approximately 75% VR1-positive neurons also contain the $P2X_3$ receptor immunoreactivity (Guo *et al.*, 1999). VR1-like proteins are colocalized with the $P2X_3$ receptor immunoreactivity in both sensory neuron somata and their central processes in the inner part of lamina II. Electrophysiological studies have demonstrated that 70 % of the capsaicin-sensitive small DRG neurons functionally express homomeric $P2X_3$ receptors (Ueno *et al.*, 1999). Moreover, the number of $P2X_3$-stained neurons is markedly reduced in DRGs of neonatal rats receiving neurotoxic capsaicin treatment (Vulchanova *et al.*, 1998). This profound

colocalization between $P2X_3$ and VR1 is likely to endow IB4 neurons with a unique signaling role in nociceptive transmission.

1.4.6.4 Regulation of $P2X_3$ expression by trophic factors

Neurotrophins such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) are crucial for survival of nociceptive neurons during development. It appears that they also play an important role in the maintenance of biochemical and electrophysiological phenotype in sensory neurons in adult animals (see review: Lindsay, 1992). Administration of exogenous neurotrophic factors after nerve injury (axotomy) has been shown to have a selective beneficial effect on the survival and phenotypic expression of primary sensory neurons in dorsal root ganglia and motoneurons in the spinal cord (see review: Terenghi, 1999). Neuropeptide-containing DRG neurons are found to be sensitive to nerve growth factor (NGF) and express the high-affinity receptor trk A (tyrosine kinase A) (see review: Lindsay, 1992). Intrathecal infusion of NGF for 7 days reversed the down-regulation of CGRP- and SP-containing neurons, but not somatostatin expression following nerve injury. The lack of effect of NGF on somatostatin-containing neurons is consistent with the absence of high-affinity trk A receptors in somatostain-positive neurons (Verge *et al.*, 1995). Sensitivity to ATP in isolated DRG neurons appears not regulated by NGF (Bevan & Winter, 1995), possibly due to the absence of trk A receptors on IB4-positive $P2X_3$-containing cells (Bradbury *et al.*, 1998).

It has been found that IB4-positive neurons express the glial cell line-derived neurotrophic factor (GDNF) receptor complex and respond to GDNF *in vitro* and *in*

vivo (Molliver *et al.*, 1997; Bennett *et al.*, 1998). Evidence shows that IB4-positive neurons switch from dependence on NGF to dependence upon GDNF during development (Molliver *et al.*, 1997). GDNF supportsthe survival of IB4-positive, but not CGRP-containing sensory neurons in culture. Given intrathecally, GDNF can reverse axotomy-induced down-regulation of the $P2X_3$ receptor in IB4-positive neurons (Bradbury *et al.*, 1998). GDNF can also reverse the slowing of conduction velocity in a subset of small DRG neurons following axotomy (Bennett *et al.*, 1998). Thus, it appears that IB4-positive neurons may be rescued by exogenous GDNF following nerve injury.

1.5 P2X RECEPTORS ARE IMPLICATED IN NOCICEPTIVE PATHWAYS

1.5.1 General remarks

Skin contains mechanoreceptors, thermoreceptors and nociceptors. Nociceptors are pain-sensing neurons and belong to the class of Aδ- and C- afferents that detect chemical, thermal and mechanical stimuli at noxious levels. Nociceptors are also present in muscle and joints. These nociceptors transduce noxious sensation into depolarizations to trigger action potentials, which propagate to central terminals of primary afferents in the dorsal horn and trigger release of neurotransmitters to postsynaptic dorsal horn neurons. These are followed by the excitation of high order sensory neurons in the CNS. Early studies showed that ATP evoked pain sensation at human blister bases and initiated nerve action potentials when applied to rabbit ear blister bases (Bleehen & Keele, 1977; Bleehen, 1978). These experiments suggested that ATP might act as a pain mediator on cutaneous nociceptors. Studies of isolated

sensory neurons have demonstrated that ATP activates an inward cationic current, suggesting a receptor-mediated mechanism underlying the algogenic process of ATP (Jahr & Jessell, 1983; Kristal *et al*, 1983; Bean, 1990; Bean *et al.*, 1990). Subsequently, molecular cloning, tissue distribution and electrophysiological studies of P2X receptors in sensory neurons strongly support the notion that ATP, as a chemical mediator, may activate nociceptors via P2X receptor subtypes (Chen *et al.*, 1995; Collo *et al.*, 1996; Robertson *et al.*, 1996). Burnstock and colleagues then proposed that ATP and ATP-gated ion channels might play an important role in nociceptive transmission (Burnstock, 1996a; Burnstock & Wood, 1996).

1.5.2 ATP excites isolated sensory neurons

ATP-gated ionic channels were first identified in dissociated sensory neurons (Jahr & Jessell, 1983; Krishtal *et al.*, 1983). Subsequent investigations of ATP-responses in isolated sensory neurons confirmed the presence of functional P2X receptors in DRG neurons, nodose and trigeminal ganglion neurons (Bean, 1990; Bean *et al.*, 1990; Li *et al.*, 1993; Khakh *et al.*, 1995; Robertson *et al.*, 1996; Cook *et al.*, 1997). Three distinct P2X receptor phenotypes have been defined in excitable cells including smooth muscle cells, autonomic and sensory neurons (Evans & Kennedy, 1994; Buell *et al.*, 1996a; Evans & Surprenant, 1996). These phenotypes include i) a full sensitivity to both ATP and α,β-meATP and rapid desensitization of evoked currents ii) a full sensitivity to both ATP and α,β-meATP and slow desensitization of evoked currents iii) a full sensitivity to ATP but not α,β-meATP and slow desensitization kinetics.

Over 80% of adult DRG neurons respond to both ATP and α,β-meATP with a rapidly-inactivating inward current (Burgard *et al.*, 1999; Grubb & Evans, 1999). A similar response has been observed in neonatal DRG neurons, a subset of trigeminal ganglion neurons and smooth muscle cells (Evans & Kennedy, 1994; Robertson *et al.*, 1996; Cook *et al.*, 1997). $P2X_3$ receptor mRNA and proteins are concentrated in DRG and trigeminal ganglion neurons (Chen *et al.*, 1995; Bradbury *et al.*, 1998; Vulchanova *et al.*, 1998). Taken together, these results suggest that the rapidly-inactivating ATP-activated response in dorsal root and trigeminal ganglion neurons is mediated by homomeric $P2X_3$ receptors. The unique expression pattern of the $P2X_3$ receptor in small-sized sensory neurons strongly pointed to the involvement of $P2X_3$ receptors in nociception, because most of the small sensory neurons are nociceptors (Chen *et al.*, 1995).

A small proportion of DRG neurons responds to both ATP and α,β-meATP with a slowly-inactivating inward current (Burgard *et al.*, 1999; Grubb & Evans, 1999). A similar sustained response has also been reported in nodose ganglion neurons and a subset of trigeminal ganglion neurons (Lewis *et al.*, 1995; Cook *et al.*, 1997). The heteromeric $P2X_{2/3}$ receptor has been suggested to be the dominant receptor subtype which mediates the sustained response activated by α,β-meATP in these sensory neurons (see below). Approximately 10% of DRG neurons give rise to a biphasic response consisting of transient and sustained components, which might be attributed to the expression of both homomeric $P2X_3$ and heteromeric $P2X_{2/3}$ receptors. It appears that at least two populations of P2X receptors, $P2X_3$ and $P2X_{2/3}$ are formed in variable proportions in dorsal root and trigeminal ganglion neurons

(Cook *et al.*, 1997; Burgard *et al.*, 1999; Grubb & Evans, 1999). However, the homomeric $P2X_2$ receptor is not the dominant P2X subtype in mammalian sensory neurons because currents characterized by sensitivity to ATP (insensitivity to α,β-meATP) and slow desensitization kinetics have not been directly observed in sensory neurons. In contrast, slowly-desensitizing currents activated by ATP, but not α,β-meATP are the properties of P2X receptors in PC12 cells, superior cervical and pelvic ganglion neurons (Nakazawa *et al.*, 1990; Cloues *et al.*, 1993; Khakh *et al.*, 1995; Zhong *et al.*, 1998).

Nodose ganglia are sensory vagal ganglia which receive sensory input from visceral organs including the trachea, lung, heart, liver, stomach, gut and urinary bladder. In nodose ganglion neurons, both ATP and α,β-meATP evoke a slowly-inactivating current, which shares no similarity with homomeric $P2X_{1-7}$ receptors (Khakh *et al.*, 1995; Lewis *et al.*, 1995). However, coexpression of $P2X_2$ and $P2X_3$ receptor subtypes gives rise to a similar slowly-inactivating response to α,β-meATP (Lewis *et al.*, 1995). It has been strongly suggested that heteropolymerization of $P2X_2$ and $P2X_3$ subunits creates ion channels that are similar in their properties to those observed in nodose ganglion neurons. These results suggest that P2X receptors are multimers, in which subunits can form as either homo- or heteropolymers. Nonetheless, ATP responses in sensory neurons vary from cell to cell in single ganglia, indicating the presence of variable proportions of either P2X homomers or heteromers, or both (Thomas *et al.*, 1998; Burgard *et al.*, 1999; Grubb & Evans, 1999). In the present study (Chapter 3), I have investigated the properties of P2X receptors formed by coexpression of $P2X_2$ and $P2X_3$ subunits in *Xenopus* oocytes and explored

the mechanism of the variability of ATP responses in sensory neurons by changing the injected cRNA ratio for $P2X_2$ and $P2X_3$ subunits.

1.5.3 ATP excites primary afferent terminals

To address whether P2X receptor subtypes are directly involved in primary sensory transduction, studies on intact preparations and *in vivo* are needed. Functional P2X receptors have been found on peripheral terminals of primary afferents supplying the rat knee joint, jejunum and tongue (Dowd *et al.*, 1998, Kirkup *et al.*, 1999; Rong *et al.*, 2000). A subpopulation of C- and Aδ- nociceptive afferents innervating the knee joint was excited when ATP and α,β-meATP were applied intra-arterially or intra-articularly in the anaesthetized rat (Dowd *et al.*, 1998). However, no difference was found in the α,β-meATP-induced rapid excitation afferents supplying normal and inflamed joints. Intra-arterial administration of ATP and α,β-meATP evoked excitation of afferent nerves supplying the jejunum in the anaesthetized rat, which could be blocked by P2 antagonists suramin and PPADS, but not Ca^{2+} channel toxins ω-conotoxin MVIIA and SVIB (Kirkup *et al.*, 1999). Administration of adenosine intravenously or intra-arterially failed to produce a similar excitatory effect. Therefore, the burst of action potentials of intestinal afferent nerves following administrations of ATP and α,β-meATP was unlikely to be mediated by adenosine and its membrane receptors. It has been proposed that the increase in mesenteric afferent nerve activity is a functional consequence of the activation of P2X receptors on mesenteric nerve terminals (Kirkup *et al.*, 1999).

ATP and α,β-meATP preferentially activated general sensory afferents (trigeminal branch of the lingual nerve) rather than special sensory (taste) nerves (the chorda tympani nerve) in tongue-trigeminal nerve preparations (Rong *et al.*, 2000). Arterial injections of ATP and α,β-meATP selectively induced a rapid dose-dependent increase in lingual nerve activity, which could be antagonized by P2 antagonists suramin and PPADS and also abolished by pretreatment with capsaicin. In addition, in the skin-nerve preparation, ATP induced heat sensitization of nociceptors by elevating the intracellular Ca^{2+} level, possibly via P2X receptors in the DRG neurons (Kress & Guenther, 1999). These observations support the notion that ATP and P2X receptors play an important role in primary sensory signaling and may also account for the hypersensitivity of inflamed tissues.

1.5.4 ATP evokes nociception in conscious animals and human

Animal behavioural studies indicate that the concentrations of endogenous ATP are capable of exciting nociceptors in inflamed skin, and exogenous ATP produced nocifensive behaviour in normal skin after injectioninto rat hindpaw (Bland-Ward & Humphrey, 1997; Hamilton *et al.*, 1999b). Subplantar α,β-meATP injections evoked a transient episode of hindpaw lifting and licking behaviour, which was desensitized by local pretreatment with α,β-meATP and blocked by ipsilateral injections of bupovacaine (a local anaesthetic). A similar pain-related behaviour was not evoked by ATP and α,β-meATP in capsaicin pretreated animals. Taken together, these findings suggest that subplantar administration of α,β-meATP causes paw lifting and licking

behaviour as a result of the direct activation of P2X receptors on the cutaneous afferents (Bland-Ward & Humphrey, 1997; Hamilton *et al.*, 1999b).

The nociceptive effect of ATP is markedly augmented in the presence of inflammation or inflammatory mediators (Sawynok & Reid, 1997; Hamilton *et al.*, 1999b). ATP and α,β-meATP significantly potentiated the second phase response (flinching behaviour) induced by formalin. This pro-nociceptive effect of ATP and α,β-meATP was blocked by P2 receptor antagonists suramin and PPADS (Sawynok & Reid, 1997). Furthermore, formalin-induced pain behaviour was markedly reduced in $P2X_3$-receptor-null mutants, though behavioural responses to noxious mechanical and thermal stimuli were normal (Cockayne *et al.*, 2000; Souslova *et al.*, 2000). The painful effects of ATP and its analogues have also been investigated in three inflammatory models induced by intraplantar injections of carrageenan, local irradiation with ultraviolet B and simultaneous injections of prostaglandin E_2 (Hamilton *et al.*, 1999b). In all models the nocifensive behaviour induced by α,β-meATP was greatly enhanced and the concentration of α,β-meATP required to produce pain-related activity significantly reduced. These *in vivo* experiments provide further evidence that ATP may function as a peripheral mediator of nociception, particular in pathophysiological conditions. In addition, intrathecal administration of α,β-meATP caused thermal hyperalgesia, which was completely blocked by intrathecal pretreatment with P2X receptor antagonist PPADS (Tsuda *et al.*, 1999a). These authors argued that the effect of α,β-meATP was mediated by P2X receptors at central terminals of sensory afferents in the spinal dorsal horn. It is possible that α,β-meATP-induced glutamate release from afferent central terminals leads to the

generation of thermal hyperalgesia. These data suggest that ATP and P2X receptors may play an important presynaptic role in the spinal sensory synapse.

Bleehen and Keele (1977) did the first psychophysiological study in humans, which showed that ATP was a painful stimulus when applied to blister bases. Most recently, the action of ATP has been quantified in healthy volunteers (Hamilton *et al.*, 2000). ATP was administrated into the skin using iontophoresis, which caused a dose-dependent pain sensation. Persistent inotophoresis of ATP led to desensitization of evoked pain, which has led to the suggestion that $P2X_3$ receptor subtype may be involved in the primary nociceptive transduction. Pretreatment with topical capsaicin significantly reduced the pain caused by ATP. Thus, ATP is capable of producing pain sensation on the human skin via capsaicin-sensitive sensory neurons. Taken together, it is possible that ATP, released in the vicinity of peripheral nociceptive terminals, activates $P2X_3$ receptors on nearby nociceptive afferents and initiates pain signaling in animals and humans.

1.5.5 P2X receptor mediates sensory transmission in the CNS

Neurotransmitters released by nociceptors that activate dorsal horn cells include the excitatory amino acid, glutamate, and several peptides such as substance P (SP), CGRP and vasoactive intestinal polypeptide (VIP). Evidence shows that ATP is released from central terminals of afferent nerves (Holton & Holton, 1954; Sweeney *et al.*, 1989) and may act as a neurotransmitter to activate second order sensory neurons in the spinal cord. The spinal cord expresses all mRNA except that of the $P2X_3$ subtype, whilst only $P2X_4$ and $P2X_6$ transcripts are found throughout the spinal cord

(Collo *et al.*, 1996). $P2X_2$ and $P2X_4$ receptor proteins are extensively concentrated in the dorsal horn neurons at all levels of spinal cord (Lê *et al.*, 1998b; Vulchanova *et al.*, 1998; Kanjhan *et al.*, 1999). Indeed, ATP elicits fast excitatory potentials when applied to dorsal horn neurons (Jahr & Jessel, 1983; Fyffe & Perl, 1984; Salter & Henry, 1985; Bardoni *et al.*, 1997). ATP has been shown to activate a subpopulation of dorsal horn neurons in culture (Jahr & Jessell 1983; Bardoni *et al.*, 1997). *In vivo* experiments have shown that ATP had diverse effects on functionally identified fibers in the dorsal horn of the spinal cord (Fyffe & Perl, 1983; Salter & Henry, 1985). When applied iontophoretically to neurons in the spinal cord *in vivo*, ATP selectively excited a subset of spinal neurons. It was also found that many primary afferents excited by ATP were mechanoreceptive. Multireceptive fibers, which received excitatory input from both nociceptors and mechanoreceptors, also responded to ATP. More recently, excitatory synaptic transmission mediated by P2X receptors was found in a restricted subset of lamina II neurons in the spinal cord slices (Bardoni *et al.*, 1997).

1.6 DEVELOPMENT OF P2X AGONISTS AND ANTAGONISTS

1.6.1 Agonists

Although the cloning and heterologous expression of P2X and P2Y receptors have helped in subtyping P2 receptors, the development of subtype-selective agonists and antagonists remains critical in the classification and functional studies of P2 receptors. So far, native P2X subtypes are defined mainly according to the time course and relative potency of various nucleotide agonists. Some of the useful agonists are stable

ATP analogues including 2-MeSATP, α,β-meATP and β,γ-meATP. 2-MeSATP is almost as potent as ATP at recombinant $P2X_{1-7}$ receptors (see reviews: King 1998; North & Surprenant, 2000). α,β-meATP is well known for it's selectivity for Group 1 receptors ($P2X_1$ and $P2X_3$). It also activates heteromeric $P2X_{2/3}$ receptors at far lower concentrations than those needed for agonists of Group 2 receptors including $P2X_2$, $P2X_4$ and $P2X_5$. Distinguishing between the $P2X_1$ and $P2X_3$ receptors is difficult because these two P2X receptors have a similar pharmacology and kinetics (see reviews: King, 1998; North & Surprenant, 2000). In some cases, immunohistochemical techniques have been used in conjunction with pharmacological studies to determine the native P2X subtype (Cook *et al.*, 1997; Bo *et al.*, 1999; Rong *et al.*, 2000).

It has been argued that isomer L-β,γ-meATP shows about 30-fold selectivity for $P2X_1$ over $P2X_3$ receptors (Evans *et al.*, 1995; Trezise *et al.*, 1995; Rae *et al.*, 1998). It seems possible to distinguish between $P2X_1$ and $P2X_3$ receptors in biological tissues and intact animals on the basis of selectivity of α,β-meATP and L-β,γ-meATP. Behavioural studies have shown pro-nociceptive effects of α,β-meATP in rats and mice following intrathecal administration (Driessen *et al.*, 1994; Tsuda *et al.*, 1999a). In contrast, intrathecal injections of L-β,γ-meATP failed to evoke pain-related behaviour in mice (Tsuda *et al.*, 1999a). It seems likely that $P2X_3$ receptors on either postsynaptic or presynaptic sites mediate α,β-meATP-induced nociception. However, isolated dorsal horn neurons were insensitive to α,β-meATP (Bardoni *et al.*, 1997), a result comparable with that of immunohistochemical studies (Bradbury *et al.*, 1998; Vulchanova *et al.*, 1998). In addition, intrathecal administration of α,β-meATP had

no effect on the electrically evoked response in dorsal horn neurons in anaesthetized rats (Stanfa *et al.*, 2000). Therefore, it is the $P2X_3$ receptor in central terminals of sensory afferents, which mediates the pain-related behaviour induced by intrathecal applications of α,β-meATP.

Diadenosine polyphosphates (Ap_nA, where n=2-6) were recently recognized as useful tools in determining the P2X receptor subtype. However, there is considerable variability in potency and efficacy of diadenosine polyphosphates at recombinant $P2X_{1-4}$ receptors among published values. The activity of diadenosine polyphosphates was found to increase with increasing numbers of phosphate groups at the rat $P2X_1$ receptor, where Ap_6A was a full agonist, Ap_5A and Ap_4A were partial agonists, whereas Ap_3A was a weak agonist, and Ap_2A had no effect (Wildman *et al.*, 1999a). However, Ap_4A and Ap_5A were found to be full agonists whilst Ap_6A a partial agonist at the human $P2X_1$ receptor (Evans *et al.*, 1995; Bianchi *et al.*, 1999). Interestingly, Ap_4A was found to be the only diadenosine polyphosphate which acted as a full agonist at the rat $P2X_2$ receptor expressed in oocytes (Wildman *et al.*, 1999a), whilst none of diadenosine polyphosphates was effective at the rat $P2X_2$ receptors when expressed in astrocytoma cells (Bianchi *et al.*, 1999). Nevertheless, Ap_4A, Ap_5A and Ap_6A were full or nearly full agonists at the rat $P2X_3$ receptor expressed in both oocytes and astrocytoma cells (Bianchi *et al.*, 1999; Wildman *et al.*, 1999a). All diadenosine polyphosphates tested are much less effective than ATP at rat and human $P2X_4$ receptors (Bianchi *et al.*, 1999; Wildman *et al.*, 1999a).

1.6.2 Antagonists

It has been strongly emphasized that antagonists, rather than agonists, are the preferred tools for pharmacological classification (Kenakin *et al.,* 1992). Unfortunately, much of the pharmacological classification of P2X and P2Y receptors still rests on relative agonist potencies and kinetics of evoked responses. In the following sections, the antagonist activity of suramin, PPAD, TNP-ATP and their structurally related compounds at native and recombinant P2X receptors will be described.

1.6.2.1 Suramin and its analogues

Various smooth muscle preparations have been used in studies of the antagonist activity of putative P2X and P2Y antagonists. ATP binds both P2X and P2Y receptors in smooth muscle and causes contraction and relaxation, respectively (see reviews: Burnstock & Kennedy, 1985; Evans & Kennedy, 1994). Suramin has an inhibitory action not only in the tissues where ATP produces contraction via P2X receptors, but also in the tissues where ATP causes a relaxation through P2Y receptors (see review: Ralevic & Burnstock, 1998). Suramin inhibited α,β-meATP-evoked contraction in the mouse, rat and guinea-pig vas deferens (Dunn & Blakeley, 1988; Mallard *et al.*, 1992; Bailey & Hourani, 1994), the guinea-pig urinary bladder (Hoyle *et al.*, 1990; Bailey & Hourani, 1994) and the rabbit ear artery (Leff *et al.*, 1990). Suramin behaved as a competitive antagonist in a vascular preparation (Leff *et al.*, 1990), but produced a noncompetitive antagonism in urinary bladder preparations (Hoyle *et al.*, 1990; Bailey & Hourani, 1994). In addition, it significantly antagonized relaxant responses evoked by exogenous ATP and electrical stimulation of the intramural purinergic nerves in the

taenia coli (Hoyle *et al.*, 1990). A similar inhibition of relaxant responses by suramin has also been observed in the rabbit isolated thoracic aorta, an action that can be explained by the inhibition of ecto-nucleotidases as well as the inhibition of endothelium P2Y receptors (Ziyal *et al.*, 1997).

Suramin is also an effective P2X antagonist in sensory neurons. However, rapidly-inactivating and slowly-inactivating P2X currents showed different sensitivity (Grubb & Evans, 1999; Petruska *et al.*, 2000). Suramin blocked rapidly-inactivating ATP-activated currents in rat DRG neurons, yielding a pIC_{50} value of 6.5 (Grubb & Evans, 1999). Of the α,β-meATP-sensitive DRG neurons, suramin (10 μM) inhibited slowly-inactivating ATP-evoked currents by 66%, whilst it blocked rapidly-inactivating currents by 84% (Petruska *et al.*, 2000). It was found to block slowly-inactivating ATP-evoked currents by 86% in α,β-meATP-insensitive neurons.

Suramin has also been tested in several pain models. For example, it was found to suppress nociceptive-related behaviour evoked by intrathecal applications of α,β-meATP (Driessen *et al.*, 1994). In addition, nocifensive behaviour in response to subcutaneous injections of formalin could be markedly reduced by the pre-treatment with suramin (Sawynok & Reid, 1997). However, the analgesic effect of suramin in animal pain models may not be solely attributed to its antagonism at P2X receptors because of its complicated actions on multiple neurotransmitters. Evidence has shown that suramin inhibits various molecules and a variety of transmitter-gated ion channels at concentrations similar to those that produce an inhibitory effect at P2X receptors (see review: Ralevic & Burnstock, 1998). For example, suramin at micromolar concentrations inhibited GABA and glutamate receptors in rat hippocampal neurons

(Nakazawa *et al.*, 1995). Thus, caution should be taken when interpreting the effects of suramin in biological tissues and intact animals.

Several suramin analogues including 8,8'-(carbonylbis(imino-3,1-phenylene carbonylimino))bis-(1,3,5-naphthalenetrisulphonic acid) (NF023) and 8,8'-(carbonylbis(imino-4,1-phenylene carbonylimino-4,1- phenylene carbonylimino))bis-(1,3,5-naphthalenetrisulphonic acid) (NF279) have been investigated on native P2X and P2Y receptors, and recombinant P2X receptors. NF023 selectively blocked the contraction induced by α,β-meATP, presumably via $P2X_1$-like receptors, in the rabbit saphenous artery, rabbit vas deferens and rat mesenteric artery (Lambrecht, 1996; Ziyal *et al.*, 1997). It was more potent than suramin and had a reasonable selectivity for P2X receptors over ecto-nucleotidases (Bültmann *et al.*, 1996; Ziyal *et al.*, 1997). In contrast, NF023 was less potent than suramin in blocking P2Y receptors, which were thought to mediate relaxation in the rat duodenum and guinea-pig taenia coli (Bültmann *et al.*, 1996; Lambrecht, 1996). NF023 also blocked P2Y mediated relaxation in rat mesenteric artery, but failed to block endothelium-dependent relaxation in rabbit aorta artery (Lambrecht, 1996; Ziyal *et al.*, 1997). This difference is likely due to distinct P2Y subtypes expressed in the vascular smooth muscle and vascular endothelium (Corr & Burnstock, 1994).

NF279 is a weak antagonist at P2Y receptors in the guinea-pig taenia coli, which makes it a selective antagonist at P2X receptors (Damer *et al.*, 1998). When tested at heterologously expressed P2X receptors, NF023 was found to be a surmountable antagonist at rat and human $P2X_1$ receptors with IC_{50} values in a sub-micromolar range (Soto *et al.*, 1999). It also blocked $P2X_3$, $P2X_{2/3}$ and $P2X_2$ receptors

in a micromolar concentration range. Thus, neither NF023 nor NF297 is a subtype-selective antagonist at P2X receptors. Although NF023 has been suggested to be a useful tool in distinguishing rapidly-desensitizing agonist responses mediated by $P2X_1$ from those mediated by $P2X_3$ receptors (Soto *et al.*, 1999), in general, suramin and its analogues are nonselective P2 antagonists, while the latter shows some of the improvements in potency and selectivity.

1.6.2.2 PPADS

Pyridoxal 5'-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) was first introduced as a selective P2X antagonist (Lambrecht *et al.*, 1992; Ziganshin *et al.*, 1993; McLaren *et al.*, 1994). It produced a concentration-dependent inhibition of the purinergic component of contractions evoked by sympathetic nerve stimulation in the guinea-pig vas deferens and rabbit urinary bladder (Ziganshin *et al.*, 1993; McLaren *et al.*, 1994). It also caused a substantial inhibition of contractions evoked by exogenous α,β-meATP, leaving responses to noradrenaline, carbachol and histamine in the vas deferens unaltered (McLaren *et al.*, 1994). However, in the rabbit urinary bladder, PPADS caused a small but significant suppression of the maximal response of carbachol, which suggested that PPADS might be a weak antagonist at the muscarinic receptor (Ziganshin *et al.*, 1993). PPADS is a slowly-reversible antagonist at recombinant $P2X_1$, $P2X_2$, $P2X_3$ and $P2X_5$ receptors (IC_{50}, 1-2 μM) and a weak antagonist at $P2X_4$, $P2X_6$ and $P2X_7$ receptors (IC_{50}, 10-500 μM) (see review; King 1998).

PPADS was also found to be an effective antagonist in blocking inward currents evoked by ATP and α,β-meATP in rat and bullfrog DRG neurons (Grubb & Evans, 1999; Ueno *et al.*, 1999; Li, 2000). PPADS inhibited rapidly-inactivating ATP-activated currents in rat DRG neurons in a concentration-dependent manner (pIC_{50}, 6.4) (Grubb & Evans, 1999). A close inspection of the nature of PPADS antagonism has revealed that it inhibited ATP-activated currents in a complex noncompetitive manner (Li, 2000). It has been suggested that the slowly-reversible inhibition induced by PPADS results from an action at an allosteric site on P2X receptors. In behavioural studies, PPADS completely blocked thermal hyperalgesic responses evoked by intrathecal administration of α,β-meATP in mice (Tsuda *et al.*, 1999a). In addition, intrathecal pretreatment with PPADS significantly suppressed nociceptive behaviour induced by subplantar injections of formalin and capsaicin (Tsuda *et al.*, 1999b), an action which was thought to be the consequence of inhibition of spinal P2X receptors.

PPADS is now known as a nonselective antagonist at P2X and P2Y receptors (see review: Ralevic & Burnstock, 1998). Studies have shown that PPADS failed to discriminate adequately between P2X and P2Y receptors in some preparations (Brown *et al.*, 1995; Windscheif *et al.*, 1995; Lambrecht, 1996). PPADS antagonized α,β-meATP-induced contractions in certain vascular and visceral smooth muscles with a pA_2 value ranging from 6.02 to 6.41 (Lambrecht, 1996), whilst it inhibited P2Y-receptor-mediated relaxant responses in the guinea-pig taenia coli, rat duodenum and certain artery preparations with a pA_2 value between 4.59 and 5.46 (Brown *et al.*, 1995; Windscheif *et al.*, 1995; Lambrecht, 1996). Recently, structurally related compounds of PPADS were the subjects of detailed pharmacological study

(Lambrecht *et al.*, 2000). One of them is pyridoxal-5'-phosphate-6-(2'-naphthylazo-6'-nitro-4',8'-disulfonate) (PPNDS), which potently inhibited α,β-meATP-activated isometric contractions in the rat vas deferens and ATP-activated currents at the recombinant $P2X_1$ receptor (Lambrecht *et al.*, 2000). However, the potency for PPNDS at P2Y receptors in the longitudinal smooth muscle of guinea pig ileum was not markedly different from that of PPADS. Therefore, both PPADS and PPNDS are potent antagonists at P2X receptors, particular $P2X_1$ subtype, though they are not highly selective for P2X over P2Y receptors.

1.6.2.3 TNP-ATP

Trinitrophenyl-ATP (TNP-ATP) is a potent antagonist in blocking ATP-activated currents at the recombinant $P2X_1$, $P2X_3$ and $P2X_{2/3}$ receptors (IC_{50} about 1 nM), but is less effective at the recombinant $P2X_2$, $P2X_4$ and $P2X_7$ receptors (IC>1μM) (Virginio *et al.*, 1998). It has been shown that the $P2X_{1/5}$ receptor, formed by coexpression of $P2X_1$ and $P2X_5$ receptor subtypes, was much less sensitive to TNP-ATP than was the $P2X_1$ receptor (Haines *et al.*, 1999). TNP-ATP also potently inhibited rapidly-inactivating α,β-meATP-activated currents in rat DRG neurons (pIC_{50}: 9.5) (Grubb & Evans, 1999; Dunn *et al.*, 2000). These results indicate that TNP-ATP is a powerful pharmacological tool for the study of native P2X receptors. On the basis of sensitivity to TNP-ATP, the presence of two populations of P2X receptors, $P2X_2$ and $P2X_{2/3}$, has been revealed in rat nodose and guinea-pig superior cervical ganglion neurons (Thomas *et al.*, 1998; Zhong *et al.*, 2000). An *in vivo* study has shown that thermal hyperalgesia induced by intrathecal applications of α,β-meATP could be completely

blocked by pretreatment of TNP-ATP given intrathecally (Tsuda *et al.*, 1999a). In addition, intrathecal pretreatment with TNP-ATP significantly inhibited nociceptive behaviour induced by intraplantar injections of capsaicin and formalin (Tsuda *et al.*, 1999b). These findings suggest that TNP-ATP-sensitive spinal P2X receptors, possibly $P2X_3$ subtype, may be involved in the generation of thermal hyperalgesia and nocifensive activity induced by local capsaicin and formalin.

CHAPTER 2

GENERAL METHODOLOGY

2.1 *XENOPUS LAEVIS*

Xenopus laevis, the South African claw-toed frog is a member of the family of *pipidae*. The name of *Xenopus laevis* came from the ancient Greek, *Xen* meaning strange, *pus* meaning foot and *laevis* meaning smooth. *Xenopus* is regarded as a primitive frog with no teeth or tongue and is almost completely aquatic. The female of the species is nearly twice as large as the male when fully grown and is distinguished by a posterior tail-like bud.

Adult female *Xenopus laevis* frogs weighing 200-300g were supplied by Blade Biologicals (Kent, U.K.) and Xenopus 1 (U.S.A.), and were allowed to acclimatize in quarantine tanks for a minimum of 1 month before experimental use. Frogs were kept in 390 litre tanks of tap water equipped with a continuous overflow filtration system at 21-23°C. Frogs were exposed to a twelve hour artificial light and dark cycle to help break the natural breeding pattern and prevent seasonal variation in the oocyte quality.

2.2 *XENOPUS* OOCYTE EXPRESSION SYSTEM

2.2.1 General remarks

Each ovary of the adult female frog consists of about 24 lobes containing oocytes of six developmental stages classified by appearance and biochemical differences, known as stage I-VI (Dumont, 1972, see Photograph 2.1). During stage I to III, oocytes are small and lack any discrimination between the dark brown animal hemisphere and white vegetal hemisphere. At stage IV, demarcation between the animal and vegetal poles is clear. Oocytes at stage VI are fully grown, being 1.2-1.3 mm in diameter. Usually, oocytes of stage V and VI are used for receptor expression and functional

studies because mature oocytes allow a high expression density of foreign proteins and can accept volumes of up to 100 nl into the 700-900 nl cell volume. Each oocyte is surrounded by a vitelline membrane and follicular cell layer. Evidence has shown that endogenous ATP-activated ion channels are expressed in the follicular layer (King *et al.*, 1996a; 1996b). ATP evoked transient and sustained currents in ~50% follicular oocytes, but not in defolliculated oocytes. Therefore, the follicular cell layer was removed when recombinant ATP-gated ionic and metabolic channels are studied. This procedure also provides a closer contact of bathing solution with the oocyte membrane and eliminates other endogenous receptors expressed in the follicular cell layer.

Miledi and colleagues first demonstrated that oocytes injected with messenger ribose nucleic acids (mRNA) from cat and human brain functionally expressed a variety of ligand- and voltage-gated ion channels (Miledi *et al.*, 1982; Gundersen *et al.*, 1984). In subsequent studies, oocytes were injected with capped ribose nucleic acids (cRNA) synthesized from cloned cDNAs isolated from biological tissues (McCaman *et al.*, 1988; Bartel *et al.*, 1989). It has been found that foreign cRNAs, inserted into plasmid vectors containing viral transcription promotors, are efficiently translated and resultant proteins incorporated into the oocyte plasma membrane. Thus, mRNA/cRNA receptor proteins can be expressed heterogeneously in oocytes for pharmacological and functional studies. To date, the *Xenopus laevis* oocyte has proved to be an ideal expression system because it has few endogenous channels. Other advantages of the oocyte expression system include its large cell size (up to 1.3 mm in diameter), long survival (up to 2 weeks) and simple storage conditions. In addition to being used for electrophysiological and pharmacological studies of

receptors of interest, the oocyte expression system has been used for a variety of other purposes, including studies of chromatin assembly, cell cycling and assembly of multimers. Indeed, oocytes have been used successfully for the determination of the quaternary structure of the P2X receptor (Nicke *et al.*, 1998).

2.2.2 Preparation of *Xenopus* oocytes

The Animals Scientific Procedures Act (1986) states that a donor frog may only be used for oocytes extraction on two occasions. In this study, frogs were only used once using non-recovery procedures in order to minimize surgical and attendant costs. Preparation of oocytes, injection of mRNA and electrophysiological measurements have been described in detail by Stühmer (1992). *Xenopus laevis* were anaesthetized with the methane sulfonate salt of 3-aminobenzoic acid ethyl ester (tricaine 0.1% w/v) for about 20 min. When the withdrawal response evoked by squeezing the toe web and righting reflex were inhibited, frogs were killed by decapitation and the spinal cord was pithed. This procedure followed the guidelines of the Home Office Schedule-1 humane amphibia killing procedure (revised February 1997). A midline incision was made in the abdomen using a surgical scalpel and ovarian lobes were removed by blunt dissection. Ovarian lobes were washed several times, before storing (at 4 °C), in Barth' solution pH (7.45) 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 $\mu g\ l^{-1}$.

Under a dissecting microscope, mature oocytes (stage V and VI) were mechanically removed from the inner epithelial cell lining of the ovarian sacs and defolliculated by a two-step process. Firstly, isolated oocytes were treated with

collagenase (Type 1A, 2 mg ml^{-1} in Ca^{2+}- free frog Ringer's solution) for 2-3 hr at 18°C. Once the enzymatic treatment was complete, oocytes were washed thoroughly in Ca^{2+}-free Ringer's solution to remove collagenase and any lysed cells. Secondly, collagenase-treated oocytes were left in a double strength Ca^{2+}-free Ringer's solution for approximately 20 min to shrink oocytes and facilitate the removal of the follicular cell layer. The enveloping follicle cell layer was removed mechanically using fine forceps. Defolliculated oocytes were thoroughly washed and stored in Barth's solution at 4°C, ready for cRNA injections. Healthy mature oocytes were round with dark brown and white hemispheres, and could tolerate repeated impalement by injection micropipettes and recording microelectrodes.

2.2.3 Synthesis of capped RNAs from cloned DNAs

In vitro synthesis of cRNA from cloned cDNAs was made by Dr. A. Townsend-Nicholson (UCL). cDNAs encoding $P2X_{1-4}$ and VR1 receptors were kind gifts from Dr. G. Buell (formerly Glaxo, Geneva), Dr. D. Julius (UCSF, U.S.A.), Professor J. Wood (UCL) and Dr. X. Bo (UCL). Natural mRNAs normally translate best, whereas it is now known that the performance of most synthetic mRNAs can be enhanced when transcribed from the vector pSP64T (Krieg & Melton, 1984), where the cDNA is flanked by sequences from the *Xenopus* β-globin cDNA. The synthesis of cRNAs from linearized DNA templates was described in detail by Goldin & Sumikawa (1992). cDNAs for $P2X_{1-4}$ and VR1 receptors were subcloned into pcDNA3 (Invitrogen) plasmid and then cut with a restriction enzyme (NotI) to linearize the coding region. After linearization, cDNAs were extracted with phenol-chloroform-

isoamyl alcohol, then precipitated with ethanol, and re-suspended in RNase-free water for use as a transcription template. Purified transcripts were suspended in 1 mM Tris-HCl buffer solution (pH 6.5). The cRNA was aliquoted in autoclaved Eppendorf tubes and stored at -80 °C before use.

2.2.4 Oocyte microinjection

Microinjection techniques of cRNA into oocytes were previously described in detail (Goldin & Sumikawa, 1992; Stühmer, 1992). Figure 2.1 schematically shows the microinjection of cRNAs and consequent production of exogenous proteins in the oocyte membrane. The oocyte microinjection protocol requires an environment that is relatively RNase-free. Aseptic techniques were applied to avoid the breakdown of cRNAs during handling. Microinjection pipettes (Drummond Scientific Company, PA, USA) were pre-treated with diethyl pyrocarbonate solution (DEPC, Sigma, 0.2% v/v) for 1 h and autoclaved (121 °C) for 30 min and then baked (220°C) for 4 hours. These borosilicate pipettes had an outer diameter of 1.17 mm and an inner diameter of 0.68 mm and a length of 20 cm. Microinjection pipettes were pulled using a vertical pipette puller (David Kopf Instruments, California, U.S.A.). The tip of the microinjection pipette was broken back using fine forceps to produce a tip diameter of approximately 5-15 μM and then back-filled with autoclaved coloured mineral oil to allow the visualization of the interface between oil and cRNAs. The prepared micropipette was then attached to a 10 μl digital Drummond micropipette dispenser, which was attached to a Narishige micromanupulator. The injection stage was prepared from microscope slides covered by a strip of Nescofilm. The Nescofilm-

cover was removed immediately before the injection. cRNA (1-2 μl), previously aliquoted in Eppendorf tubes was sucked up and expelled on the Nescofilm stage using a 10 μl pipette. The droplet of cRNA was then sucked up into the microinjection pipette and was ready for injections.

Defolliculated oocytes were injected (40 nl) cytosolically with cRNA encoding rat $P2X_{1-4}$ receptors, either individually or together. To express rat $P2X_{1-4}$ receptors separately, cRNAs for $P2X_1$ (1 μg μl^{-1}), $P2X_2$ (0.02 μg μl^{-1}), $P2X_3$ (1 μg μl^{-1}) and $P2X_4$ (1 μg μl^{-1}) were injected individually into defolliculated oocytes. In coexpression experiments, oocytes were coinjected with cRNAs for both $P2X_2$ and $P2X_3$ receptor subunits. In an attempt to mimic the spectrum of agonist responses that are seen in sensory neurons (see Section 1.5.2), varying concentrations of cRNAs for $P2X_2$ and $P2X_3$ receptors were injected. It is known that cRNAs for $P2X_2$ and $P2X_3$ receptors exhibit different translational efficiencies in oocytes. However, the relative expression density may reflect the amount of cRNAs injected if the amount of one cRNA is kept fixed whilst that of the other is changed. In this study (Chapter 3), the cRNA concentration for $P2X_3$ (1 μg μl^{-1}) was kept fixed, while that for $P2X_2$ was altered (2, 10, 20, and 50 ng μl^{-1}). Therefore, oocytes were injected with cRNAs for $P2X_2$ and $P2X_3$ at 4 concentration ratios: 1:500, 1:100, 1:50 and 1:20. After injection, the oocytes were incubated at 18°C in Barth's solution (pH 7.45) for 24 h ($P2X_1$ or $P2X_3$ receptors) or 48 h ($P2X_2$ or $P2X_4$ or $P2X_{2/3}$ receptors) to allow full expression and then stored at 4 °C for up to 12 days.

cRNA for VR1 receptors was synthesized from NotI-linearized VR1 cDNA templates (a kind gift from Dr. D. Julius, UCSF, U.S.A.). Oocytes injected with

cRNA for VR1 receptors (1 μg μl^{-1}, 40 nl) were examined for sensitivity to capsaicin, ATP and protons. To investigate potential crosstalk between $P2X_3$ and VR1 receptors, $P2X_3$ and VR1 receptors were expressed together in oocytes. To generate low expression density of VR1 receptors in coinjected oocytes, the VR1 cRNA concentration was lowered from 1 μg μl^{-1} to 0.5 and 0.2 μg μl^{-1}. cRNA-injected oocytes were incubated at 18° C in Barth's solution (pH 7.45) for 96h to allow full expression of VR1 receptors (Caterina *et al.*, 1997) and then stored at 4° C for up to 12 days.

2.3 TWIN-ELECTRODE VOLTAGE-CLAMP RECORDINGS

Twin-electrode voltage-clamp is a conventional technique for whole-cell recordings made from oocytes expressing ion channels. The voltage electrode (ME1) was connected via a Ag/AgCl wire to a X1LU headstage (unity gain preamplifier). A reference bath electrode (Ag/AgCl pellet) was also connected to the preamplifier. The voltage signal (Vm) was recorded differentially between these two inputs, which determined the amount of current passed through the current electrode (ME2). The current electrode (ME2) was connected via a Ag/AgCl wire to a X10 MGL headstage (clamping amplifier). Command potentials (10 mV for 20 msec) were used to tune the clamping amplifier. The phase lag was set fully anticlockwise (as recommended in The Axon Guide) and time constant set between 0.2 and 2 msec. The amplifier gain was adjusted to obtain a rectangular voltage trace for the command potential and fast capacitative current transients (without oscillation) for the current trace.

2.3.1 Whole-cell recordings from cRNA-injected oocytes

Electrophysiological recording techniques for the oocyte have been described in detail (Stühmer, 1992). A cRNA-injected oocyte was placed in a recording chamber and constantly perfused (at 5 ml min^{-1}) with Ringer's solution containing (mM): NaCl 110, KCl 2.5, HEPES 5, $BaCl_2$ 1.8, pH 7.5. Here, Ba^{2+} replaced Ca^{2+} in Ringer's solution to prevent the activation of Ca^{2+}-activated Cl^- channel, a major endogenous channels in oocytes. A suction pipette in the recording chamber was used to control the level of perfusion fluid and remove waste. The oocyte was viewed in the recording chamber with a Prior microscope (7-40x, World Precision Instruments INC. Florida, U.S.A.). The membrane of the oocyte was penetrated by two microelectrodes, made from 1.5 mm O.D. borosilicate glass (Clark Electromedical Instruments, Reading, U.K.) and prepared using a vertical puller (David Kopf Instruments, California, USA). Each microelectrode had a tip resistance of 1-2 MΩ when filled with 3.0 M KCl. The oocyte was impaled equatorially with both electrodes. The upper ends of microelectrodes were plugged with vaseline to prevent evaporation of the KCl solution in the electrode.

A twin-electrode amplifier (Axoclamp 2B, Axon Instruments, CA, USA) was used for voltage-clamp recordings in oocytes. For healthy oocytes, the resting membrane potential was in the range of –40 to –80 mV. Insertion of the current electrode brought a small reduction in membrane potential. On insertion of both electrodes, a small depolarizing current pulse (40 nA, 0.2 Hz, 1000 ms) was applied for 20 min to monitor the recovery of membrane potential and estimate the input resistance. The input resistance was in a range of 1-2 MΩ. Subsequently, a desired holding

potential –30 to –50 mV (unless stated otherwise) was set. All recordings were made at room temperature (18° C). Holding currents to maintain a desired clamp potential were between +20 to –40 nA. Higher holding currents suggested a leaky cell membrane. No further study was carried out if the holding current was over 40 nA during experiments. Electrophysiological data were recorded on a chart recorder (Gould 2200s, Ilford, U.K.).

2.3.2 Technical difficulties in voltage clamping of the oocyte

Two technical difficulties in voltage clamping of oocytes arise because of the large size of these cells. Firstly, the time constant (τ) of charging the membrane capacitance is slow for big cells, which have a large capacitance (C_m). The time constant is determined by the equation: $\tau = R_s \cdot C_m$, where R_s is the series resistance. For channels that activate and inactivate very quickly, important kinetic information will be lost or distorted during the settling time of clamp. Because of its large size, several milliseconds are required for the membrane to be clamped to the desired value (Stühmer & Parekh, 1995). This has no complication in the present study because drug-incduced membrane currents occurred slowly in oocytes expressing P2X receptors or VR1 receptors (>> 10 ms). Secondly, high levels of receptor expression can result in membrane currents well beyond 10 μA. With currents of this magnitude, substantial voltage errors arise due to potential drop and failure of voltage-clamp. In the present study, the amplitude of agonist-activated currents at P2X or VR1 receptors were in the range of 0.2 to 2 μA. Thus, clamping voltage errors brought about by currents of high amplitude were negligible in the present study. In addition,

endogenous ion channels are of importance when the heterologously expressed receptors give rise to small currents (<100 nA). However, endogenous currents were inconsequential in this study.

Two other disadvantages of the oocyte system include different post-translational modifications from native cells and inability to control the intracellular ionic composition by electrode dialysis. One further concern is that oocytes at stage IV are not perfect spheres. The membrane capacitance is 6.5 times larger than expected for a lipid bilayer (1 $\mu F\ cm^{-2}$) estimated for most other cells and would suggest oocytes membrane possesses many microvilli (Dascal *et al.*, 1987; Baumgartner *et al.*, 1999). This membrane property further limits the clamp settling speed and distorts kinetics of tested channels. Despite these limitations, the oocyte is still a useful and convenient expression system.

2.4 PREPARATION AND ELECTROPHYSIOLOGICAL RECORDINGS OF CHROMAFFIN CELLS

2.4.1 Chromaffin cell preparation

Chromaffin cells were obtained from the adrenal medullae of 17-day old male Sprague-Dawley rats and 17-day old male Duncan Hartley guinea-pigs. Briefly, rats and guinea-pigs were killed by inhalation of a rising concentration of CO_2 and cervical dislocation. The adrenal glands were rapidly dissected out, cut transversely into 2-4 pieces and the darker cortex trimmed away from the medullary tissue and discarded. The adrenal medullae were minced and dissociated by incubation in 4 ml Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution with 10 mM HEPES pH 7 buffer (HBSS) (Life

Technologies, Paisley, U.K.) containing 1.5 mg/ml collagenase (Class-II, Worthington Biochemical Corporation, Reading, U.K.) and 6 mg/ml bovine serum albumin (Sigma, Poole, U.K.) at 37°C for 40 min. The adrenal tissue was subsequently incubated in 4 ml HBSS containing 1 mg/ml trypsin (Sigma) at 37°C for 15 min and then triturated with a fire polished glass pipette followed by centrifugation at 900 r.p.m. for 5 min. The dispersed cells were re-suspended in Dulbeccos's modified Eagle's medium (DMEM) (Life Technologies) supplemented with 10% bovine serum, 2 mg/ml $NaHCO_3$, 5.5 mg/ml glucose, 200 IU/ml penicillin and 200 μg/ml streptomycin. Cells were plated in 35-mm culture dishes (Falcon) coated with collagen, or treated with 10 mg/ml laminin (Sigma) at room temperature for 1 h. Cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and used after 1-7 days.

2.4.2 Electrophysiological recording from adrenal chromaffin cells

Whole-cell voltage-clamp recording was carried out at room temperature using an Axopatch 200B amplifier (Axon Instruments, California, U.S.A.). Patch pipettes were pulled from thin-walled glass capillary tubes with fine filament (GC 150 TF; Clark Electromedical Instruments, Reading, U.K.), using a two-stage puller (PP-830, Narishige, Tokyo, Japan), fire polished and filled with a solution containing (mM): citric acid 56, $MgCl_2$ 3, CsCl 10, NaCl 10, HEPES 40, EGTA 0.1, TEACl 10, pH 7.2 (adjusted with CsOH). A Cs^+ based pipette solution was used to prevent any complications arising from the activation of voltage- or Ca^{2+}- activated K^+ currents. These pipettes had a resistance of 2-5 MΩ when measured in the bath solution containing (mM): NaCl 154, KCl 4.7, $MgCl_2$ 1.2, $CaCl_2$ 2.5, glucose 5.6, HEPES 10,

pH 7.4. Drugs were applied rapidly through a seven-barrel manifold composed of fused glass capillaries inserted into a common outlet tube with a tip diameter of ~ 200 μm (Dunn *et al.*, 1996). Solutions were delivered by gravity flow from independent reservoirs placed above the preparation. The bath was continuously perfused with external solution at a flow rate of 0.5 ml/min. Suramin, cibacron blue 3GA (Reactive blue 2) or PPADS were applied for 2 minutes prior to and during the ATP application. Unless indicated otherwise, membrane potential was held at –70 mV. A 2 min interval between agonist applications was found to be sufficient to obtain reproducible responses to ATP. Furthermore, there was no indication of any interactions between DMPP and ATP responses when they were separated by a 2 min interval. Signals were filtered at 2 KHz and either stored on a digital tape recorder (Biologic DTR 1205) or recorded using a Gould TA240 chart recorder.

2.5 SOLUTIONS AND DRUGS

All common salts were AnalaR grade (Aldrich Chemicals, U.K.). Adenosine 5′-triphosphate disodium salt (ATP) was purchased from Boehringer (Mannheim, Germany). Adenosine-5′-*O*-(3-trio)triphosphate (ATPγS), P^1, P^5-diadenosine pentaphosphate (Ap_5A), α,β-methylene ATP (α,β-meATP), β,γ-methylene ATP (β,γ-meATP), Reactive blue-2 (RB-2), uridine 5′-triphosphate (UTP), 8-methyl-N-vanillyl-6-nonenamide (capsaicin), monensin and 5'-adenylic acid deaminase (from *Aspergillus sp.*) were purchased from Sigma Chemical Co. (St Louis, U.S.A. or Poole, U.K.). Diadenosine polyphosphates (Ap_3A, Ap_4A, Ap_5A) and phosphodiesterase (EC.3.1.15.1) (from *Crotalus durissus*) were purchased from Boehringer (Mannheim,

Germany). 2-methylthioadenosine triphosphate tetrasodium (2-MeSATP), pyridoxal-5'-phosphate-6'-azophenyl-2′,4′-disulphonic acid (PPADS) were purchased from RBI-SEMAT (U.K.) and 2′,3′-*O*-trinitrophenyl-ATP (TNP-ATP) from Molecular Probes. Suramin was a gift from Bayer plc (Berkshire, U.K.). Solutions of agonists and antagonists were prepared daily from a stock solution (10 or 100 mM, stored frozen) made up in extracellular bathing solution.

Solutions were delivered by gravity flow from independent reservoirs placed above the preparation. Applications of agonists were separated by a 5 min interval for $P2X_2$ receptors, and a 20 min interval for other P2X receptor subtypes and VR1 receptors. With $P2X_2$ and $P2X_3$ expressed together, applications of agonist were separated by 20 min intervals, unless otherwise stated. At the concentrations stated in the text, P2 antagonists TNP-ATP, suramin, PPADS, RB-2, diinosine polyphosphate (Ip_nI, n=3, 4 and 5) were applied for 1 min prior to, and during agonist applications. Zn^{2+} (at the concentrations showed in the text) was added simultaneously with agonist of P2X receptors. The pH of solutions containing agonists and antagonists was adjusted to 7.45 with 1 N NaOH or 1N HCl immediately before experiments. The acid pH (5.5 and 6.5) and alkaline pH (8.0) of bathing Ringer's solutions was adjusted by addition of either 1.0 N HCl or 1.0 N NaOH before experiments.

2.6 DATA ANALYSIS

Data were expressed as either a percentage of the maximum current evoked by ATP, or by α,β-meATP, or as a percentage of the first response observed in the absence of an antagonist. In the study of antagonism, at least two control agonist applications

were carried out prior to antagonist superfusion. EC_{50} values for agonists were taken from Hill plots using the transform log (*I*/*I*max-*I*), where *I* is 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 v2.05, GraphPad). Data are presented as mean ± s.e.mean. Student's *t* test was used; *p* values ≤ 0.05 were considered significant, n refers to the number of cells tested.

2.7 LIST OF ABBREVIATIONS

ATP: adenosine 5'-triphosphate

ATPγS: adenosine-5´-*O*-3-thio-triphosphate

Ap_5A: P^1, P^5-diadenosine pentaphosphate

Capsaicin, 8-methyl-N-vanillyl-6-nonenamide

cRNA: capped ribose nucleic acid

DIDS: 4,4´-diisothiocyanostilbene-2,2´-disulphonic acid

DMEM: Dulbeccos's modified Eagle's medium

DMPP: dimethylphenylpiperazinium iodide

DRG: dorsal root ganglion

EGTA: ethylene glycol- bis[β-aminoethylether]-N,N,N',N'-tetraacetic acid

HBSS: Hank's balanced salt solution

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

I_{ATP}: ATP-activated membrane current

IC_{50}: concentration causing 50% reduction of agonist response

Ip_3I: diinosine triphosphate

Ip_4I: diinosine tetraphosphate

Ip_5I: diinosine pentaphosphate

α,β-meATP: α,β-methylene ATP

β,γ-meATP: β,γ-methylene ATP

L-β,γ-meATP: L isomer of β,γ-methylene ATP

2-MeSATP: 2-methylthio ATP

mRNA: messenger RNA

KN-62: 1-[N,*O*-bis(5-isoquinolinesulphonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine

MRS 2220: cyclic pyroxidine-$\alpha^{4,5}$-monophosphate-6-azophenyl-2′,5′-disulphonic acid

NF023: 8,8'-(carbonylbis(imino-3,1-phenylene carbonylimino))bis-(1,3,5-naphthalenetrisulphonic acid)

NF279: 8,8'-(carbonylbis(imino-4,1-phenylene carbonylimino-4,1- phenylene carbonylimino))bis-(1,3,5-naphthalenetrisulphonic acid)

n_H: Hill coefficient

pA_2: negative log of antagonist concentration causing a 2 fold decrease in agonist potency

pEC_{50}: negative log of agonist concentration causing 50% of maximum effect

pIC_{50}: negative log of antagonist concentration causing 50% reduction of agonist response

PPADS: pyridoxal-5'-phosphate-6-azophenyl-2',4'-disulfonic acid

P5P: pyridoxal-5'-phosphate

RB-2: Reactive blue-2

TNP-ATP: 2′,3′-*O*-(2,4,6-trinitrophenyl)-ATP

UTP: uridine 5′-triphosphate

V_H: holding potential

VR1 receptor: vanilloid receptor type 1 receptor

Photograph 2.1 *Xenopus* Oocytes of different stages of maturity. In stage V and VI, oocytes are pigmented dark brown in the animal hemisphere. The vegetal hemisphere shows the yellow colour of the egg yolk.

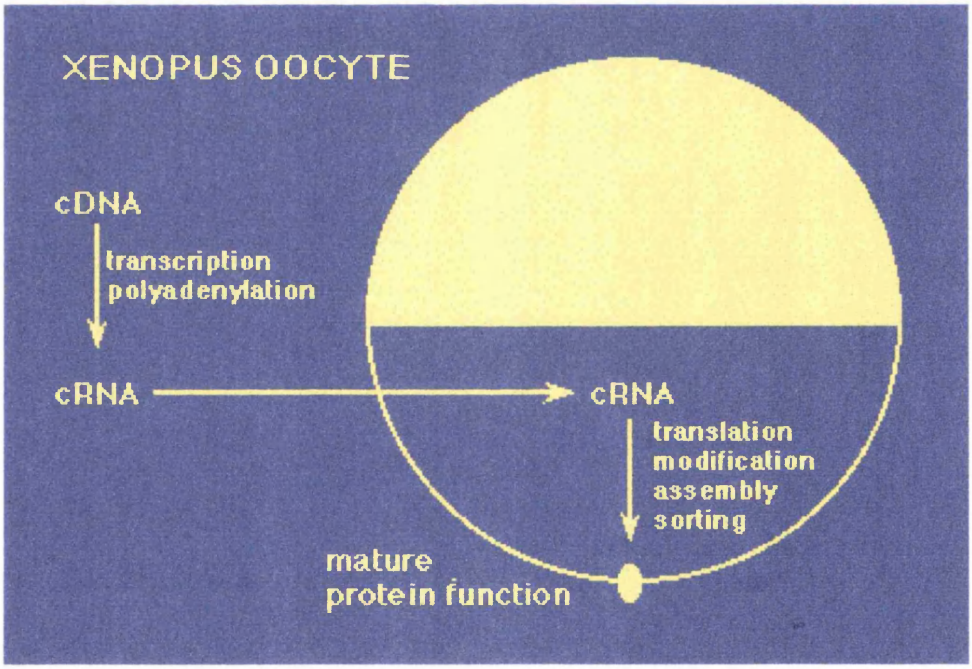


Figure 2.1 Schematic representation of cRNA microinjection into the *Xenopus* oocytes.

SECTION A

CHAPTER 3

COEXPRESSION OF $P2X_3$ AND $P2X_2$ RECEPTOR SUBUNITS IN VARYING AMOUNTS GENERATES HETEROGENEOUS POPULATIONS OF P2X RECEPTORS THAT EVOKE A SPECTRUM OF AGONIST RESPONSES COMPARABLE TO THAT SEEN IN SENSORY NEURONS

3.1 ABSTRACT

Using voltage-clamp procedures on *Xenopus* oocytes, agonist-evoked ionic currents by P2X receptors resulting from the coexpression of $P2X_2$ and $P2X_3$ subunits were compared against the agonist responses of homomeric $P2X_2$ and $P2X_3$ receptors. With the quantity of $P2X_3$ mRNA kept constant and quantity of $P2X_2$ mRNA progressively increased, expressed P2X receptors changed from a $P2X_3$-like receptor to a $P2X_2$-like receptor. In all cases, however, agonist-evoked responses comprised biphasic (fast and slow) currents - the former showing the properties of $P2X_3$ receptors and latter consistent with the presence of $P2X_2$ and $P2X_{2/3}$ receptors. Using desensitisation procedures, the $P2X_3$-like fast current was selectively removed to allow the slow current to be studied in isolation. $P2X_{2/3}$ receptors were then characterised by slowly inactivating inward currents that were reproducible within 30 seconds of washout and whose pharmacological profile (*selective* agonists, Ap_5A > α,β-meATP >> β,γ-meATP > UTP; antagonists, TNP-ATP >> suramin ≥ RB-2) contrasted with the profile of $P2X_2$ receptors (Ap_5A, α,β-meATP, β,γ-meATP and UTP inactive; antagonists, RB-2 > TNP-ATP > suramin). Thus, these experiments reveal that coexpression of two P2X subunits which of themselves can generate functional homomeric receptors results in a complex population of heterogeneous P2X receptors – in this case $P2X_2$, $P2X_3$ and $P2X_{2/3}$ receptors. Depending on the relative levels of P2X subunit coexpression, the operational profile of the resultant P2X receptors can change from one phenotype to another. This spectrum may explain the variability of agonist responses in small sensory neurons that also express $P2X_2$ and $P2X_3$ subunits in different amounts.

3.2 INTRODUCTION

P2X receptors represent a family of ATP-gated ion-channels that play a significant role in fast excitation and synaptic transmission in many excitable tissues (Ralevic and Burnstock, 1998). As far as sensory neurons are concerned, P2X receptors have been implicated in the direct excitation of primary afferent nerve fibres (Bland-Ward and Humphrey, 1997; Cook *et al.*, 1997; Dowd *et al.*, 1998; Burgard *et al.*, 1999; Hamilton and McMahon, 2000), in ascending excitation of second order sensory neurons in the dorsal horn of the spinal cord and dorsomediolateral nuclei of the brainstem (Li & Perl, 1995; Bardoni *et al.*, 1997; Gu and MacDermott, 1997; Scisclo *et al.*, 1997; Tsuda *et al.*, 1999b) and in the excitation of higher order neurons in the CNS (Driessen *et al.*, 1998; Ralevic *et al.*, 1999).

Thus far, cDNA sequences for seven P2X subunit proteins ($P2X_{1-7}$) have been cloned from mammalian tissues. These P2X subunit proteins can combine to form ion-channels, as homomeric and heteromeric assemblies of three, possibly four, P2X subunits (Kim *et al.*, 1997; Nicke *et al.*, 1998; Torres *et al.*, 1999). Transcripts for six subunits ($P2X_{1-6}$) have been found in sensory neurons (Chen *et al.*, 1995; Collo *et al.*, 1996; Cook *et al.*, 1997). However, immunohistochemical studies reveal a predominance of $P2X_3$-like protein in the cell bodies of sensory ganglia and associated sensory nerve endings in skin and lamina II of the spinal cord. $P2X_3$-like immunoreactivity frequently co-localises with $P2X_2$-like immunoreactivity in sensory neurons (Cook *et al.*, 1997; Bradbury *et al.*, 1998; Vulchanova *et al.*, 1998; Xiang *et al.*, 1998). Small sensory DRG neurons containing $P2X_3$-immunopositive material also contain specific biochemical markers for nociceptor cells (Chen *et al.*, 1995; Bradbury *et al.*, 1998).

It is well known that sensory neurons show a marked variability in the time course of responses to P2X receptor agonists. Agonist-evoked ion currents in many rat DRG and trigeminal ganglion neurons are known to activate and inactivate rapidly, in a manner similar to agonist responses of homomeric $P2X_3$ receptors (Chen *et al.*, 1995; Cook *et al.*, 1997; Rae *et al.*, 1998). Such fast agonist responses are absent in sensory neurons of $P2X_3$-null (-/-) animals (Cockayne *et al.*, 2000). Rat nodose ganglia and bullfrog DRG neurons often respond to P2X agonists with slowly inactivating ionic currents that are similar to the responses of heteromeric $P2X_{2/3}$ receptors (Bean, 1990; Khakh *et al.*, 1995; Lewis *et al.*, 1995). Furthermore, some DRG neurons produce composite responses with both rapidly and slowly decaying ion currents (Burgard *et al.*, 1999; Grubb and Evans, 1999).

These differences in the operational profile of native P2X receptors may be due to different levels of expression of $P2X_2$, $P2X_3$, and $P2X_{2/3}$ receptors in individual sensory neurons (Burgard *et al.*, 1999). Here, I have investigated the operational profiles of the recombinant P2X receptors generated by co-expressing varying amounts of $P2X_2$ and $P2X_3$ subunits in *Xenopus* oocytes. The resultant spectrum of operational profiles was attributed to a heterogeneous mixture of homomeric and heteromeric P2X receptors and this mixture might conveniently explain the range of operational profiles of sensory neurons that also express $P2X_2$ and $P2X_3$ receptor subunits in varying amounts.

3.3 METHODS

Preparation of Oocytes and Expression of Recombinant P2X Receptors

Briefly, defolliculated oocytes (stages V and VI) were injected (40 nl) cytosolically with capped ribonucleic acid (cRNA) encoding either rat $P2X_2$ or rat $P2X_3$ receptor subunits. In coexpression experiments, oocytes were injected with a mixture of cRNAs prepared

at 4 concentration ratios (1:500, 1:100, 1:50 and 1:20), by mixing $P2X_2$ cRNA (2, 10, 20, and 50 μg ml^{-1}) with an equal volume of $P2X_3$ cRNA (1 mg ml^{-1}). 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 sulphate 50 μg l^{-1} for 48 h to allow full receptor expression and then stored at 4° C for up to 12 days.

Solutions and Electrical Recording of cRNA-Injected Oocytes

Briefly, 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 3 M KCl. Oocytes were perfused constantly (at 5 ml min^{-1}) with Ringer's 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) and at a holding potential of –50 mV (unless stated otherwise). Electrophysiological data were recorded on a chart recorder (Gould 2200S; Ilford, UK).

Solutions were delivered by gravity flow from independent reservoirs placed above the recording chamber. Applications of agonists were separated by a 20 min interval, unless otherwise stated. The P2 receptor antagonists TNP-ATP (0.003-1 μM), suramin (0.01-100 μM), PPADS (0.3-100 μM), RB-2 (0.03-100 μM) and Ip_5I (0.01-100 μM) were applied for 1 min prior to, and during, agonist application. When constructing concentration-response curves for P2X agonists, data were normalized to the agonist response evoked by 100 μM α,β-meATP. EC_{50} values for P2X agonists were taken from Hill plots, where the transform log (I/I_{max}-I) was used (I being 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). Data are presented as mean ± s.e.mean for the given number of observations (n). Student's unpaired *t* test was used and *p* values ≤ 0.05 were considered significant.

3.4 RESULTS

Three groups of cRNA-injected defolliculated oocytes were tested. The first and second groups comprised oocytes injected with $P2X_2$ or $P2X_3$ cRNAs, respectively. The third group of oocytes were injected with a fixed amount of $P2X_3$ cRNA and varying amounts of $P2X_2$ cRNA. The first and second groups behaved as previously observed for homomeric $P2X_2$ and $P2X_3$ receptors (King, 1998). The third group of oocytes showed a spectrum of operational profiles that ranged from predominantly $P2X_3$-like to predominantly $P2X_2$-like.

Use of α,β-meATP to Distinguish Types of P2X Receptors

α,β-meATP reliably distinguishes homomeric $P2X_2$ receptors from homomeric $P2X_3$ receptors (Fig. 3.1A). α,β-meATP (100 μM) did not activate any detectable current at $P2X_2$ receptors, whilst a rapidly activating and rapidly inactivating current was evoked at $P2X_3$ receptors. Sham-injected control oocytes did not produce any detectable responses to α,β-meATP (100 μM).

Application of α,β-meATP to oocytes coinjected with mixed cRNAs gave rise to biphasic responses comprising an initial rapidly activating and rapidly inactivating current (I_1) followed by a slowly rising, slowly inactivating component (I_2). The relative amplitudes of fast (I_1) and slow (I_2) currents varied with the concentration ratio of the P2X cRNAs injected (Fig. 3.1B). As the amount of $P2X_2$ cRNA was elevated

(from a ratio of 1:500 to 1:20), there was a steady increase in the relative fraction of the slowly inactivating component (I_2) at the expense of the rapidly inactivating component (I_1) which was progressively incorporated into, and ultimately obscured by, the slower event.

Some homomeric P2X receptor ion-channels possess binary permeability properties, which can result to biphasic ion currents under certain conditions (Khakh *et al.*, 1999). Other heteromeric P2X receptors ($P2X_{1/5}$ and $P2X_{2/6}$) also produce biphasic ion currents that involve complex kinetics of channel inactivation (Haines *et al.*, 1999; King *et al.*, 2000). However, the fast (I_1) and slow (I_2) components of α,β-meATP-evoked currents appeared to be mediated by two different sets of P2X receptors, since the former could be selectively desensitized by repetitive applications of α,β-meATP (40 s apart) without any significant change in the latter component (Fig. 3.1C). This desensitisation procedure was used later to study the slow current in isolation. Homomeric $P2X_3$ receptors also were found to desensitise fully with repeated applications of α,β-meATP (40 s apart) (data not shown).

Potency and Efficacy of α,β-meATP at P2X Receptors

Concentration/response (C/R) curves for the fast current (I_1) evoked by α,β-meATP were reliably determined for those oocytes injected with mixed cRNAs ratios of 1:500 and 1:100 (Fig. 3.2A). Here, EC_{50} values (and Hill coefficients) for agonism were 1.7 ± 0.3 μM (0.8 ± 0.1; n=7) and 1.6 ± 0.4 μM (0.8 ± 0.2; n=5), respectively. For oocytes injected with mixed cRNAs ratios of 1:50 and 1:20, the amplitude of the fast current to α,β-meATP could not be easily resolved from the slow current (I_2) over the full range of agonist concentrations used and, accordingly, it was not possible to determine EC_{50}

values here. In contrast, α,β-meATP yielded an EC_{50} value of 1.9 ± 0.3 μM (0.8 ± 0.1; n=6) at homomeric $P2X_3$ receptors (Fig. 3.2A).

Slow currents (I_2) evoked by α,β-meATP were more easily monitored in oocytes injected with each of the 4 ratios of mixed P2X cRNAs. Here, EC_{50} values (and Hill coefficients) for agonism were: 8.6 ± 1.2 μM (0.9 ± 0.1; n=7) at 1:500 ratio; 9.5 ± 1.0 μM (0.8 ± 0.1; n=6) at 1:100 ratio; 10.2 ± 1.1 μM (1.0 ± 0.1; n=3) at 1:50 ratio; 10.3 ± 1.1 (0.8 ± 0.1, n=3) at 1:20 ratio (Fig. 3.2B). None of the EC_{50} values for slow responses were significantly different for each mixture of cRNAs used, but were 5-fold higher than corresponding EC_{50} values for the fast response. This difference again supports the notion that two P2X receptors mediated the fast and slow responses to α,β-meATP - most likely homomeric $P2X_3$ and heteromeric $P2X_{2/3}$ receptors, respectively. Under the experimental conditions used, homomeric $P2X_3$ did not give rise to slow responses to α,β-meATP whilst homomeric $P2X_2$ receptors were not activated by this synthetic nucleotide (see Fig. 3.1A).

The above data revealed that EC_{50} values for either fast or slow currents to α,β-meATP are unaffected by altering the mixture of injected P2X cRNAs. However, closer inspection of the amplitude of fast and slow currents revealed a marked difference in agonist efficacy. For a test concentration of 3 μM α,β-meATP (applied at a constant holding potential of –50 mV), the mean amplitude of the fast current (I_1) was approximately 800 nA when a small amount of $P2X_2$ cRNA was injected into oocytes (1:500 ratio), and was significantly lower, approximately 250 nA, when more $P2X_2$ cRNA was injected (1:20 ratio) (Fig. 3.2C). The converse occurred for the amplitude of the slow current (I_2) (Fig. 3.2C). Since the amount of injected $P2X_3$ cRNA was kept constant in these experiments and only the amount of $P2X_2$ cRNA was changed, the observed differences in α,β-meATP efficacy appeared to involve a decrease in the

number of fast-activating $P2X_3$ ion-channels and concomitant increase in the number of slowly-activating $P2X_{2/3}$ ion-channels, presumably as more $P2X_2$ subunits competed for $P2X_3$ subunits to generate heteromeric $P2X_{2/3}$ receptors.

Slow Currents to ATP and α,β-meATP Involve Different P2X Receptors

ATP and other nucleotides were tested on oocytes coinjected with mixed cRNAs (1:500 ratio), then tested again on homomeric $P2X_2$ receptors. EC_{50} values for these nucleotides at different P2X receptors are given in Table 3.1. ATP, 2-MeSATP, ATPγS evoked slow currents at homomeric $P2X_2$ receptors, and also produced slow ion currents in oocytes co-expressing $P2X_2$ and $P2X_3$ subunits. The potency of ATP and 2-MeSATP, but not ATPγS, was lower at homomeric $P2X_2$ receptors than at the P2X receptor population formed by $P2X_2$ and $P2X_3$ subunit coexpression. Furthermore, 4 nucleotides (α,β-meATP, β,γ-meATP, Ap_5A and UTP) were inactive at homomeric $P2X_2$ receptors but were agonists at presumptive $P2X_{2/3}$ receptors (Table 3.1).

Inspection of C/R curves for the above nucleotides showed that ATP, 2-MeSATP and ATPγS had an apparent greater efficacy than α,β-meATP at presumptive heteromeric $P2X_{2/3}$ receptors. In contrast, β,γ-meATP, Ap_5A and UTP showed similar or lower efficacy than α,β-meATP (Fig. 3.3 A,B). Thus, only those nucleotides able to stimulate homomeric $P2X_2$ receptors seemed to have a greater efficacy at heteromeric $P2X_{2/3}$ receptors. Otherwise, nucleotides that did not activate $P2X_2$ receptors showed no better efficacy than α,β-meATP at presumptive $P2X_{2/3}$ receptors. These findings could be explained if $P2X_2$ and $P2X_{2/3}$ receptors were simultaneously generated when $P2X_2$ and $P2X_3$ subunits were co-expressed in oocytes, and if both receptor subtypes contributed towards the slow ion currents evoked by agonists common to each subtype (*i.e.* ATP, 2-MeSATP, ATPγS).

The presence of two distinct P2X receptors were confirmed in desensitisation experiments where ATP and α,β-meATP were applied successively to generate two slow currents. Saturating concentrations of α,β-meATP (100 μM), applied until the evoked slow response had completely desensitized, were always followed by a second slow current when oocytes were challenged with a saturating concentration of ATP (100 μM) (Fig. 3.3C). Where ATP was applied first, however, a successive application of α,β-meATP failed to activate an additional slow response (Fig. 3.3C). These results are compatible with α,β-meATP being an agonist of heteromeric $P2X_{2/3}$ receptors alone, while ATP acts as an agonist of both homomeric $P2X_2$ and heteromeric $P2X_{2/3}$ receptors. Thus, the residual slow response to ATP following desensitisation of the slow-response to α,β-meATP was mediated by $P2X_2$ receptors alone, while the failure of α,β-meATP to generate an additional current after ATP was due to the latter activating then inactivating $P2X_{2/3}$ receptors.

Antagonism of the Heteromeric $P2X_{2/3}$ Receptor

A way was devised to study $P2X_{2/3}$ receptors in isolation, using α,β-meATP to activate $P2X_{2/3}$ receptors and employing a desensitizing procedure of two agonist pulses (40 s apart) to inactivate all homomeric $P2X_3$ receptors present (as shown in Fig. 3.1C). The blocking activity of a series of P2 receptor antagonists was tested on the residual slow response evoked by the second pulse of α,β-meATP. Suramin (0.01-100 μM), RB-2 (0.03-100 μM), PPADS (0.03-100 μM) and TNP-ATP (0.001-1 μM) caused a concentration-dependent inhibition of $P2X_{2/3}$ receptors, whereas Ip_5I (0.01-100 μM) was inactive. IC_{50} values are given in Table 3.2. The heteromeric $P2X_{2/3}$ receptor was remarkably sensitive to TNP-ATP (IC_{50}, 11 nM), a feature shared with the homomeric $P2X_3$ receptor (IC_{50}, 0.3 nM). Except for suramin, all antagonists including TNP-ATP

appeared to work in non-competitive manner although their blocking actions were slowly reversible after washout. For suramin, a pA_2 value of 5.9 ± 0.4 was determined at the heteromeric $P2X_{2/3}$ receptor.

Modulation of the Heteromeric $P2X_{2/3}$ Receptor

The same procedure of α,β-meATP agonism and twin agonist pulses (40 s apart) were used again to study $P2X_{2/3}$ receptors in isolation, and assess the modulatory effects of extracellular H^+ and Zn^{2+} ions. Under these circumstances, alteration in the extracellular pH (pH 8.0-6.5) caused only a modest change in agonist potency at $P2X_{2/3}$ receptors (Fig. 3.4A). These findings contrasted with the strong effect of pH on agonist potency at $P2X_2$ receptors and weak inhibitory effect of H^+ on agonism of $P2X_3$ receptors (see Table 3.3).

Extracellular Zn^{2+} (1-100 μM) applied simultaneously with α,β-meATP (1 μM) caused a modest potentiation of slow responses (Fig. 3.4B), with a maximal effect of 89 ± 29% (n = 4) above control responses. This finding contrasted with the strong potentiation by Zn^{2+} ions (100 μM) of ATP responses at $P2X_2$ receptors by some 1320 ± 90 % (n = 4), and its weak inhibitory action against $P2X_3$ receptors in reducing control responses by 17 ± 2 % (n = 6).

Table 3.1

Agonist potency at homomeric and heteromeric P2X receptors

Nucleotide	[a]$P2X_2$	[b]$P2X_3$	[c]$P2X_2 + P2X_3$ (I_2)
ATP	4.3 ± 0.8	1.4 ± 0.3	1.2 ± 0.3
2-MeSATP	6.9 ± 1.2	0.2 ± 0.03	0.8 ± 0.2
ATPγS	8.4 ± 1.9	1.5 ± 0.4	9.4 ± 1.3
α,β-meATP	inactive	1.9 ± 0.3	8.6 ± 1.2
β,γ-meATP	>300	9.2 ± 2.4	>100
Ap_5A	inactive	0.6 ± 0.1	1.1 ± 0.3
UTP	inactive	> 100	> 100

Agonist potency given in terms of EC_{50} value (μM) (mean ± s.e.mean, n = 4-7).

[a] Determined from the slow responses in oocytes expressing $P2X_2$ subunits alone, at pH 7.4.

[b] Determined from the fast responses in oocytes expressing $P2X_3$ subunits alone.

[c] Determined from peak of slow responses (I_2) in oocytes co-expressing $P2X_2$ and $P2X_3$ subunits.

Table 3.2

Antagonist potency at homomeric and heteromeric P2X receptors

Antagonist	[a]$P2X_2$	[b]$P2X_3$	[c]$P2X_2 + P2X_3$ (I_2)
PPADS	1.6 ± 0.1	0.22 ± 0.05	2.3 ± 0.4
RB-2	0.4 ± 0.1	45 ± 11	5.2 ± 0.9
SURAMIN	10.4 ± 1.2	4.1 ± 0.7	2.4 ± 0.4
TNP-ATP	1.1 ± 0.2	0.00029±0.00004	0.0112±0.0025
Ip_5I	inactive	2.8 ± 0.7	inactive

Antagonist potency given in terms of IC_{50} value (μM) (mean ± s.e.mean, n = 4-7).

[a] Determined against slow responses to ATP (10 μM).

[b] Determined against fast responses to α,β-meATP (10 μM), except Ip_5I which was tested against ATP (3 μM).

[c] Determined against slow responses to α,β-meATP (10 μM) at $P2X_{2/3}$ receptors.

Table 3.3

Effect of pH on agonist potency at P2X receptors

Receptor	pH 6.5	pH 7.5	pH 8.0
[a]$P2X_2$	1.3 ± 0.2	16.2 ± 1.4	28 ± 2
[b]$P2X_3$	2.1 ± 0.5	1.4 ± 0.3	1.4 ± 0.2
[c]$P2X_{2/3}$	1.8 ± 0.4	8.6 ± 1.2	8.3 ± 1.1

Agonist potency given in terms of EC_{50} value (μM) (mean ± s.e.mean n = 4-6) for the stated extracellular pH values.

[a] Determined from slow responses to ATP at $P2X_2$ receptors.

[b] Determined from fast responses to ATP at $P2X_3$ receptors.

[c] Determined from slow responses to α,β-meATP at $P2X_{2/3}$ receptors.

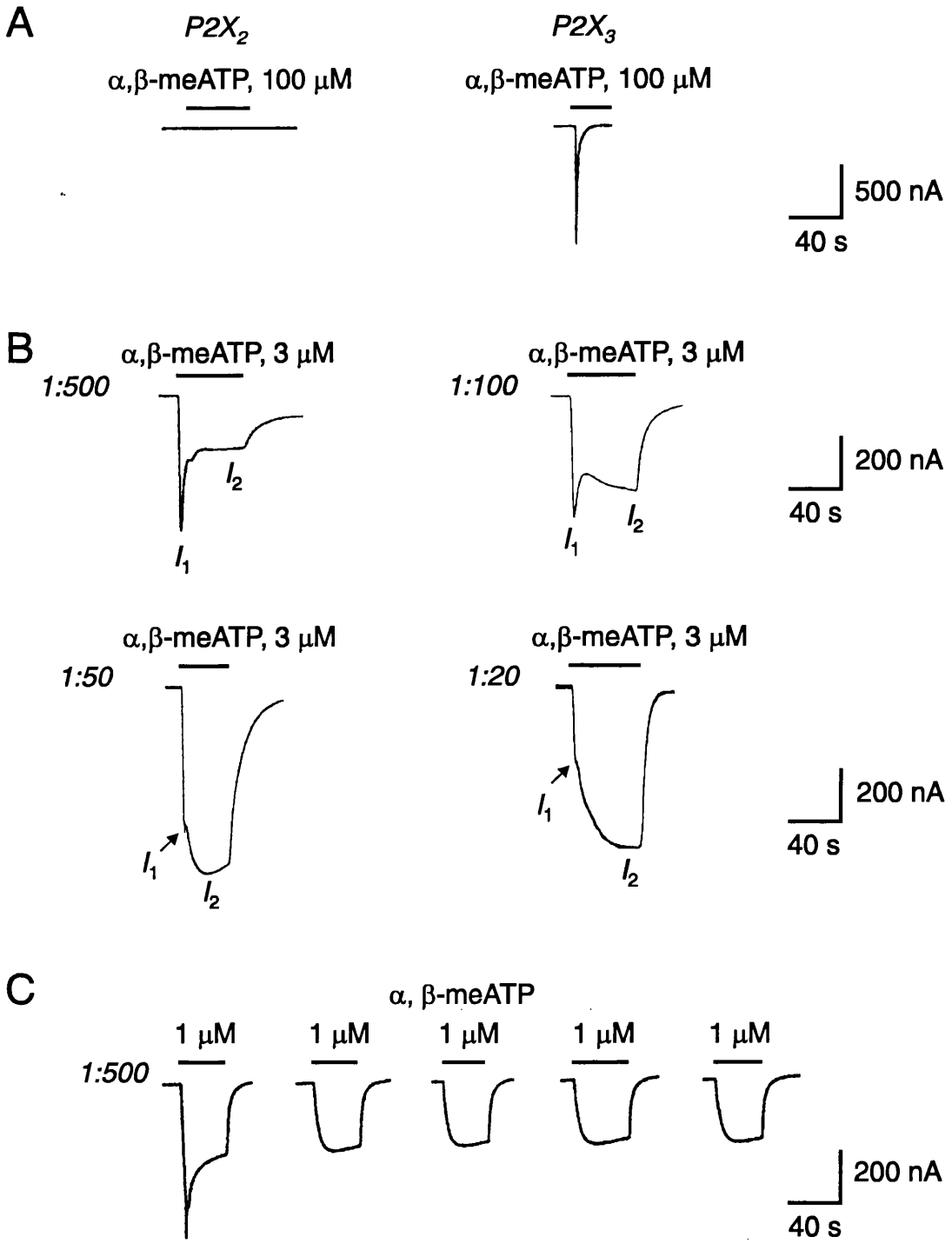


Figure 3.1 In A, α,β-meATP (100 μM) evoked whole-cell inward currents at homomeric $P2X_3$ receptors, but not homomeric $P2X_2$ receptors. In B, whole-cell currents evoked by α,β-meATP (3 μM) in oocytes injected with $P2X_2$ and $P2X_3$ cRNAs (at concentrations of 1:500, 1:100, 1:50 and 1:20). Each recording consisted of fast (I_1) and slow (I_2) inward currents. In C, consecutive applications of α,β-meATP (1 μM, applied 40s apart) evoked inward currents where the initial fast current became desensitized after the first application of the agonist, whilst the amplitude of slow current was unchanged. (A-C, V_h =-50 mV).

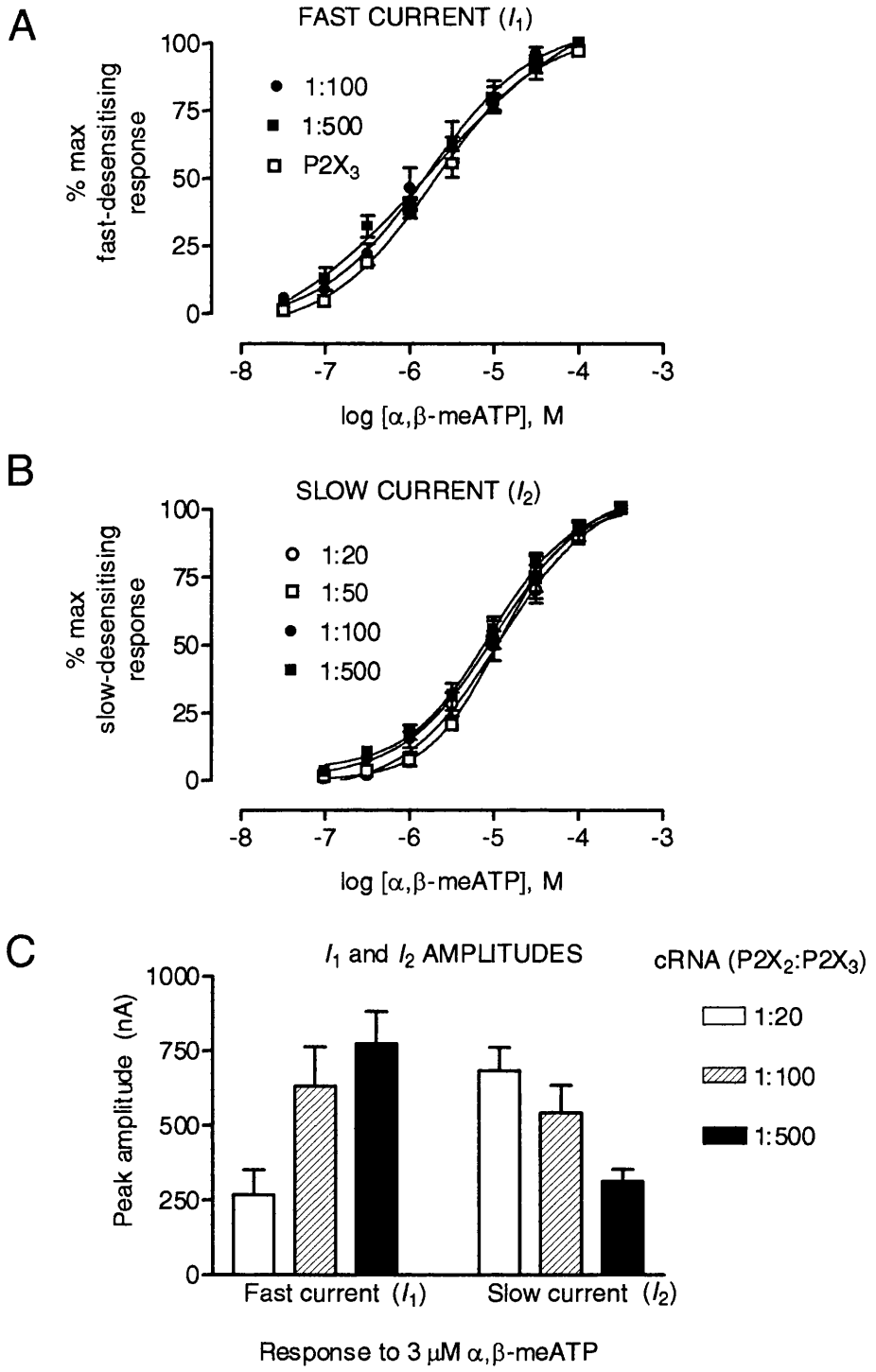


Figure 3.2 In A, concentration/response (C/R) curves are shown for fast currents (I_1) activated by α,β-meATP in oocytes injected with cRNA ratios of 1:500 and 1:100, and oocytes expressing homomeric $P2X_3$ receptors. Here, the agonist was equipotent for fast responses (I_1) and responses of $P2X_3$ receptors. In B, C/R curves are shown for slow currents (I_2) activated by α,β-meATP in oocytes injected with cRNA ratios of 1:500, 1:100, 1:50 and 1:20. Changing the amounts of cRNAs injected failed to influence agonist potency for slow responses (I_2). In C, the mean amplitudes of fast (I_1) and slow (I_2) currents are shown for oocytes injected with cRNAs at 1:20, 1:100, and 1:500 ratios. Here, agonist efficacy for I_1 and I_2 responses was highly dependent on the relative amounts of cRNA injected.

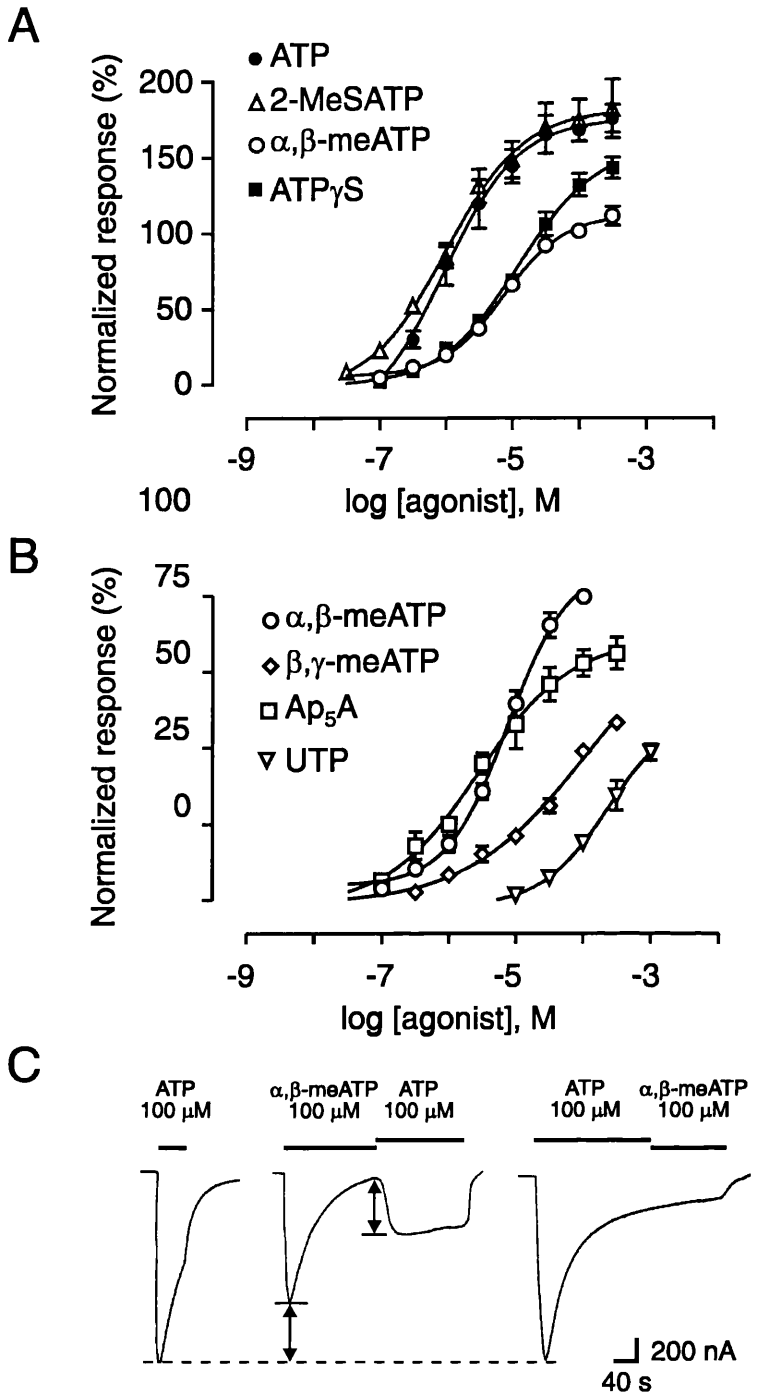


Figure 3.3 In A, concentration-response (C/R) curves are shown for slow currents (I_2) activated by a series of nucleotides at P2X receptors formed by coexpression of $P2X_2$ and $P2X_3$ subunits. The maximum (or efficacy) response to ATP, 2-MeSATP and ATPγS was markedly greater than that of α,β-meATP. In B, C/R curves for slow currents activated by a second series of nucleotides at P2X receptors formed by $P2X_2$ and $P2X_3$ subunits coexpression. The efficacy of Ap_5A, β,γ-meATP and UTP was similar to, or less than, that of α,β-meATP. In C, the successive application of α,β-meATP and ATP activated two independent slow currents. The sum of these two slow currents (see arrows) equalled the amplitude of an earlier response to ATP, where α,β-meATP desensitized had not yet occurred.

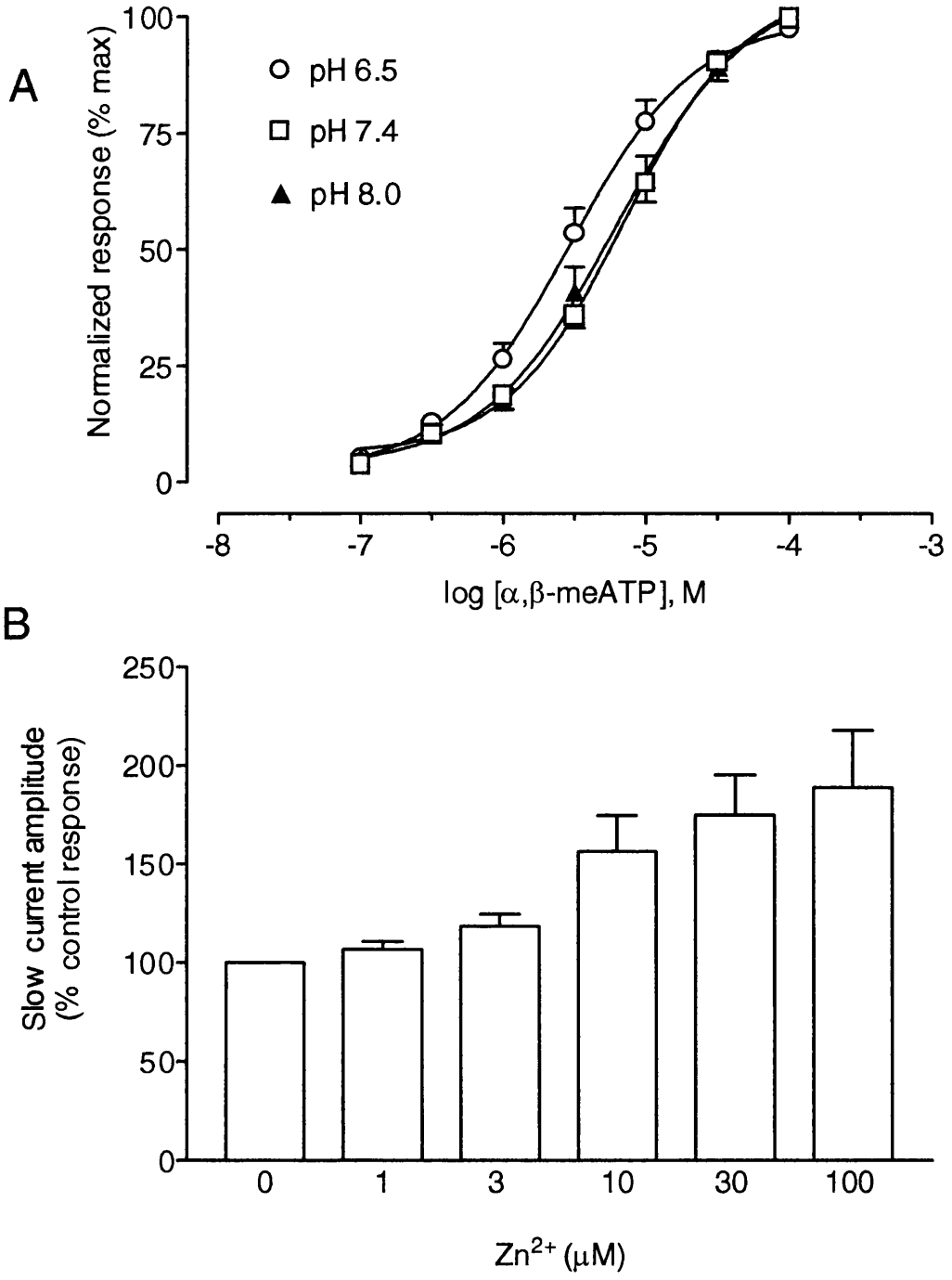


Figure 3.4 In A, concentration-response (C/R) curves are shown for slow currents (I_2) activated by α,β-meATP at $P2X_{2/3}$ receptors for the given extracellular pH levels. In B, effect of extracellular Zn^{2+} ions on the amplitude of slow currents activated by α,β-meATP (1 μM) at $P2X_{2/3}$ receptors.

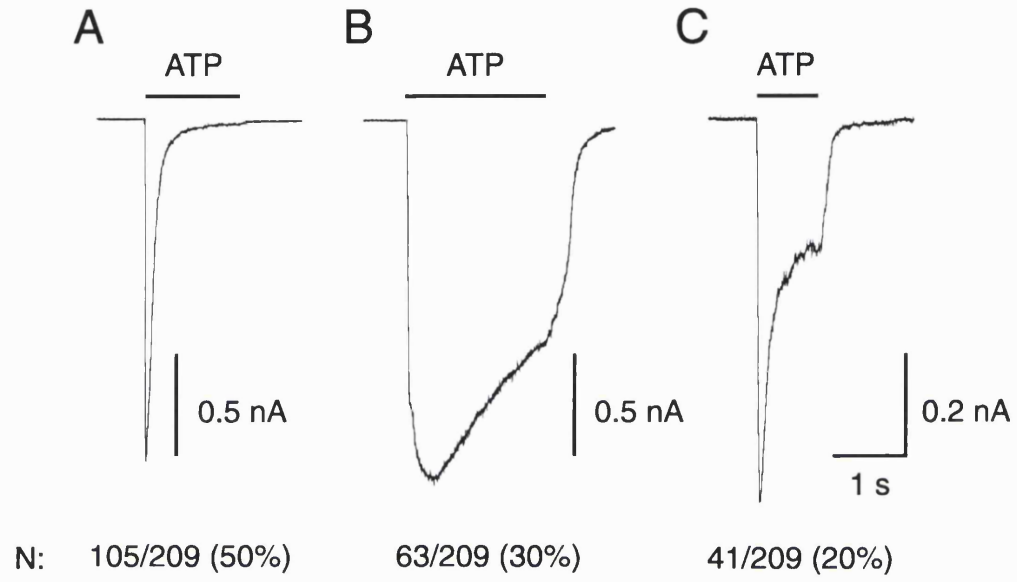


Figure 3.5 Whole-cell inward currents activated by ATP (10 μM) in rat neonatal DRG neurons. Three phenotypes were observed in 209 cells studied under patch-clamp conditions (V_h = -60 mV). (Reproduced with permission from Dr. P.M. Dunn, who carried out the survey of ATP-activated currents in rat DRG neurons).

3.5 DISCUSSION

The present study was carried out for two reasons. First, I wished to characterise the pharmacological profile of the $P2X_{2/3}$ receptor, about which surprisingly little is known apart from α,β-meATP agonism (Lewis *et al.*, 1995) and TNP-ATP antagonism (Thomas *et al.*, 1998). Second, I wished to shed further light on the variability of P2X responses in sensory neurons, in which $P2X_2$ and $P2X_3$ subunits commonly occur and where $P2X_{2/3}$ receptors may play a role in sensory transmission.

Sensory neurons show a marked variability in the time course of responses to P2X receptor agonists. Grubb and Evans (1999) reported that the majority (80%) of adult rat DRG neurons responded to nucleotides with a $P2X_3$-like phenotype, and the remainder possessed non-inactivating P2X receptors of undetermined phenotype. Burgard and colleagues (1999) reported similar findings in adult rat DRG neurons, where 70% of IB4-labelled nociceptors showed fast-desensitizing $P2X_3$-like agonist responses and remaining 30% showed either mixed (fast and slow responses) or slow responses. To exemplify these phenotypes, I carried out a brief survey of ATP responses in rat neonatal DRG neurons and found qualitatively and quantitatively similar results (see Fig. 3.5). In this exploratory survey, 50% of small DRG neurons produced fast $P2X_3$-like ATP responses, 30% gave slowly desensitizing responses and 20% mixed responses. Burgard and colleagues (1999) have attributed the last phenotype to mixed P2X receptors resulting from the coexpression of $P2X_2$ and $P2X_3$ subunits, and this observation motivated me to study the pharmacological properties of heteromeric $P2X_{2/3}$ receptors as fully as possible.

Coexpression of $P2X_2$ and $P2X_3$ subunits in approximately equal amounts in HEK 293 cells was reported, in the first study of its kind, to generate a hybrid $P2X_{2/3}$ receptor that showed the agonist profile of $P2X_3$ receptors and inactivation properties of $P2X_2$

receptors (Lewis *et al.*, 1995). However, I have found that the outcome of P2X subunit coexpression is not straightforward. Where $P2X_3$ expression was kept constant and the level of $P2X_2$ expression gradually increased, the resultant P2X receptors changed their phenotype from predominantly $P2X_3$-like to a mixed phenotype of $P2X_2$, $P2X_3$ and $P2X_{2/3}$ receptors. Thus, coexpression of two P2X subunits, where each can independently form functional homomeric receptors, seems to generate a heterogeneous population of P2X receptors – in this case $P2X_2$, $P2X_3$ and $P2X_{2/3}$ receptors. This finding suggests that the process of P2X receptor assembly occurs in a stochastic manner, the probability of forming either homomeric or heteromeric receptors being dependent on the relative numbers of the two types of P2X subunit available for receptor assembly.

For each of the four levels of $P2X_2/P2X_3$ subunit coexpression studied, the resultant population of recombinant P2X receptors gave rise to complex agonist responses. These responses involved an initial fast current that, I believe, was mediated by a small population of homomeric $P2X_3$ receptors. This conclusion was based in part on the finding that α,β-meATP was equipotent for the $P2X_3$-like fast current and homomeric $P2X_3$ receptor itself, and α,β-meATP caused prolonged desensitisation in both cases. Also, other $P2X_3$ receptor agonists (*e.g.* Ap_5A) evoked fast currents in those oocytes co-expressing $P2X_2$ and $P2X_3$ subunits. Additionally, I have already shown that Ip_5I blocks the $P2X_3$-like fast current, blocks $P2X_3$ receptors and also blocks the fast current of DRG sensory neurons with similar IC_{50} values (King *et al.*, 1999; Dunn *et al.*, 2000). Lastly, the amplitude of the $P2X_3$-like fast current was reduced as more $P2X_2$ subunits were expressed - a consequence, I believe, of the reduction in the numbers of $P2X_3$ receptors as their constituent subunits were incorporated instead into $P2X_{2/3}$ receptors.

The initial $P2X_3$-like fast current was followed by a slow current that, in all probability, involved $P2X_{2/3}$ receptors when α,β-meATP was the agonist, and both $P2X_2$ and $P2X_{2/3}$ receptors when ATP was the agonist. Significantly, other studies of $P2X_2$ and $P2X_3$ coexpression have also alluded to the dual formation of $P2X_2$ and $P2X_{2/3}$ receptors (Thomas *et al.*, 1998; Ueno *et al.*, 1998). A slow current to α,β-meATP is a recognized signature of $P2X_{2/3}$ receptors (Lewis *et al.*, 1995; Ueno *et al.*, 1999), but surprisingly little else can be said with certainty about its agonist profile. Agonism of $P2X_{2/3}$ receptors was re-assessed recently (Bianchi *et al.*, 1999), although these investigators may not have taken into account the dual formation of $P2X_2$ and $P2X_{2/3}$ receptors in their expression system.

The $P2X_{2/3}$ receptor was further characterised in my experiments by an agonist potency order of Ap_5A > α,β-meATP >β,γ-meATP > UTP, which took into consideration that none of these nucleotides can activate $P2X_2$ receptors. In all probability, ATP, 2-MeSATP and ATPγS are agonists at $P2X_{2/3}$ receptors also, but a true assessment of their potency will require the discovery of selective $P2X_2$ receptor antagonists to strip away the complication of co-activating $P2X_2$ and $P2X_{2/3}$ receptors. The agonist potency order for homomeric $P2X_3$ receptors was Ap_5A > α,β-meATP > β,γ-meATP > UTP (setting aside data for ATP, 2-MeSATP and ATPγS), which matched the rank order for heteromeric $P2X_{2/3}$ receptors although the EC_{50} values for these two P2X subtypes are not identical (see Table 3.1).

The antagonist potency order at $P2X_{2/3}$ receptors was TNP-ATP > PPADS = suramin > RB-2, and Ip_5I inactive. These antagonists were tested only on slow currents to α,β-meATP and, therefore, any interaction of these blocking agents with $P2X_2$ and $P2X_3$ receptors can be discounted. TNP-ATP was potent in the near nanomolar concentration range, in agreement with an earlier study (Thomas *et al.*, 1998).

The possibility existed that the heteromeric $P2X_{2/3}$ receptor occurred in two forms. Assuming a trimeric assembly, the heteromeric complex could comprise two $P2X_2$ subunits and one $P2X_3$ subunit or one $P2X_2$ subunit and two $P2X_3$ subunits. My experiments could not directly resolve this issue. However, my analysis of the inhibition curve (n_H ~1) suggested only a single population of heteromeric $P2X_{2/3}$ receptors. Nevertheless, such analysis was open to interpretation and was somewhat inconclusive. PPADS, suramin and RB-2 also blocked the $P2X_{2/3}$ receptor in micromolar concentrations, where the potency order (PPADS = suramin > RB-2) differed from that for homomeric $P2X_2$ receptors (RB-2 > PPADS > suramin) and $P2X_3$ receptors (PPADS > suramin > RB-2). Regarding my negative results with Ip_5I, it is of note that slow currents to α,β-meATP in sensory neurons (nodose and DRG) are similarly unaffected by this dinucleotide (Dunn *et al.*, 2000), and provides indirect proof that such slow responses probably involve native $P2X_{2/3}$ receptors.

The potentiating effect of extracellular H^+ and Zn^{2+} was modest at recombinant $P2X_{2/3}$ receptors compared to their reported facilitatory actions on $P2X_2$ receptors (Wildman *et al.*, 1998). Regarding the effects of H^+ ions, our results revealed a 4-fold increase in agonist potency at $P2X_{2/3}$ receptors in changing from pH 8.0 to 6.5, and a 21-fold increase for $P2X_2$ receptors. Regarding the effects of Zn^{2+} ions, agonist activity was enhanced about 2-fold at $P2X_{2/3}$ receptors, but 14-fold at $P2X_2$ receptors. Both H^+ and Zn^{2+} ions potentiate ATP-activated slow currents in nodose ganglia, which express $P2X_2$ and $P2X_3$ subunits (Li *et al.*, 1996b). From my present data, it is likely that any H^+ and Zn^{2+} based potentiation of ATP-activated responses in sensory ganglia will involve both $P2X_2$ and $P2X_{2/3}$ receptors.

In summary, I have carried out a sequential pharmacological survey of the possible homomeric and heteromeric P2X receptors that could be generated by coexpression of $P2X_2$ and $P2X_3$ subunits in the same cell. My experiments indicate that, where cell types naturally express more than one P2X subunit, it is likely that a mixture of homomeric and heteromeric receptors will be present in these cells. Where the $P2X_{2/3}$ receptor was studied in isolation, I found that the pharmacological profile is

comparatively unique and does not seem to be governed by either of the constituent $P2X_2$ and $P2X_3$ subunits. Thus, α,β-meATP (but not the structurally related β,γ-meATP) is a potent agonist at $P2X_{2/3}$ receptors - a profile that does not fit either homomeric $P2X_2$ or $P2X_3$ receptors. Also, the potency of the antagonists tested here on $P2X_{2/3}$ receptors, particularly TNP-ATP, RB-2 and the inactive Ip_5I, did not mirror our findings with homomeric $P2X_2$ and $P2X_3$ receptors. These pharmacological findings indicate that the therapeutic value of P2X subunit-selective agonists and antagonists (a popular objective at this point in time) might not be entirely appropriate, and that selective agonists and antagonists for those P2X heteromultimers found in tissues need also be sought.

SECTION A

CHAPTER 4

INHIBITION OF ATP-GATED $P2X_3$ RECEPTORS BY CAPSAICIN-ACTIVATED VR1 RECEPTORS COEXPRESSED IN *XENOPUS* OOCYTES

4.1 ABSTRACT

The potential for interaction between $P2X_3$ and VR1 receptors, coexpressed in *Xenopus* oocytes, was investigated under twin electrode voltage-clamp conditions. ATP evoked a pH-insensitive transient inward current via rat $P2X_3$ receptors, whereas capsaicin produced a pH-sensitive sustained inward current via rat VR1 receptors. Neither agonist showed cross-activating or inactivating properties. When applied simultaneously, ATP and capsaicin evoked a biphasic inward current comprising a $P2X_3$-like response followed by a VR1-like response, the potency and efficacy of each agonist being unaffected by the presence of the other. When agonists were applied successively, evoked responses were non-additive and resulted in a one-way inhibition of ATP responses due to a reduction in the number of $P2X_3$ receptors available for activation. The degree of inhibition was related to the amplitude of the VR1-like response and, in turn, dependent on the level of VR1 receptor expression, the capsaicin concentration in the superfusate, extracellular pH and, to a limited extent, holding potential. Substitution of ion-channel permeants (Na^+ and Ba^{2+}) failed to abolish one-way inhibition, and altering the direction of current flow also failed. Monensin, which inhibits the internalisation of P2X receptors, failed to inhibit one-way inhibition of ATP responses as well. My results suggest that VR1 and $P2X_3$ receptors interact physically within the membrane in a state-dependent manner, to prevent the opening of the $P2X_3$ receptor. The one-way inhibition observed in the present study may have a functional bearing on the signalling properties of nociceptors where $P2X_3$ and VR1 receptors are involved in pain signalling.

4.2 INTRODUCTION

Extracellular ATP applied to a blister base evokes the sensation of pain in humans (Bleehen & Keele, 1977) and, when injected into the rat hindpaw, elicits signs of pain (lifting and licking) (Bland-Ward & Humphrey, 1997). Electrophysiological and behavioural studies suggest that ATP-gated ion-channels found in primary afferent neurons, particularly the $P2X_3$ receptor, can initiate nociception (Krishtal *et al.*, 1983, 1988; Bean, 1990; Chen *et al.*, 1995; Burnstock & Wood, 1996; Cook *et al.*, 1997; Bland-Ward & Humphrey, 1997; Dowd *et al.*, 1998; Hamilton *et al.*, 1999b; Burnstock, 2000; Cockayne *et al.*, 2000; Piper & Docherty, 2000; Souslova *et al.*, 2000). $P2X_3$ receptors are found in small-sized sensory neurons in spinal ganglia (DRG) and co-exist with biochemical markers of nociceptive neurons, *e.g.* IB4 lectin (Chen *et al.*, 1995; Collo *et al.*, 1996; Bradbury *et al.*, 1998; Vulchanova *et al.*, 1998; Xiang *et al.*, 1998). Although transcripts for $P2X_{1,2,4\text{-}6}$ subunits are also expressed in DRG cells (Collo *et al.*, 1996; Vulchanova *et al.*, 1998; Xiang *et al.*, 1998), the principal P2X receptor phenotype here is $P2X_3$-like which is defined by fast inactivating inward currents to α,β-meATP and ATP (Chen *et al.*, 1995; Robertson, *et al.*, 1996; Rae *et al.*, 1998; Burgard *et al.*, 1999; Grubb & Evans, 1999).

The vanilloid VR1 receptor is also found in IB4-labelled sensory neurons (Wood & Docherty, 1997; Guo *et al.*, 1999; McCleskey & Gold, 1999; Szallasi & Blumberg, 1999). The VR1 receptor is activated by capsaicin and other substances, including anandamide (Zygmunt *et al.*, 1999; Jerman *et al.*, 2000), and by noxious heat and H^+ ions (Caterina *et al.*, 1997; Tominaga *et al.*, 1998), each evoking slowly inactivating inward currents. Extracellular capsaicin evokes a painful sensation that is believed due to the direct excitation of VR1 receptors in peripheral sensory nerve endings (Bevan & Szolcsanyi, 1990; Holzer, 1991; Liu & Simon, 1994; Oh *et al.*, 1996; Wood & Docherty, 1997; Szallasi & Blumberg, 1999; Piper & Docherty, 2000). VR1 transcripts

are present in small- and medium-sized sensory neurons in rat DRG (Caterina *et al.*, 1997; Guo *et al.*, 1999).

Available evidence suggests $P2X_3$ and VR1 receptors might interact on a functional level, thereby altering the perception of noxious stimuli and the actions of biogenic agents at nociceptive nerve fibres. A significant proportion (>70%) of small DRG cells contain both VR1- and $P2X_3$-like immunoreactive material (Guo *et al.*, 1999). About the same proportion (70%) of rat DRG neurons responding to capsaicin also elicited $P2X_3$-like responses to α,β-meATP (Ueno *et al.*, 1999). Capsaicin activation of the rat lingual nerve greatly reduced multiunit discharges evoked by α,β-meATP (Rong *et al.*, 2000). A one-way desensitisation of a subset of native P2X receptors was observed following the activation of vanilloid receptors in rat DRG cells (Piper & Docherty, 2000). Thus, I have investigated this potential for receptor-receptor interaction by coexpressing rat $P2X_3$ and rat VR1 subtypes in *Xenopus* oocytes and monitoring the outcome of their simultaneous or consecutive activation with ATP and capsaicin under voltage-clamp conditions.

4.3 METHODS

Preparation of oocytes and expression of $P2X_3$ and VR1 receptors

Xenopus laevis were anaesthetized with Tricaine (0.2%, $w.v^{-1}$) and killed by decapitation. Mature oocytes (stages V and VI) were taken from ovarian lobes, defolliculated by a two-step process that involved collagenase treatment (Type I, 2 $mg.ml^{-1}$ in Ca^{2+}-free Ringer's solution) and removing the follicle cell layer with fine forceps. In preliminary experiments, defolliculated oocytes were injected (40 nl) cytosolically with cRNA ($1\mu g.\mu l^{-1}$) encoding either rat $P2X_3$ or rat VR1 receptors. In coexpression experiments, oocytes were injected (40 nl) with a mixture of cRNAs of $P2X_3$ subunit ($1\ \mu g.\mu l^{-1}$) and VR1 subunit ($1\ \mu g.\mu l^{-1}$). The concentration of cRNA for

VR1 was lowered to 0.5 μg μl^{-1} and 0.2 μg μl^{-1} in some experiments (to reduce the level of VR1 receptor expression), whilst the concentration of cRNA for $P2X_3$ remained fixed (at 1 $\mu g.\mu l^{-1}$). RNA-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 sulphate 50 $\mu g.l^{-1}$ for 96 h to allow full receptor expression, and then stored at 4° C for up to 7 days.

Solutions and electrical recording of cRNA-injected oocytes

Membrane currents were recorded under voltage-clamp conditions using a twin-electrode amplifier (Axoclamp 2B). Microelectrodes had a resistance of 1-2 MΩ when filled with 3 M KCl. Oocytes were perfused constantly (at 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 capsaicin-activated current exhibits variable rates of desensitisation and strong tachyphylaxis, each of which depends on the presence of external Ca^{2+} (Koplas *et al.*, 1997). In the present study, Ba^{2+} was used as a substitute for Ca^{2+} in the extracellular solution to reduce the desensitization of capsaicin responses. The amplitude of capsaicin and ATP responses were reproducible when each agonist was applied at intervals more than 20 minutes apart. Agonists were applied until the evoked response reached peak amplitude. All recordings were made at room temperature (18°C) and at a holding potential of –30 mV (unless stated otherwise). Electrophysiological data were recorded on a chart recorder (Gould 2200s).

Agonist solutions were delivered by gravity flow from independent reservoirs placed above the preparation. EC_{50} values for agonists were taken from Hill plots, using the transform log $(I/I_{max}-I)$, where I is the peak current evoked by each concentration of agonist. The Hill coefficient was taken from the slope of Hill plots. Concentration-response curves were fitted by non-linear regression analysis using

commercial software (Prism v2.05, GraphPad). Data are presented as mean ± standard error of the mean (SEM). Student's *t* test was used; *p* values ≤ 0.05 were considered significant.

4.4 RESULTS

ATP and capsaicin are independent agonists of their receptors

Under voltage-clamp conditions (V_h = -30 mV), ATP (1 μM) evoked rapidly inactivating inward currents in oocytes expressing $P2X_3$ receptors alone. ATP responses were unaffected by the presence of capsaicin in the superfusate (Fig 4.1A). In complementary experiments, capsaicin (1 μM) produced slowly inactivating inward currents in oocytes expressing VR1 receptors alone, and these responses were unaffected by ATP (Fig. 4.1B). When the two receptors were coexpressed, the simultaneous application of ATP and capsaicin elicited biphasic responses that comprised an initial transient ($P2X_3$-like) current followed by a sustained (VR1-like) current. The amplitude of each current was not noticeably different from control responses to either ATP or capsaicin alone (Fig. 4.1C).

ATP responses at $P2X_3$ receptors were monitored over a wider concentration range (0.03-300 μM), in the absence and presence of capsaicin (10 μM). Capsaicin failed to change ATP potency (EC_{50}: 1.3 ± 0.3 μM *vs* 1.2 ± 0.4 μM, n=6), or alter the maximum ATP response (Fig. 4.2A). Capsaicin responses at VR1 receptors were also studied over a wider concentration range (0.1-30 μM), in the absence and presence of ATP (10 μM). ATP failed to change capsaicin potency (EC_{50}: 0.86 ± 0.07 μM *vs* 0.88 ± 0.05 μM, n=6), or alter the maximum capsaicin response (Fig. 4.2B). Thus, neither agonist showed cross-activating or inactivating properties in preliminary experiments.

The activation of some ion-channels can be affected by extracellular pH. Here, extracellular pH had a modest effect on ATP potency at $P2X_3$ receptors (Fig. 4.2C) and, failed to evoke a membrane response. EC_{50} values for ATP activation were: pH 8.0, 1.3±0.4 μM; pH 7.5, 1.3±0.3 μM; pH 6.5, 2.0 ± 0.3 μM; pH 5.5, 2.7 ± 0.5 μM (n=6). Thus, a 2-fold reduction in ATP potency resulted from changing extracellular pH from 8.0 to 5.5, in agreement with previous reports (Stoop *et al.*, 1997; Wildman *et al.*, 1999c). Capsaicin (10 μM) had no effect on ATP potency at the four levels of extracellular pH examined.

Capsaicin responses at the VR1 receptor were more sensitive to extracellular pH and, additionally, low levels (*i.e.* pH 5.5) also activated a slowly desensitising inward current (83 ± 36 nA, n=6), approximately 10% of the maximal capsaicin response in the same oocytes. The capsaicin potency was markedly changed by extracellular pH (Fig. 4.2D). EC_{50} values for VR1 receptor activation were: pH 8.0, 1.1 ± 0.1 μM; pH 7.5, 0.86 ± 0.07 μM; pH 6.5, 0.19 ± 0.03 μM; pH 5.5, 0.04 ± 0.01 μM (n=6). Thus, changing extracellular pH from pH 8.0 to pH 5.5 resulted in a 28-fold increase in capsaicin potency, in line with earlier although limited findings (Tominaga *et al.*, 1998; Jerman *et al.*, 2000). ATP (10 μM) had no effect on capsaicin potency at the four levels of extracellular pH examined.

One-way inhibition of ATP responses by capsaicin

$P2X_3$ and VR1 receptors were coexpressed in oocytes, and agonists applied sequentially to study the effects of activating one receptor after the other. Application of ATP (10 μM; for 60 s), followed by capsaicin (10 μM; for 40 s), elicited biphasic responses where the amplitude of each component was not significantly different from control responses to either ATP or capsaicin alone (Fig. 4.3A). With the order changed, an initial application of capsaicin (10 μM, for 60-180s) caused a significant

reduction in the ATP response (10 μM) (Fig. 4.3B). This one-way inhibition was observed in a series of 30 cells tested, was largely independent of the duration of capsaicin application (Fig. 4.3B) but highly dependent on the peak amplitude of the capsaicin response (Fig. 4.3C). Here, this inverse relationship between the amplitude of the capsaicin responses and degree of inhibition of ATP responses was fitted to a straight-line plot ($r^2 = 0.72$, $p < 0.001$, n=30).

Factors influencing the degree of inhibition of ATP responses

The degree of inhibition of ATP responses was altered by the level of VR1 receptor expression (Fig. 4.4A-C). Changes were made by lowering the amount of VR1 mRNA injected into oocytes, yet keeping the amount of $P2X_3$ mRNA fixed, thereby changing mRNA concentration ratios from 1:1 to 1:0.5 and, finally, to 1:0.2 ($P2X_3$:VR1). This procedure resulted in a step-wise decrease in the maximum capsaicin response: 1195 ± 212 nA (1:1 ratio); 670 ± 121 nA (1:0.5 ratio); 194 ± 36 nA (1:0.2 ratio) (n=6), whilst the maximum response to ATP alone was not significantly different: 1244 ± 168 nA (1:1); 1090 ± 96 nA (1:0.5); 1351 ± 184 nA, (1:0.2) (n=6). However, the degree of inhibition caused by a near-saturating concentration of capsaicin (10 μM) on the following ATP response (10 μM) was progressively reduced from 80 ± 3% (1:1), to 59 ± 5% (1:0.5) and 23 ± 16% (1:0.2) (n=6). Inhibition of $P2X_3$ receptors seemed to depend on the number of available VR1 receptors.

The inhibition of ATP responses following VR1 receptor activation (capsaicin, 10 μM) was attributed to an apparent reduction in maximum responses whilst agonist potency was unchanged (Fig. 4.5). For data using the 1:1 mRNA ratio, the maximum response to ATP was reduced by 70 ± 3% (n=5) whilst ATP potency was not significantly different (EC_{50} values: 1.3 ± 0.1 μM, *VR1 not activated*; 1.2 ± 0.1 μM, *VR1 activated*;

1.3 ± 0.3 μM, *no VR1 receptor expression;* n=5). These results suggested the number of $P2X_3$ receptors available for activation were reduced by VR1 receptor activation.

The degree of one-way inhibition of ATP responses was affected by increasing the capsaicin concentration in the superfusate. Fast currents to a near-saturating concentration of ATP (10 μM) were progressively diminished from 1442 ± 279 nA to 505 ± 158 nA (n=4) as the capsaicin concentration, and amplitude of its evoked response, progressively increased from 42 ± 17 nA (at 0.3 μM) to 1505 ± 74 nA (at 10 μM) (Fig. 4.6A,B). Thus, inhibition was related to the number of VR1 receptors activated.

In complementary experiments, the degree of inhibition of ATP responses was significantly greater when the extracellular pH was lowered from 7.5 to 6.5, a procedure that selectively enhanced capsaicin, not ATP, potency. Here, the degree of inhibition of ATP responses (1 μM) caused by capsaicin responses (1 μM) changed from 21 ± 11 % (at pH 7.5) to 59 ± 6% (at pH 6.5) (n=6; $p < 0.05$, by paired *t*-test) (see Fig. 4.7A). The results further supported the notion that the number of VR1 receptors activated determines the level of $P2X_3$ receptor inhibition.

Although both VR1 and $P2X_3$ receptors carry inward currents at negative resting potentials, the VR1 ion-channel shows pronounced outward rectification (Caterina *et al.*, 1997; Zygmunt *et al.*, 1999) whereas the $P2X_3$ ion-channel shows inward rectification (Chen *et al.*, 1995). Thus, I found that the degree of inhibition of ATP responses by VR1 activation was not linearly correlated with holding potential (Fig. 4.7B). At –10 mV, capsaicin (10 μM) inhibited the response to ATP (10 μM) by 27 ± 8% (n=4), whilst the level of inhibition increased to 43 ± 11% (n=5) at –30 mV. However, the level of inhibition of ATP responses fell to 29 ± 5% (n=5) at –50 mV. Thus, a non-linear relationship was observed between the membrane potential of

oocytes, the current carrying properties of each receptor subtype and degree of inhibition of ATP responses.

Effect of ion-channel permeants on inhibition of ATP responses

The VR1 receptor is permeable to Na^+ and Ca^{2+} (Bevan & Szolcsanyi, 1990), as well as K^+, Cs^+, and Mg^{2+} (Caterina *et al.*, 1997). In the present study, only Na^+, K^+ and Ba^{2+} were available in the extracellular solution to carry the inward current into oocytes. When extracellular Ba^{2+} was replaced with equimolar Ca^{2+} (1.8 mM), the degree of inhibition of ATP responses by VR1 receptor activation was not markedly altered (Fig. 4.8A). Where extracellular Na^+ was replaced by equimolar K^+ (110 mM; totalling 112.5 mM), VR1 receptor activation still resulted in inhibition of ATP responses (Fig. 4.8B). Thus, on a macroscopic level, the type of channel permeant did not noticeably affect the observed inhibition of $P2X_3$ receptors.

The direction of current flow through VR1 and $P2X_3$ receptors was changed momentarily by stepping the membrane potential from –30 to +40 mV (for 100 ms, every 5s) (see Fig. 4.9). Under these conditions, the ATP-evoked inward current and attendant conductance change during transient outward currents were noticeably smaller during VR1 receptor activation than in control experiments with ATP alone. Inhibition of $P2X_3$ receptors appeared to occur whether inward or outward currents were carried through VR1 (and $P2X_3$) receptors.

Effect of monensin on one-way inhibition of ATP responses

Micromolar concentrations of monensin are known to inhibit the internalisation of P2X subunits (Dutton *et al.*, 2000, Li *et al.*, 2000), by a mechanism thought to disrupt the pH gradient across endosomes (Cremaschi *et al.*, 1996). In my hands, monensin (5 μM, 20 minutes preincubation) failed to significantly alter the level of one-way

inhibition of ATP responses (47.4 ± 6.8% *vs* 49.6 ± 8.7%, n = 4) following VR1 activation (Fig. 4.10). Additionally, monensin failed to significantly alter the amplitude of capsaicin responses (1335 ± 407 nA *vs* 1192 ± 449 nA, n=6), or ATP responses (448 ± 127 nA *vs* 452 ±165 nA, n=6), where each agonist was applied alone. Furthermore, monensin had no noticeable effect on the desensitisation of either VR1 or $P2X_3$ receptors. Ethanol (0.05%), the amount present in monensin (5 μM), had no noticeable effect on ATP and capsaicin responses (data not shown).

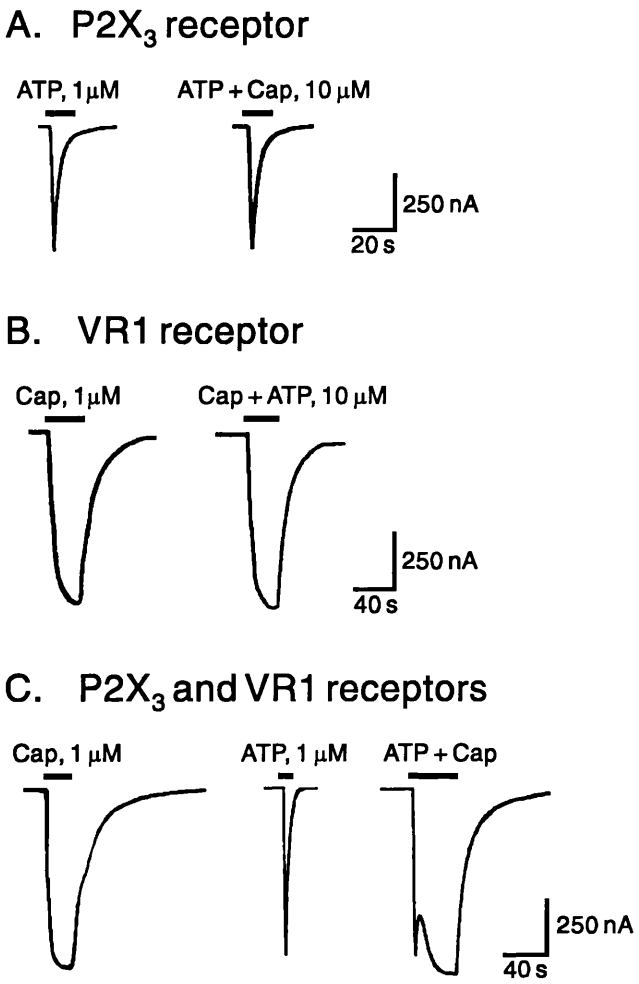


Figure 4.1. Agonist selectivity at $P2X_3$ and VR1 receptors.

A, whole-cell inward currents evoked by ATP (1 μM, for 15 s), in *Xenopus* oocyte expressing rat $P2X_3$ receptors alone, were unaffected by the presence of capsaicin (Cap, 10 μM). *B*, whole-cell inward currents evoked by capsaicin (1 μM, for 40 s), in oocyte expressing rat VR1 receptors alone, were unaffected by the presence of ATP (10 μM). C, whole-cell inward currents evoked by the successive application of capsaicin (1 μM, for 30 s) and ATP (1 μM, for 10s), then simultaneous application of capsaicin and ATP (each 1μM, for 40s), in oocyte coexpressing $P2X_3$ and VR1 receptors. (V_h = -30 mV in A-C.)

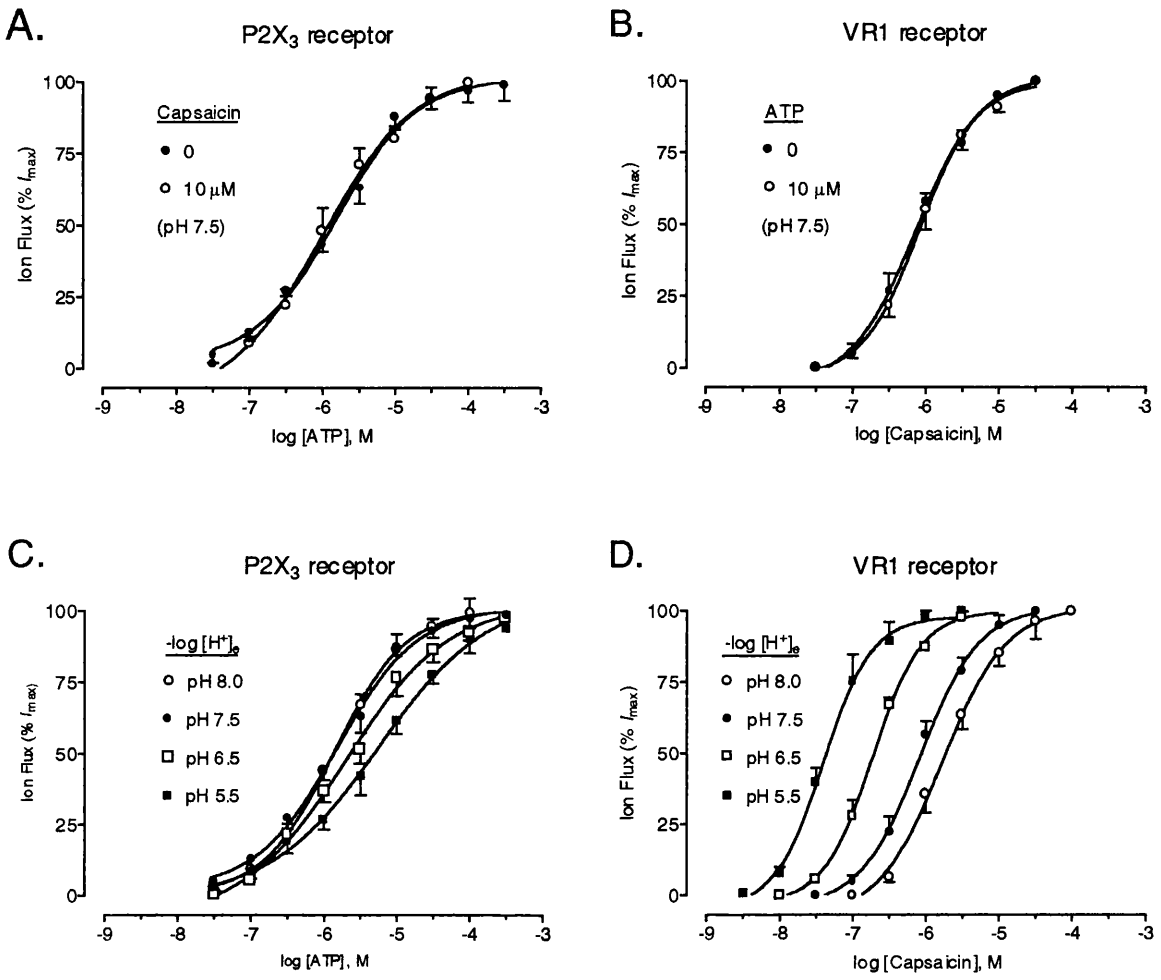


Figure 4.2 Agonist activity at $P2X_3$ and VR1 receptors. *A*, Concentration-response (C-R) relationship for ATP-activated inward current at $P2X_3$ receptors, at pH 7.5, in the absence and presence of capsaicin (10 μM). *B*, C-R relationship for capsaicin-activated inward current at VR1 receptors, at pH 7.5, in the absence and presence of ATP (10 μM). *C*, C-R relationship for ATP-activated inward currents at $P2X_3$ receptors tested at the levels of extracellular pH indicated. *D*, C-R relationship for capsaicin-activated inward currents at VR1 receptors tested at the levels of extracellular pH indicated. Curves were fitted by the Hill equation in A-D, for data given as mean±SEM for 6 cells per curve.

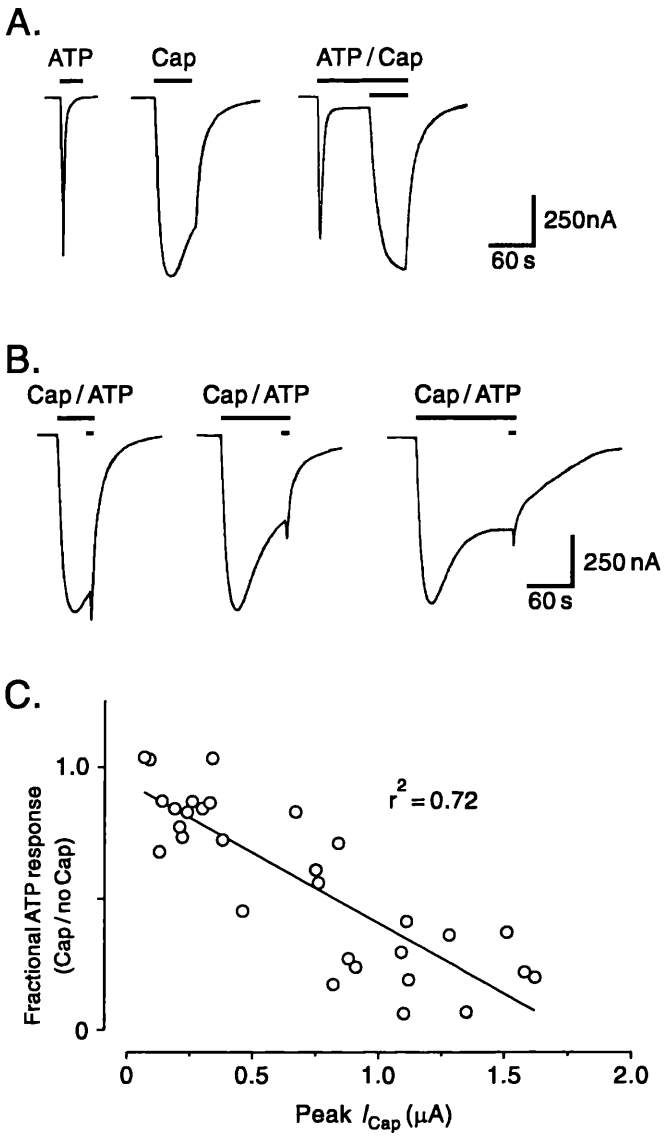


Figure 4.3 One-way inhibition of ATP responses following VR1 receptor activation. *A*, whole-cell inward currents to either ATP or capsaicin alone, then to ATP followed by capsaicin (each 10 μM), in oocytes coexpressing $P2X_3$ and VR1 receptors. *B*, whole-cell currents where ATP was applied 60, 120 and 180s after the onset of capsaicin application. (Records in *A* and *B* from the same oocyte.) Only the amplitude of ATP, not capsaicin, responses was significantly altered by coapplication of agonists. *C*, linear relationship between the degree of one-way inhibition of ATP responses (expressed as a fraction of its own control response) and peak amplitude of the response to capsaicin (10 μM) in 30 oocytes tested in this way. (V_h = -30 mV.)

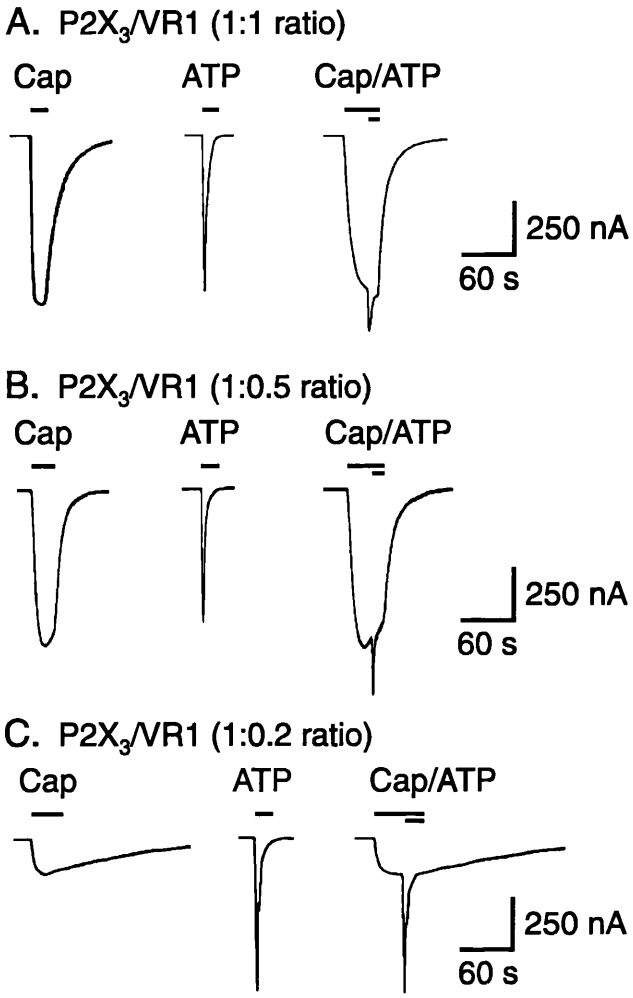


Figure 4.4 One-way inhibition of ATP responses dependent on level of VR1 receptor expression. Whole-cell inward currents were induced by either capsaicin or ATP alone, then capsaicin followed by ATP (each 10 μM), in oocytes coinjected with cRNAs encoding $P2X_3$ and VR1 receptors at respective concentration ratios of 1:1 (*A*), 1:0.5 (*B*) and 1:0.2 (*C*). ATP responses activated during VR1 receptor activation were 20±3% (1:1), 41±5% (1:0.5) and 77±16% (1:0.2) of their respective control responses. Data are expressed as mean±SEM for 5 or 6 cells. (V_h = -30 mV in A-C).

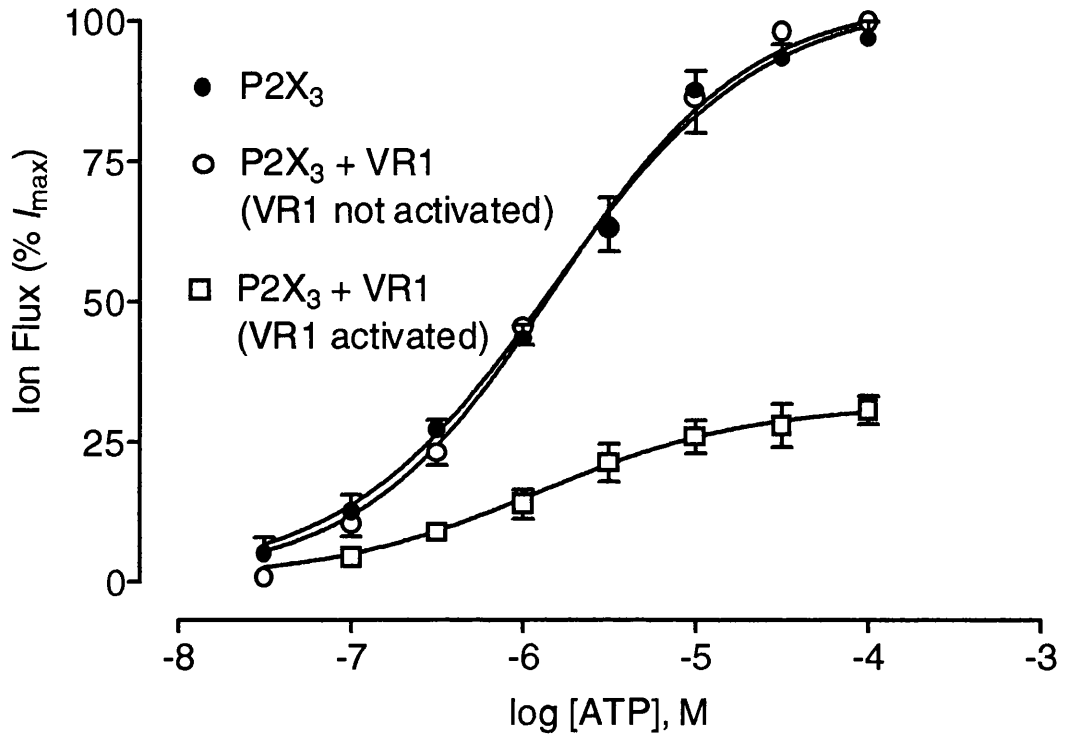


Figure 4.5 One-way inhibition due to a reduction in ATP efficacy.

C-R relationship for ATP-activated inward currents, before and during VR1 receptor activation by capsaicin (10 μM, for 60s), in oocytes coexpressing $P2X_3$ and VR1 receptors. Prior activation of VR1 receptors reduced the maximum ATP response, without altering ATP potency. The C-R curve for ATP responses is also shown for oocytes expressing $P2X_3$ receptors alone. Curves fitted to the Hill equation, and data expressed as mean±SEM (n=5).

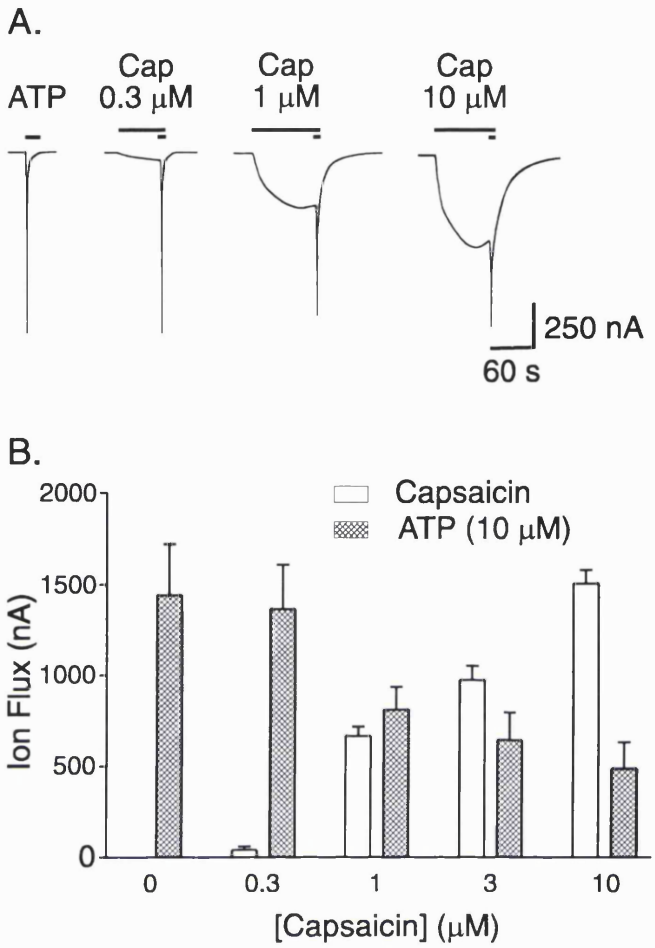


Figure 4.6 One-way inhibition of ATP responses dependent on extent of VR1 receptor activation. *A*, amplitude of inward currents to ATP (10 μM) was progressively decreased as the amplitude of the preceding capsaicin response was progressively increased by raising agonist concentration (V_h = -30 mV). *B*, cumulative data from oocytes coexpressing $P2X_3$ and VR1 receptors, showing a reciprocal relationship between the amplitude of ATP and capsaicin responses. Data expressed as mean±SEM (n=4).

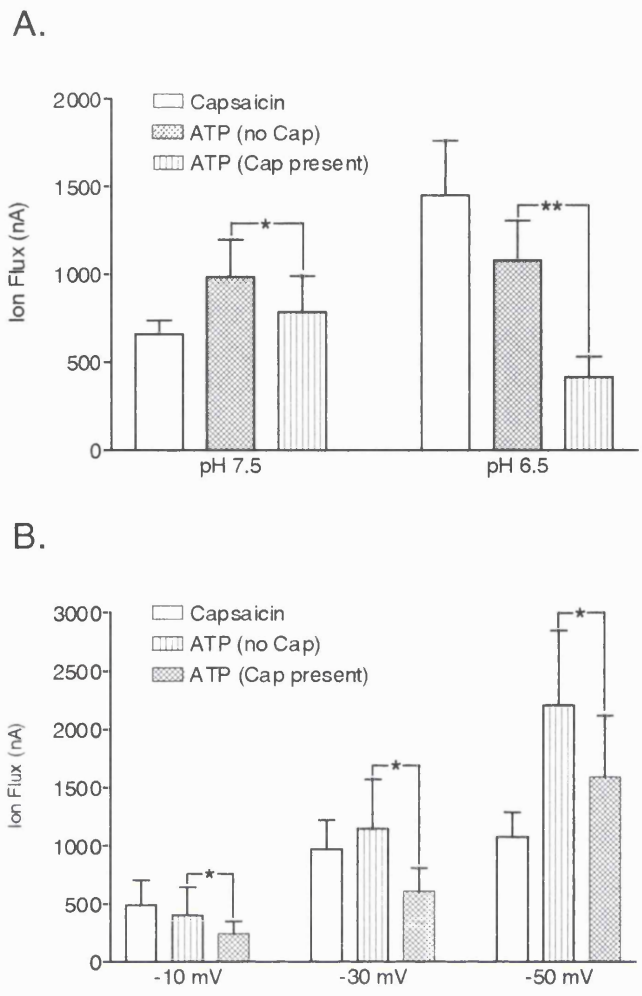


Figure 4.7 Effect of pH, and holding potential, on degree of one-way inhibition of ATP responses. *A*, amplitude of inward currents to either capsaicin or ATP alone, or to ATP in the presence of capsaicin (both 1 μM), in oocytes coexpressing $P2X_3$ and VR1 receptors and tested at extracellular pH 7.5 and pH 6.5. *B*, amplitude of inward currents to either capsaicin or ATP alone, or to ATP in the presence of capsaicin (each 1 μM), in oocytes coexpressing $P2X_3$ and VR1 receptors and tested at holding potentials of –10, -30 and –50 mV. Data as mean±SEM (n=4-6) and compared by Student's paired *t*-test (* $p < 0.05$; ** $p < 0.01$).

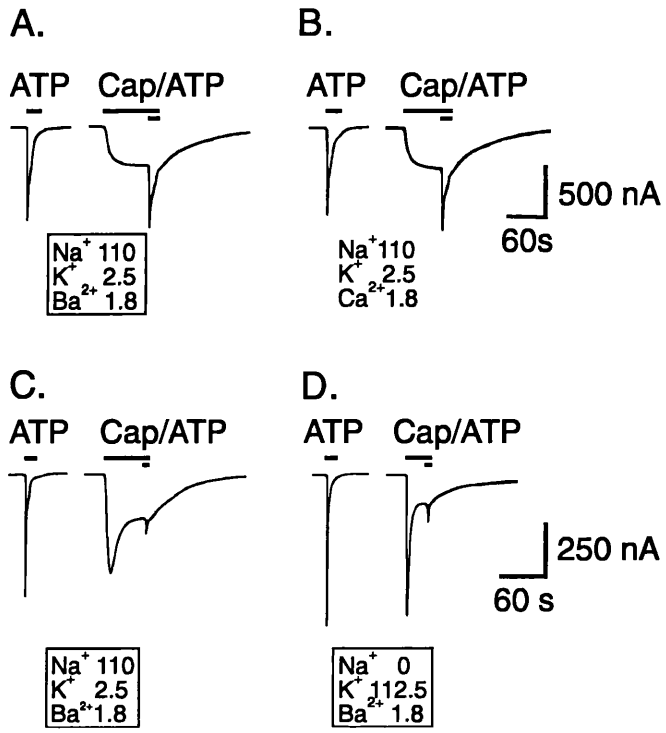


Figure 4.8 One-way inhibition of ATP-responses not dependent on ion-channel permeants. Whole-cell inward currents to ATP (10 μM), either alone or in the presence of capsaicin (1 μM), in an oocyte coexpressing $P2X_3$ and VR1 receptors and bathed in a medium containing either (A) Ba^{2+} or (B) Ca^{2+} (and other cations) at the concentrations shown (in mM). Whole-cell inward currents to ATP (10 μM), either alone or in the presence of capsaicin (1 μM), in an oocyte coexpressing $P2X_3$ and VR1 receptors and bathed in a medium containing either (C) Na^+ or (D) K^+ (and other cations) at the concentrations shown (in mM). (V_h = -30 mV in A-C, and –15 mV in D.)

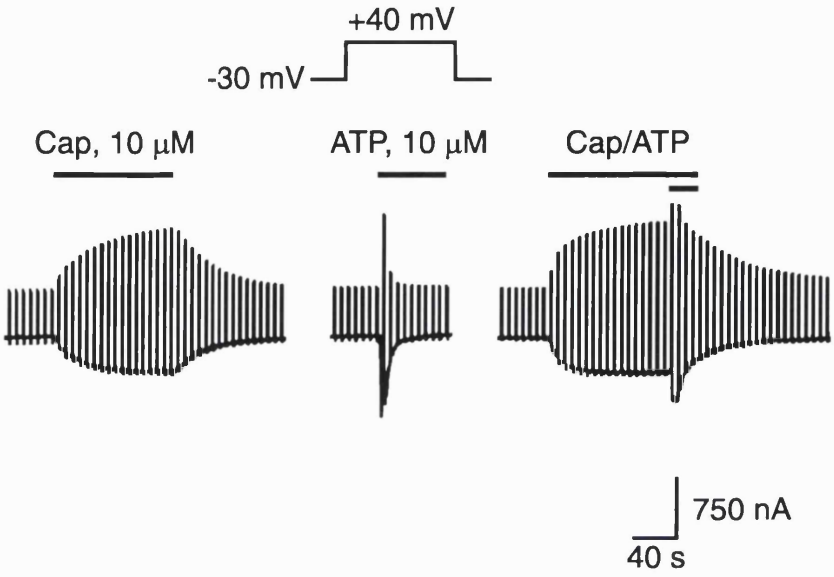


Figure 4.9 One-way inhibition of ATP responses not dependent on direction of current flow. Whole-cell inward currents to either capsaicin or ATP alone, or ATP in the presence of capsaicin (each 10 μM), in an oocyte coexpressing $P2X_3$ and VR1 receptors. Brief voltage steps (-30 mV to +40 mV; for 100 ms, every 5 s) were applied intermittently to monitor changes in membrane conductance (G_m) during each agonist response. Inward current to ATP, and attendant changes in G_m, were significantly reduced following VR1 receptor activation. (V_h = -30 mV.)

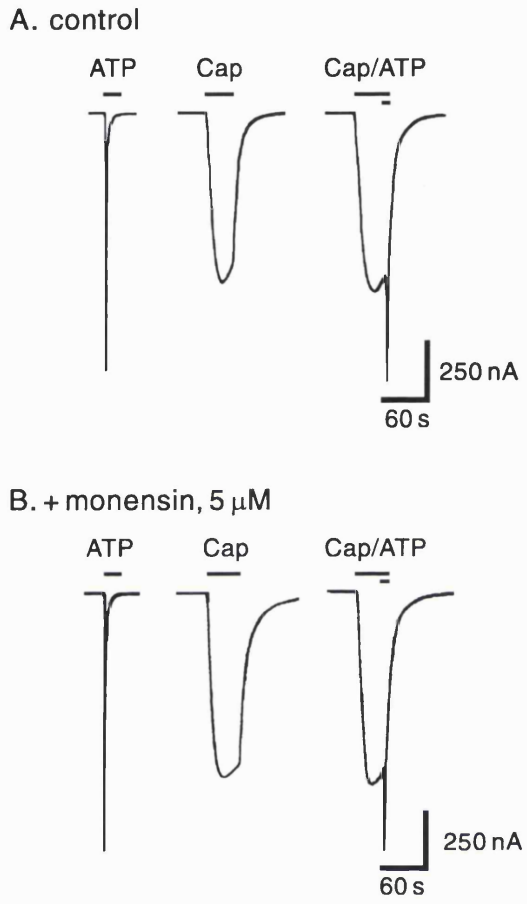


Figure 4.10 One-way inhibition of ATP responses not due to $P2X_3$ receptor internalisation. Whole-cell currents to either ATP or capsaicin alone, or ATP in the presence of capsaicin (each 10 μM), in an oocyte coexpressing $P2X_3$ and VR1 receptors and tested in the absence (*A*) or presence (*B*) of monensin (5 μM). Monensin, an inhibitor of endosome-mediated internalisation of P2X receptors, failed to inhibit one-way inhibition of ATP responses. All records from the same oocyte, held at -30 mV.

4.5 DISCUSSION

Initial observations

Capsaicin and ATP are chemical activators of peripheral nociceptors, with capsaicin acting on a vanilloid receptor (principally the VR1 subtype) and ATP on P2X receptors (principally the $P2X_3$ subtype) (Wood & Docherty, 1997). VR1 and $P2X_3$ receptors belong to unrelated gene families. The encoded VR1 subunit protein is characterised by six complete transmembrane domains and one re-entrant loop (Caterina *et al.*, 1997). The $P2X_3$ subunit features two transmembrane domains with a large extracellular loop and both N- and C-termini located intracellularly (Chen *et al.*, 1995). The principal agonists of these two receptors - the vanilloid, capsaicin, and purine nucleotide, ATP - are chemically unrelated. Thus, it was not altogether surprising to find in my preliminary experiments that ATP did not activate or modulate recombinant VR1 receptors, and capsaicin did not gate or modulate recombinant $P2X_3$ receptors. Furthermore, co-application of ATP and capsaicin evoked biphasic inward currents that could be interpreted simply as the separate and independent activation of VR1 and $P2X_3$ receptors. Thus, neither agonist showed cross-activating or inactivating properties – a conclusion shared by Piper & Docherty (2000) who have carried out similar experiments on native P2X and vanilloid receptors in adult rat DRG neurons.

One-way inhibition of ATP-responses

The main finding of this study was a one-way inhibition of $P2X_3$ receptors when VR1 receptors were activated first. There was no evidence for the converse. Concentration-responses curves for ATP, before and during VR1 receptor activation, showed that the maximum response to ATP was reduced during one-way inhibition – an effect attributed to a decrease in the number of $P2X_3$ receptor ion-channels available for activation – without a concomitant change in the EC_{50} value for ATP. I found that

the degree of one-way inhibition of ATP responses correlated well, and in a linear manner, with the amplitude of the proceeding capsaicin response. I also found that increasing the levels of capsaicin receptor expression, by inserting more VR1 cRNA into the expression system, enhanced the degree of one-way inhibition for any particular conditioning concentration of capsaicin. I further found that increasing the number of VR1 receptors activated, by increasing the concentration of capsaicin applied, would enhance the level of one-way inhibition. Reducing extracellular pH, which selectively enhances agonist potency at VR1 receptors and increases the number of VR1 receptors activated, also increased the level of one-way inhibition of ATP responses. However, increasing the driving force on ion-channel permeants, by raising the holding potential during voltage-clamp, had a complex effect on one-way inhibition. A non-linear relationship between holding potential and degree of one-way inhibition was observed, possibly because the VR1 receptor ion-channel shows a reduced conductance at increasingly negative potentials (outward rectification) (Caterina *et al.*, 1997; Zygmunt *et al.*, 1999) whilst the converse applies for the $P2X_3$ receptor (inward rectification) (Chen *et al.*, 1995). Thus, in general, my experiments suggested that one-way inhibition of ATP was highly dependent of the number of VR1 receptors activated and the amplitude of the inward current carried by these ion-channels.

Possible mechanisms for one-way inhibition

My results with ion substitution experiments failed to show that ion-channel permeants played a major role in the one-way inhibition of ATP responses. Most of our experiments were carried out with an extracellular solution containing Ba^{2+}, rather than Ca^{2+} ions, to avoid the activation of Ca^{2+}-dependent processes in oocytes. However, replacement of extracellular Ba^{2+} with equimolar Ca^{2+} ions did not

significantly enhance the degree of one-way inhibition. Others have shown that the inactivation rate constant of $P2X_2$ receptors is much faster (8-10 fold) with intracellular Ca^{2+} rather than Ba^{2+} ions (Ding & Sachs, 2000). On the other hand, Ca^{2+} influx during vanilloid receptor activation only inhibited $P2X_2$- and $P2X_{2/3}$-like receptors mediating slow currents, not $P2X_3$-like receptors mediating fast currents, in adult rat DRG neurons (Piper & Docherty, 2000). Furthermore, it has been shown previously that Ca^{2+} influx had a bearing on the inactivation rate, but not the amplitude, of ATP responses at $P2X_3$ receptors expressed in oocytes (King *et al.*, 1997a). Given that Ca^{2+} influx does not have a discernible effect on the amplitude of ATP responses at native and recombinant $P2X_3$ receptors, a role for its substitute Ba^{2+} as the root of one-way inhibition of $P2X_3$ receptors seemed unlikely.

Substitution of extracellular Na^+ with K^+ (plus re-adjustment of V_h to generate inward K^+ currents of comparable amplitude) also failed to significantly alter one-way inhibition of ATP responses. Na^+ influx is known to inhibit some native and recombinant P2X receptors (Ma *et al.*, 1999; Michel *et al.*, 1999), but it appeared that Na^+ influx made a negligible contribution to one-way inhibition in the present study. Even with Na^+ ions present in the extracellular medium, changing the direction of current flow by briefly stepping from –30 to +40 mV failed to alleviate or alter the degree of one-way inhibition. Of itself, strong depolarisation (stepping from -60 to +40 mV) had no significant effect on $P2X_3$-like responses in adult rat DRG, although this conditioning stimulus can cause a Ca^{2+}-independent inhibition of $P2X_2$- and $P2X_{2/3}$-like responses in sensory neurons (Piper & Docherty, 2000).

The notion that agonist activation of VR1 receptor could lead to the internalisation of $P2X_3$ receptors was explored briefly by studying the effects of monensin. Monensin (5 μM), which prevented the internalisation of ATP-activated and Green Fluorescent Protein-tagged P2X receptor subunits (Dutton *et al.*, 2000; Li *et al.*,

2000), failed in my study to prevent one-way inhibition. This compound also failed to significantly alter the amplitude and timecourse of either ATP or capsaicin responses, in keeping with the observations of Li *et al.* (2000). The phenomenon of one-way inhibition was not easily explained by failure of the space-clamp. First, there was little change (≤2 mV, at -30 mV) in the holding potential during the conditioning capsaicin-induced inward currents. Second, the degree of inhibition was not directly related to the holding potential (see Fig 4.7B) and the level of inhibition of ATP-evoked currents was lower at -50 mV than at -30 mV. Third, Axoclamp 2B amplifier (headstages X1LU for voltage microelectrode and X10MGL for current microelectrode) could accommodate currents up to 13 μA of amplitude (a level not reached in my experiments). Therefore, one-way inhibition was not due to saturation of the amplifier.

Evidence is gradually accumulating that a number of ligand-gated channels can interact physically with ATP-gated P2X receptors. A non-additive interaction has been demonstrated between nicotinic acetylcholine (nACh) and $P2X_2$-like receptors in isolated enteric neurons (Barajas-Lopez *et al.*, 1998; Zhou & Galligan, 1998), and between α3β4 nicotinic and $P2X_2$ ion-channels expressed in oocytes (Khakh *et al.*, 2000). Activity-dependent reciprocal inhibition has also been demonstrated between serotonin-gated 5-HT_3 receptors and $P2X_2$ receptors expressed in oocytes (Boue-Grabot *et al.*, 2000). As yet, I have no direct evidence for crosstalk between VR1 and $P2X_3$ receptors, although there is evidence that dopamine D5 and $GABA_A$ receptors do interact at the protein-protein level (Liu *et al.*, 2000), and the same has been implicated for 5-HT_3/$P2X_2$ and α3β4/$P2X_2$ receptor interactions.

Functional implications of one-way inhibition

Although the molecular basis of the interaction between $P2X_3$ and VR1 receptors remains unclear, it seems increasingly likely that this receptor-receptor interaction will have functional implications. Capsaicin activation of sensory neurons is followed by a refractory period (Holzer, 1991), where the responsiveness to other nociceptive stimuli is significantly diminished (Szallasi & Blumberg, 1996; Nolano *et al.*, 1999). In an associated set of experiments carried out in our laboratory, it has been found that the responsiveness of nociceptive nerve fibres to α,β-meATP (a $P2X_3$ receptor agonist) was either greatly diminished or blocked when capsaicin was applied to the peripheral end of rat lingual nerve (Rong *et al.*, 2000). It was surprising to me that no interaction was observed between capsaicin receptors and $P2X_3$-like receptors in the cell bodies of

adult rat DRG neurons (Piper & Docherty, 2000). I have found that the degree of inhibition of $P2X_3$ receptor is highly dependent on the level of VR1 receptor expression, the amplitude of the capsaicin response, the concentration of capsaicin used and, to some extent, on the holding potential at which experiments were conducted. It is possible that the different findings in Piper and Docherty (2000) were due in part to their voltage-clamp experiments conducted solely at –60 mV, perhaps a low expression level of capsaicin receptors at the cell body, and relatively low concentrations of capsaicin (0.5 μM) tested. Given the extensive colocalisation of $P2X_3$ and VR1 receptors on nociceptive IB4-labelled nerve fibres of dorsal roots (Guo *et al.*, 1999), it is tempting to speculate that native VR1 receptors do influence purinergic signalling through $P2X_3$ receptors at sites where the VR1 receptor density is higher than observed at the cell body (*i.e.* the peripheral and central nerve endings).

SECTION B

CHAPTER 5

DIINOSINE PENTAPHOSPHATE (IP_5I) IS A POTENT ANTAGONIST AT RECOMBINANT RAT $P2X_1$ RECEPTORS.

5.1 ABSTRACT

The antagonist activity of a series of diinosine polyphosphates (Ip_nI, where n = 3, 4, 5) was assessed against ATP-activated inward currents at rat $P2X_{1-4}$ receptors expressed in *Xenopus* oocytes and studied under voltage-clamp conditions. Diinosine polyphosphates were prepared by the enzymatic degradation of their corresponding diadenosine polyphosphates (*e.g.*, Ap_5A into Ip_5I) using 5´-adenylic deaminase, and purified using reverse-phase chromatography. Against ATP-responses at $rP2X_1$ receptors, the potency order for antagonism was (pIC_{50}): Ip_5I (8.5) > Ip_4I (6.3) > Ip_3I (>4.5). Ip_5I (10-100 nM) caused a concentration-dependent rightwards displacement of the ATP concentration-response curve without reducing the maximum ATP effect. However, the Schild plot was non-linear which indicated Ip_5I is not a competitive antagonist. Blockade by micromolar concentrations of Ip_5I was not surmountable. Ip_4I also behaved as a non-surmountable antagonist. Against ATP-responses at $rP2X_3$ receptors, the potency order for antagonism was (pIC_{50}): Ip_4I (6.0) > Ip_5I (5.6) > Ip_3I (>4.5). Blockade by Ip_4I (pA_2, 6.75) and Ip_5I (pA_2, 6.27) was surmountable at micromolar concentrations. Diinosine polyphosphates failed to inhibit ATP-responses at $rP2X_2$ receptors, whereas agonist responses at $rP2X_4$ were reversibly potentiated by Ip_4I and Ip_5I. None of the parent diadenosine polyphosphates behave as antagonists at $rP2X_{1-4}$ receptors. Thus, Ip_5I acted as a potent and relatively-selective antagonist at the $rP2X_1$ receptor. This dinucleotide pentaphosphate represents a high-affinity antagonist for the $P2X_1$ receptor, at which it acts in a competitive manner at low (≤100 nM) concentrations but has more complex actions at higher (>100 nM) concentrations.

5.2 INTRODUCTION

P2X receptors are ATP-gated cation channels composed of oligomeric assemblies of three, or possibly four, receptor protein subunits (Kim *et al.*, 1997; Nicke *et al.*, 1998; Torres *et al.*, 1999). Seven P2X subunits ($P2X_{1-7}$) have been cloned thus far, the operational profiles and pharmacological characteristics of homomeric assemblies of $P2X_{1-7}$ receptors having been well documented (for reviews, see: Evans *et al.*, 1998; Humphrey *et al.*, 1998a; King, 1998). Transcripts for $P2X_{1-4}$ receptor proteins are abundant in excitable tissues (neurons, smooth muscle, cardiac muscle) and in secretory epithelia, while the distribution of $P2X_5$ and $P2X_7$ mRNA is highly restricted. $P2X_6$ transcripts are especially abundant in the neuraxis (Collo *et al.*, 1996), but it remains controversial that the $P2X_6$ subunit protein is capable of forming homomeric assemblies (Torres *et al.*, 1999).

The correspondence between homomeric P2X receptors and native P2X receptors has been hampered by a paucity of selective ligands for P2X subunits. Operational profiles of homomeric P2X receptors have been matched loosely to some examples of native P2X receptors (Evans & Surprenant, 1996), but the possibility of subpopulations of homomeric and heteromeric P2X receptors in any pool of native P2X receptors cannot be discounted. Some progress has been made with agonists showing reasonable P2X subunit selectivity, *e.g.* L-β,γ-meATP at $P2X_1$ receptors (Evans *et al.*, 1995), D-β,γ-meATP at $P2X_3$ receptors (Rae *et al.*, 1998), and activity series of the diadenosine polyphosphates (Ap_nA, where n = 2-6) at $P2X_{1-4}$ receptors (Wildman *et al.*, 1999a). However, differentiating homomeric P2X receptors by agonist activity alone is a lengthy and laborious process (Humphrey *et al.*, 1998b). Further progress has been made with P2X subunit-selective antagonists: the anion

transport inhibitor, DIDS, is relatively selective for $P2X_1$ receptors at micromolar concentrations (Evans *et al.*, 1995); the PPADS derivative, MRS 2220, is wholly selective for $P2X_1$ receptors at micromolar concentrations (Jacobson *et al.*, 1998); the suramin analogue, NF023, is a potent antagonist at $P2X_1$ receptors at submicromolar concentrations, whilst 35-138 fold less potent at species orthologues of $P2X_3$ receptors (Soto *et al.*, 1999); TNP-ATP is a potent antagonist at both $P2X_1$ and $P2X_3$ receptors at nanomolar concentrations (Virginio *et al.*, 1998); KN-62 is a potent antagonist of $P2X_7$ receptors, although it too shows differential activity at species orthologues of the $P2X_7$ receptor (Humphreys *et al.*, 1998). Neither suramin, PPADS nor Reactive blue 2 (RB-2) readily discriminate between P2X subunits (Evans *et al.*, 1998; King, 1998; Ralevic & Burnstock, 1998).

Diinosine polyphosphates (abbreviated Ip_nI, where *n* is the number of phosphates) comprise two ribosylated inosine molecules bridged by a phosphate chain. These dinucleotides are synthesised by deaminating diadenosine polyphosphates with the non-specific AMP-deaminase of *Aspergillus sp.* (Guranowski *et al.*, 1995, Pintor *et al.*, 1997). One member of this family, P^1,P^5-bis(5′-inosyl) pentaphosphate (Ip_5I), has already shown interesting pharmacological properties, being a potent antagonist at: *i*) a specific dinucleotide receptor for diadenosine polyphosphates in rat brain synaptosomes (IC_{50} value, 4nM); *ii*) a P2X receptor in the same preparation (IC_{50} value, 30μM); *iii*) the $P2X_1$-like receptor in guinea-pig isolated vas deferens (pA_2 value, 6.5) (Hoyle *et al.*, 1997; Pintor *et al.*, 1997). In this chapter, I describe the antagonist properties of Ip_5I and two related dinucleotides, Ip_4I and Ip_3I, on homomeric $P2X_{1-4}$ receptors. The activity profile of dinucleotide pentaphosphate reveals selectivity for the $P2X_1$ receptor subtype at nanomolar concentrations.

5.3 METHODS

Diinosine polyphosphate synthesis

Diinosine pentaphosphate (Ip_5I) was prepared by enzymatic degradation of diadenosine pentaphosphate (Ap_5A). Ip_4I and Ip_3I were also prepared in the same manner from Ap_4A and Ap_3A, respectively. 5'-adenylic acid deaminase (0.12 U) from *Aspergillus sp.* was incubated with 10 mM Ap_5A in a final volume of 1 ml of 50 mM HEPES (pH 6.5) for 90 minutes (at 37° C). Aliquots (10 µl) were taken at different times, placed in 100° C water bath for 5 minutes to stop the enzymatic reaction, and diluted 1:100 with distilled water to monitor the production of Ip_5I by HPLC techniques. After 90 minutes, the reaction was stopped by boiling the incubation medium at 100° C for 5 minutes, after which protein debris was removed by filtration through a Millex-G5 filter (0.22 µm; from Millipore). The reaction product was confirmed as Ip_5I by HPLC detection. Samples were treated with phosphodiesterase (3 mU, at 37° C) from *Crotalus durissus* (EC.3.1.15.1) (for rationale, see **5.4 Results**) then diluted 1:100 with distilled water for HPLC separation and detection of Ip_5I breakdown products.

Chromatographic procedures

The chromatographic equipment consisted of a Waters 600E delivery system, a Waters 717+ autosampler and a Waters 2487 dual wavelength absorbance detector, which were managed by Millenium 2010 software. Analyses were performed under reverse-phase chromatography conditions, equilibrating the system with 100 mM KH_2PO_4, 4% methanol, pH 6.0, at 1.5ml min^{-1}. The column was a Spherisorb ODS-2 (25 cm length, 0.4 cm diameter; from Waters). Detection was monitored at 260 nm

wavelength. For phosphodiesterase measurements, ion-pair chromatography was performed. The mobile phase conditions were 10 mM KH_2PO_4, 2 mM tetrabutyl ammonium, 15% acetonitrile, pH 7.5, at 2ml min^{-1}. The column was a Spherisorb ODS-2. Detection was performed as above.

Oocyte preparation

See Chapter 2.

Electrophysiology

ATP-evoked membrane currents (I_{ATP}) (V_h = -60 to -90 mV) were recorded from cRNA-injected oocytes using a twin-electrode voltage-clamp amplifier (Axoclamp 2B). The voltage-recording and current-recording microelectrodes (1-5 MΩ tip resistance) were filled with 3.0 M KCl. Oocytes were superfused with a Ringer's solution (5ml min^{-1}, at 18° C) containing (mM): $NaCl_2$, 110; KCl, 2.5; HEPES, 5; $BaCl_2$, 1.8, adjusted to pH 7.5. The extracellular pH was maintained at pH 7.5 in all experiments, since the potency of ATP at $P2X_{1-4}$ receptors is affected by H^+ ions (King *et al.*, 1996c; Wildman *et al.*, 1999b;c).

Solutions and drugs

All solutions were nominally Ca^{2+}-free to avoid the activation of a Ca^{2+}-dependent Cl^- current ($I_{Cl,Ca}$) in oocytes. ATP was prepared in Ca^{2+}-free Ringer's solution at the concentrations stated, and superfused by a gravity-feed continuous flow system which allowed rapid addition and washout. Ip_nI compounds were dissolved in a buffer solution (HEPES 50 mM, pH 6.5 with KOH) to give a 10 mM stock solution, then

diluted further using Ringer's solution and readjusted to pH 7.5. For inhibition curves, ATP (at the EC_{70} value at pH 7.5 (in μM): $P2X_1$, 1; $P2X_2$, 20; $P2X_3$, 3; $P2X_4$, 10) was added to the superfusate for 60-120s, then washed off with Ringer's solution for 30 minutes. After obtaining agonist responses of consistent amplitude (Fig. 5.1A), diinosine polyphosphates (Ip_nI, 0.1-100,000 nM) were added to the superfusate for 30 minutes before and during re-application of ATP. The blocking activity of Ip_nI compounds did not improve with pre-incubation periods longer than 10 minutes (Fig. 5.1B) which suggested that Ip_nI blockade was not use-dependent, although Ip_nI compounds were routinely applied throughout the full 30 minutes of the ATP-washout period. For Schild analyses, the concentration/ response (C/R) relationship was determined for ATP (0.01-300 μM), then cRNA-injected oocytes were incubated with Ip_nI compounds for 30 minutes, after which ATP C/R curves constructed again in the continued presence of Ip_nI. EC_{50} values and slopes of C/R curves were taken from Hill plots, using the transform log $(I/I_{max}-I)$ where I is the current evoked by each concentration of ATP. pA_2 values were determined by Schild analysis, using the equation $pA_2 = \log (DR-1)-\log[Ip_5I]$.

5.4 RESULTS

Production of Ip_nI

5'-adenylic acid deaminase of *Aspergillus sp.* efficiently transformed Ap_5A into Ip_5I, fully converting the substrate into product over 90 minutes without further transformation of Ip_5I into other by-products. During intermediate times, HPLC analysis of the reaction medium revealed an additional peak with a retention time between that of the initial Ap_5A peak and final Ip_5I peak (Fig. 5.2A). This additional

peak gradually disappeared over 90 minutes and was attributed to the intermediary reaction product (Ip_5A), where only one of two adenosine moieties had been deaminated. Like results were obtained when producing Ip_4I from Ap_4A and Ip_3I from Ap_3A.

The final reaction product was confirmed as Ip_5I (as opposed to related breakdown products, *i.e.* IMP and Ip_4) in experiments where phosphodiesterase was used. This enzyme cleaves Ip_5I to yield inosine monophosphate (IMP) and the mononucleotide inosine 5'-tetraphosphate (Ip_4) (Fig. 5.2B). The retention times for these breakdown products was compared and matched against times for standard solutions of IMP and Ip_4. The absence of IMP and Ip_4 peaks (prior to enzymatic treatment at zero time) in HPLC analysis indicated Ip_5I had not been broken down during its synthesis. Similar results were observed when testing the purity of Ip_4I and Ip_3I.

Blockade of P2X receptors by Ip_5I

The pentaphosphate Ip_5I (1μM) was a potent inhibitor of ATP-responses at $rP2X_1$ receptors, and weak inhibitor of ATP-responses at $rP2X_3$ receptors (Fig. 5.3A,B and Table 5.1). Additionally, Ip_5I failed to block $rP2X_2$ receptors and potentiated ATP-responses at $rP2X_4$ receptors (Fig. 5.3C,D and Table 5.1). Thus, Ip_5I is an antagonist at Group 1 P2X receptors (as defined by Humphrey *et al.*, 1998a), yet approximately 900-fold more potent at $rP2X_1$ than $rP2X_3$ receptors. The inhibitory and facilitatory effects of Ip_5I were reversed on washout. Micromolar levels of Ip_5I had no effect on the holding current of either *Xenopus* oocytes injected with cRNA for $rP2X_{1-4}$ receptors or water-injected (control) oocytes. Thus, Ip_5I inhibition or facilitation was not due to a partial agonistic effect.

Blockade of P2X receptors by Ip_nI series

The blocking activity Ip_4I and Ip_3I was also investigated at Group 1 P2X receptors (Fig. 5.4A,B and Table 5.1). Ip_4I was 180-fold less potent than Ip_5I as an antagonist of $rP2X_1$ receptors yet, in contrast, 3-fold more potent than Ip_5I as an antagonist of $P2X_3$ receptors. The triphosphate Ip_3I was a weak antagonist of $rP2X_1$ and $rP2X_3$ receptors, only showing significant levels of blockade at high concentrations (>10 μM). The blocking actions of Ip_4I and Ip_3I were reversed on washout.

The actions of Ip_4I and Ip_3I were also studied at $rP2X_2$ and $rP2X_4$ receptors (Fig. 5.5A,B and Table 5.1). Both Ip_nI compounds, like Ip_5I, were inactive as either antagonists or potentiators at $rP2X_2$ receptors (Fig. 5.5A). At $rP2X_4$ receptors, Ip_4I was over 800-fold less potent than Ip_5I at potentiating ATP-responses (an effect reversed on washout), while Ip_3I had no effect (Fig. 5.5B and Table 5.1).

Schild analysis of Ip_5I blockade

Ip_5I (10, 30 and 100 nM) caused a rightwards displacement of the ATP concentration-response (C/R) curve, without altering the maximum agonist effect (Fig. 5.6A,B and Table 5.2). However, higher concentrations of Ip_5I (1 and 10 μM) completely blocked the agonist effects of ATP (≤1 mM) (Fig. 5.8), beyond which ATP exerts non-specific excitatory effects on *Xenopus* oocytes (Kupitz & Atlas, 1993). Thus, Ip_5I blockade was surmountable at low Ip_5I concentrations (≤100 nM), and nonsurmountable at micromolar concentrations. The antagonistic effects of Ip_5I (10-100 nM) on ATP C/R curves (EC_{50} values, Hill coefficients and pA_2 values) are summarised in Table 5.2. Schild analysis of paired C/R curves revealed that pA_2 values were not uniform,

varying between 7.70-9.23 (range of 12 determinations) for the mean pA_2 values listed (Table 5.2). This variability, in combination with nonsurmountable blockade with micromolar Ip_5I, indicated that this dinucleotide did not antagonise ATP-mediated membrane currents at $rP2X_1$ receptors in a simple competitive manner. On the other hand, high concentrations of Ip_5I (3 μM, approximate IC_{50} value) did cause a surmountable antagonism of ATP-responses at $rP2X_3$ receptors (Fig. 5.6B). EC_{50} values and Hill coefficients for ATP were (for the following Ip_5I concentrations): 0 μM, 1.4±0.4 μM (n_H, 1.13±0.12); 3μM, 9.6±2.5 μM (n_H: 1.08±0.18) (n=4). The pA_2 value for Ip_5I antagonism at $rP2X_3$ receptors was 6.27±0.22 (n = 4).

Schild analysis of Ip_4I blockade

Ip_4I (3 μM, approximate IC_{50} value) caused a nonsurmountable inhibition of ATP-response at $rP2X_1$ receptors, in the manner of non-competitive antagonist (Fig. 5.7A). EC_{50} values and Hill coefficients for ATP were (for the following Ip_4I concentrations): 0 μM, 0.38±0.06 μM (n_H, 0.84±0.17); 3 μM, 1.91±0.43 μM (n_H, 0.63±0.46) (n = 4). The maximum ATP effect was reduced by 56±7% (n = 4) by the dinucleotide. Thus, Ip_4I caused a reduction in both agonist potency and maximum response.

At $rP2X_3$ receptors, Ip_4I (3 μM, approximate IC_{75} value) caused a surmountable inhibition of ATP responses (Fig. 5.7B). EC_{50} values and Hill coefficients for ATP were (for the following Ip_4I concentrations): 0 μM, 1.0±0.3 μM (n_H, 0.82±0.11); 3 μM, 17.8±5.3 mM (n_H, 0.76±0.19) (n=4). The pA_2 value for Ip_4I antagonism at $rP2X_3$ receptors was 6.75±0.13 (n = 4).

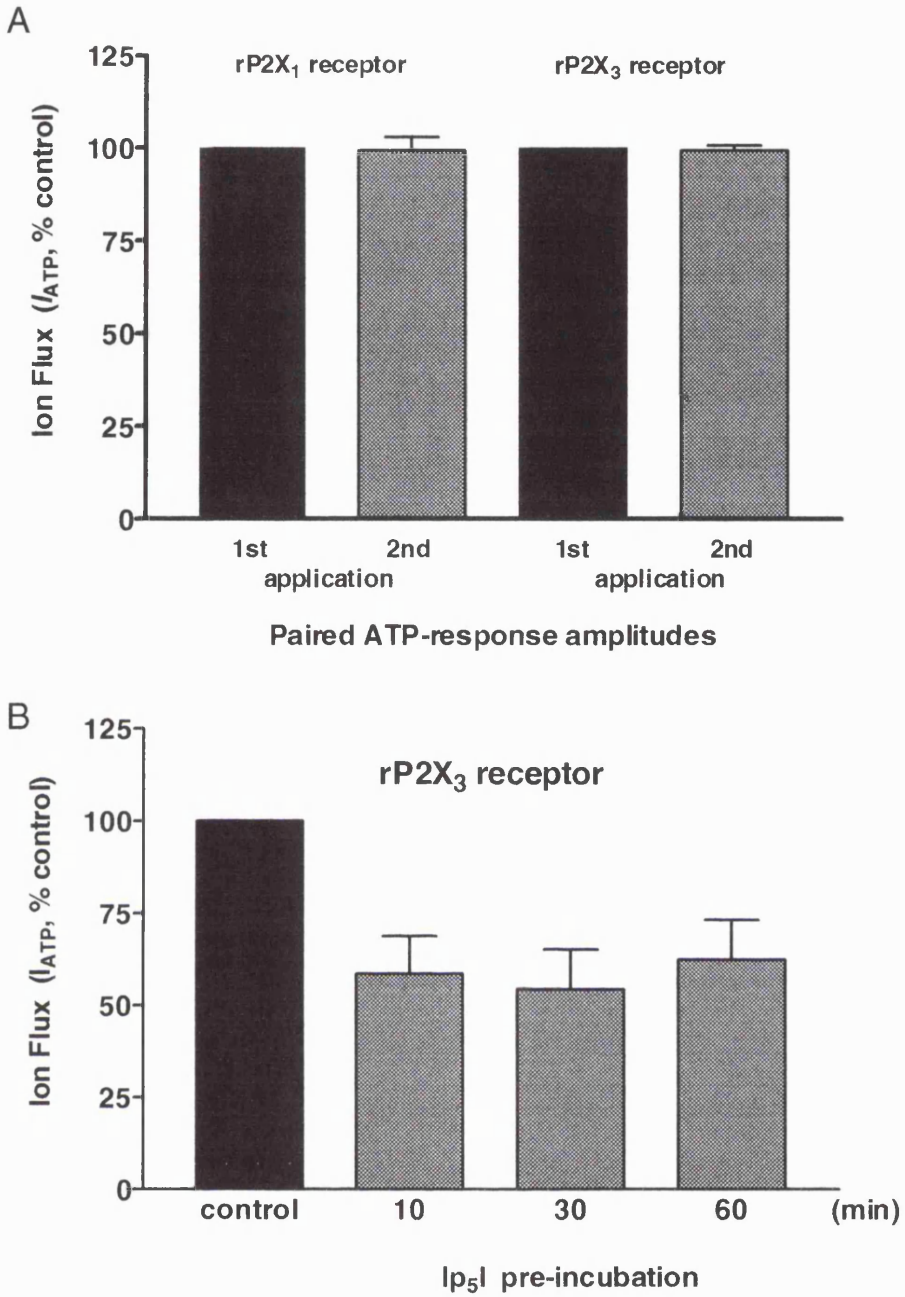


Figure 5.1 Consistency of agonist and antagonist activity. (A) Histograms of the amplitudes of paired agonist-responses at homomeric $rP2X_1$ and $rP2X_3$ receptors (using ATP: $rP2X_1$, 1 μM; $rP2X_3$, 3 μM). Washout periods of 30 min were used between first and second applications of ATP, a periodicity sufficient to yield I_{ATP} responses of consistent amplitude. Thus, Ip_nI-related antagonism could not be explained in terms of a rundown of ATP-responses at $P2X_1$ and $P2X_3$ receptors. (B) The effect of increasing the pre-incubation period (10, 30 and 60 min) to Ip_5I (3 μM) on the level of antagonism of ATP-responses (using 3 μM) at homomeric $rP2X_3$ receptors. The blocking activity of Ip_5I neither improved nor waned over 60 min pre-incubation, indicating that blockade was not use-dependent. Data: mean±s.e.mean (n=4) in A and B.

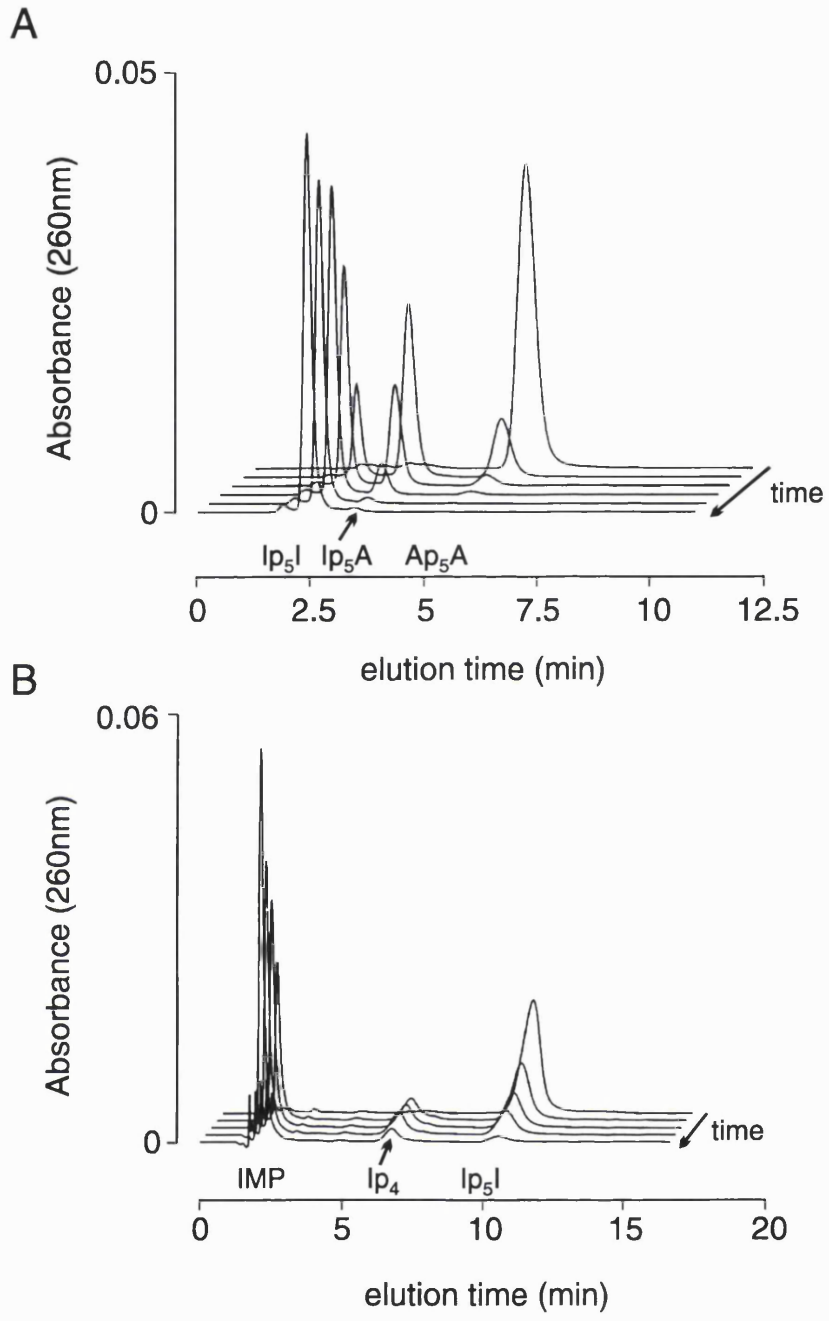


Figure 5.2 HPLC analysis of Ip_5I production. (A) A series of chromatographic profiles for the time-dependent enzymatic conversion of Ap_5A into the intermediary product Ip_5A and final product Ip_5I by adenylate deaminase (*Aspergillus sp.)* over a period of 90 min (chromatograms at: T = 0, 15', 30', 45', 60' and 90'). (B) A series of chromatographic profiles for the time-dependent production of IMP and Ip_4 by phosphodiesterase breakdown of the reaction product Ip_5I (chromatograms at: T = 0, 5', 10', 15' and 30'). Ordinate scalars (A and B) as AUFS (absorbance units full scale), as defined by Millennium 2010 software (Waters).

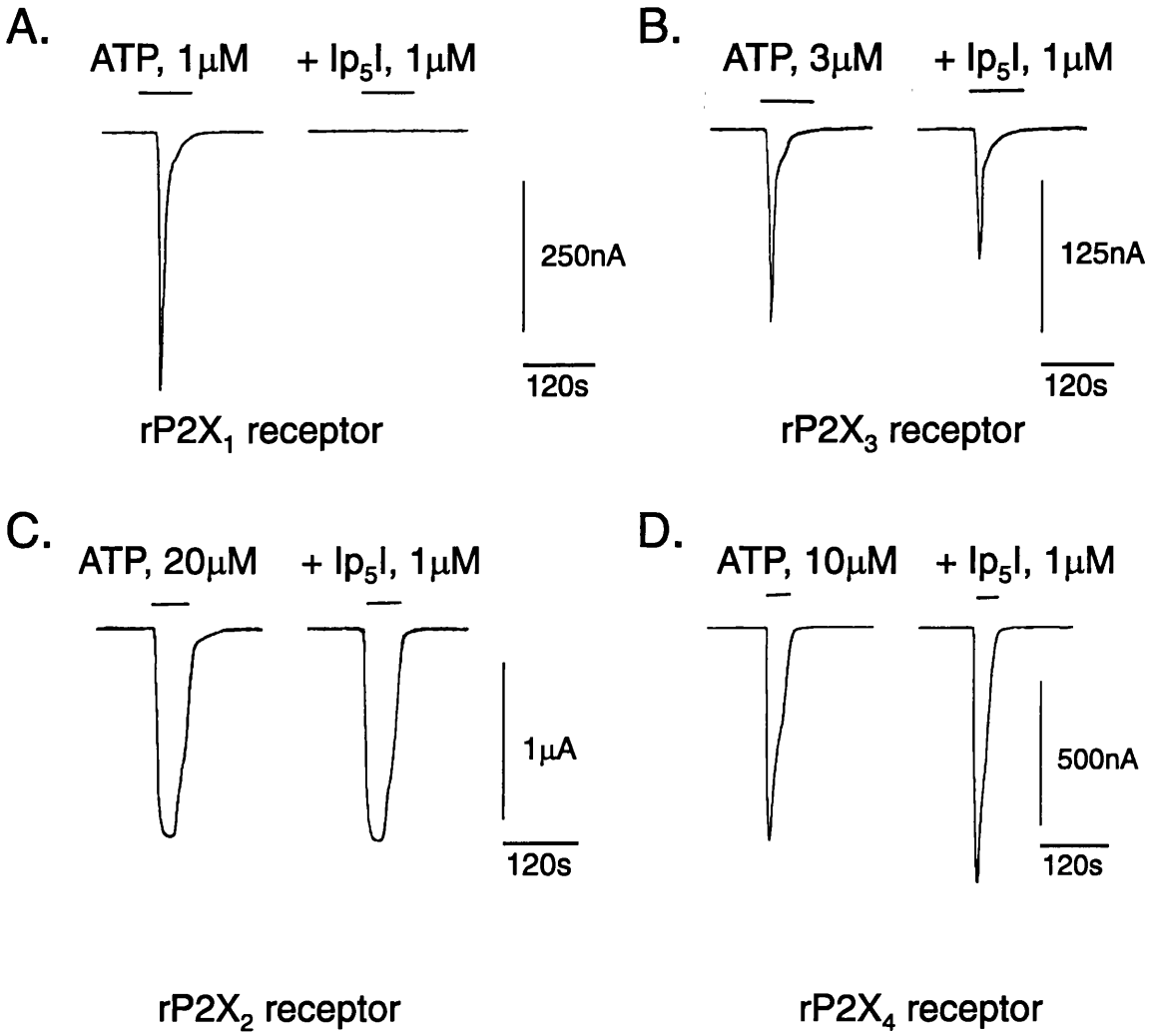


Figure 5.3 Ip_5I antagonism of ATP-responses at $rP2X_{1-4}$ receptors. ATP-activated whole-cell inward currents (I_{ATP}), before (first record) and during (second record) superfusion of micromolar Ip_5I (1 μM, 30 min pre-incubation). Paired I_{ATP} records were taken from single cRNA-injected oocytes expressing homomeric $rP2X_1$ (in A), $rP2X_3$ (in B), $rP2X_2$ (in C) and $rP2X_4$ receptors (in D). ATP was applied at a concentration equivalent to the EC_{70} value for each recombinant $rP2X_{1-4}$ receptor (see Methods). V_h = -60 mV in A-D.

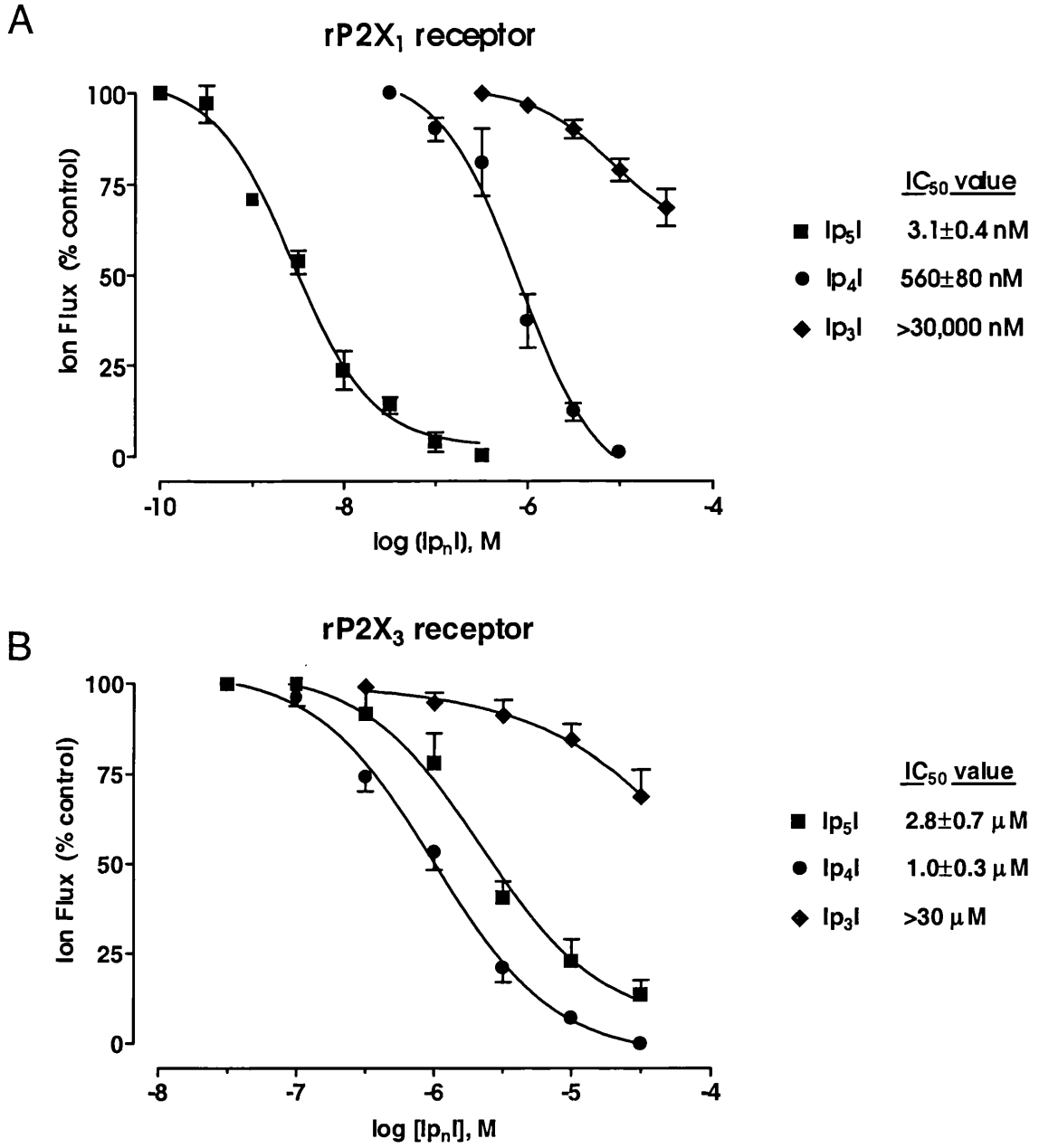


Figure 5.4 Inhibition curves for Ip_nI series at Group 1 P2X receptors. (A) Concentration-dependent inhibition of I_{ATP} (ATP, 1 μM) at homomeric $rP2X_1$ receptors by Ip_5I, Ip_4I and Ip_3I. (B) Concentration-dependent inhibition of I_{ATP} (ATP, 3 μM) at homomeric $rP2X_3$ receptors by the same diinosine polyphosphates. IC_{50} values and slopes of inhibition curves are given in Table 5.1. Data: mean±s.e.mean (n=5) in A and B.

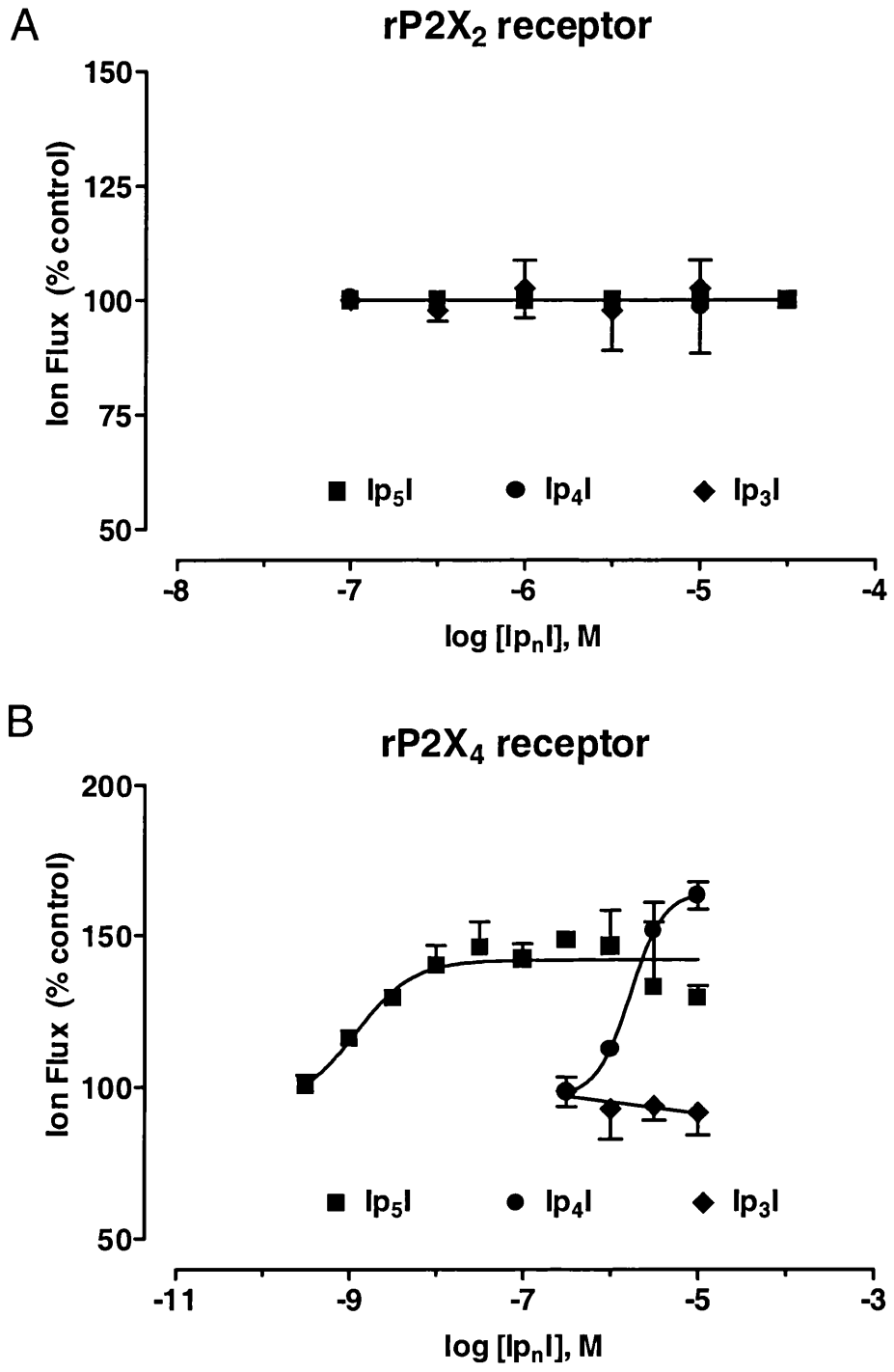


Figure 5.5 Activity of Ip_nI series at $rP2X_2$ and $rP2X_4$ receptors. (A) The IpnI series neither inhibited nor potentiated ATP-responses (using 20 μM) at $rP2X_2$ receptors. (B) Ip_5I and Ip_4I potentiated ATP-responses (using 10 μM) at $rP2X_4$ receptors. Activity indices for Ip_nI series given in Table 5.1. Data: mean±s.e.mean (n=5) in A and B.

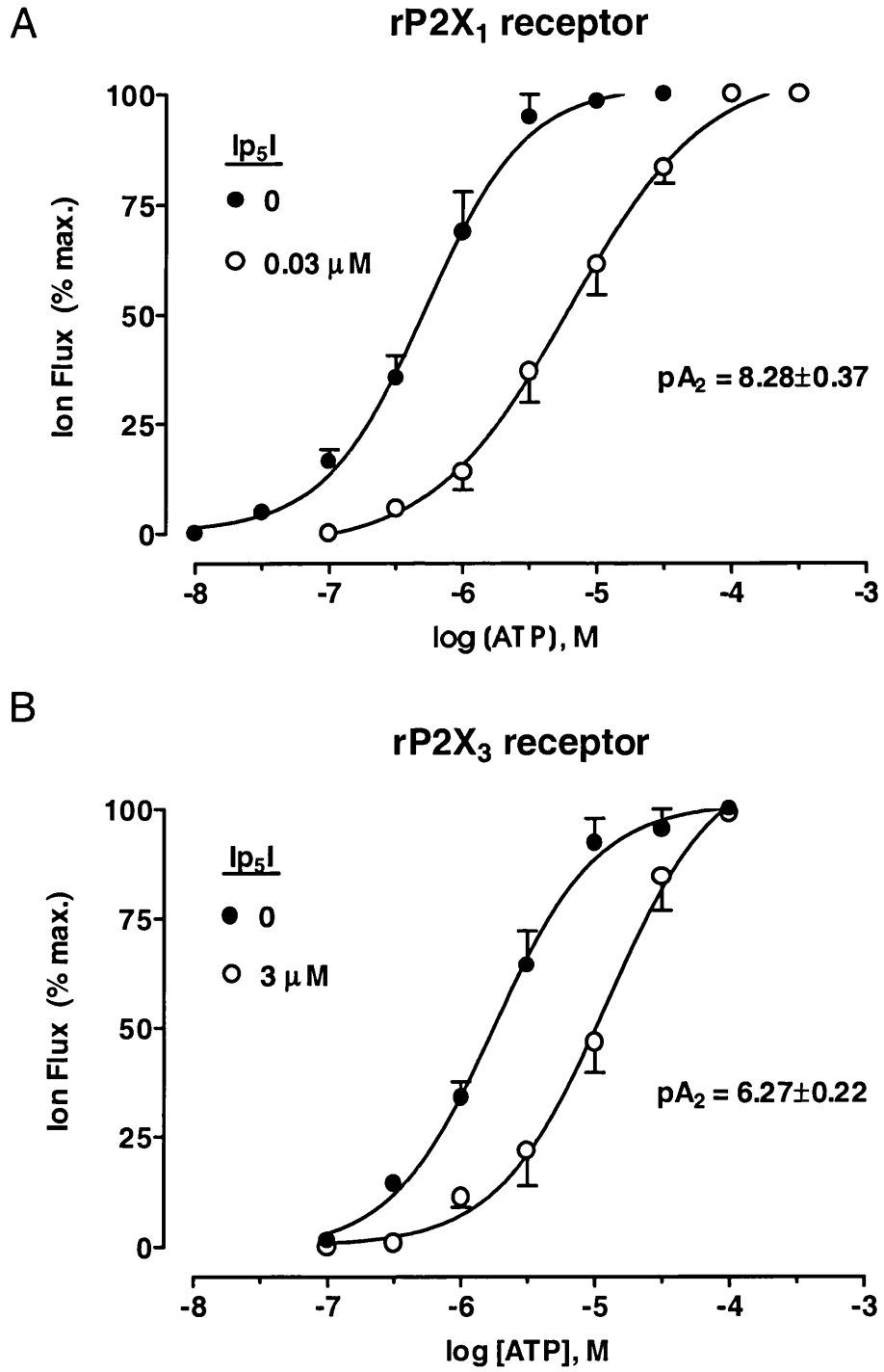


Figure 5.6 Ip_5I antagonism of ATP-responses at Group 1 P2X receptors. (A) Concentration-response curves for ATP (0.01-300 μM) at homomeric $rP2X_1$ receptors, before and during the presence of Ip_5I (30 μM). (B) Concentration-response curves for ATP (0.1-100 μM) at homomeric $rP2X_3$ receptors, before and during the presence of Ip_5I (3 μM). EC_{50} values, Hill coefficients and pA_2 values given in Table 5.2. Data: mean±s.e.mean (n=4) for paired C/R curves, in A and B.

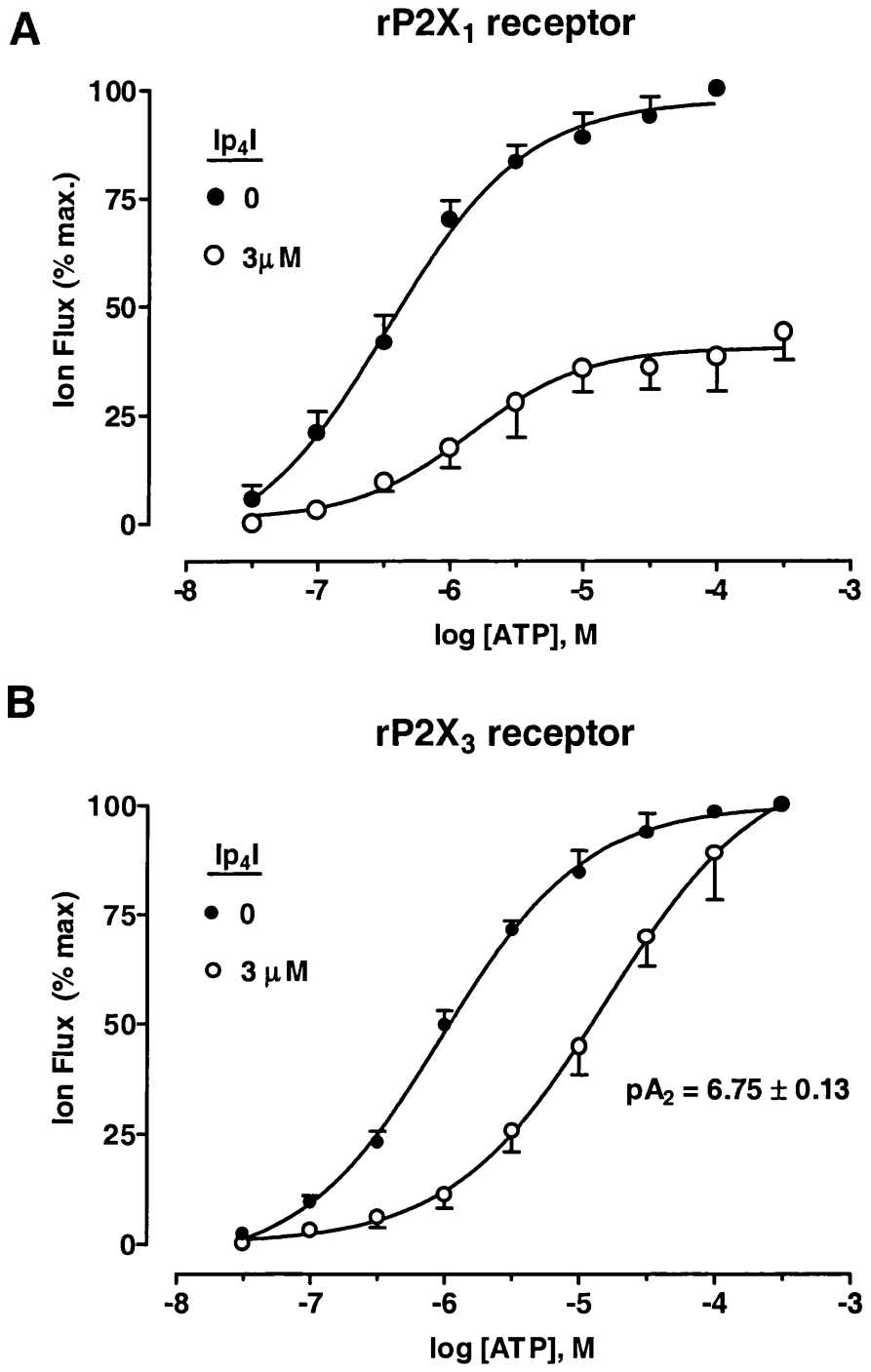


Figure 5.7 Ip_4I antagonism of ATP-responses at Group 1 P2X receptors. (A) Concentration-response curves for ATP (0.03-300 μM) at homomeric $rP2X_1$ receptors, before and during the presence of Ip_4I (3 μM). (B) Concentration-response curves for ATP (0.03-300 μM) at homomeric $rP2X_3$ receptors, before and during the presence of Ip_4I (3 μM). EC_{50} values, Hill coefficients and pA_2 values given in the text. Data: mean±s.e.mean (n=4) for paired C/R curves, in A and B.

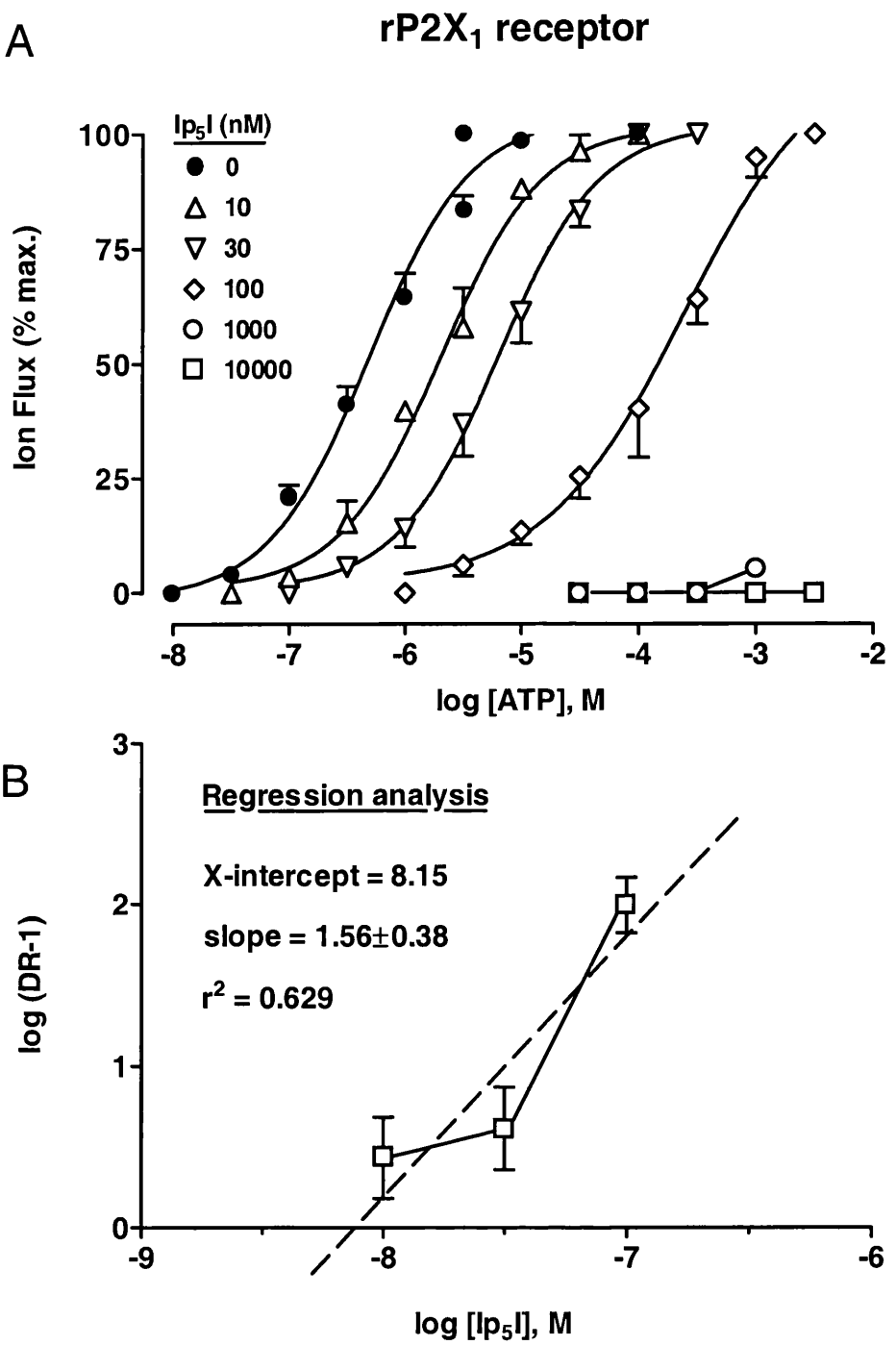


Figure 5.8 Schild analysis of Ip_5I blockade of $rP2X_1$ receptors. (A) Concentration/response (C/R) curves for the ATP-activated $rP2X_1$ receptor, in the absence and presence of Ip_5I (10-10,000 nM). The control C/R curve represented pooled data of 20 determinations, and test C/R curves determined for four experiments for each of the five concentrations of Ip_5I used (10, 30, 100, 1000 and 10,000 nM). (B) The Schild plot of C/R data according to EC_{50} values stated in Table 5.2. Linear regression (Prism v2.0, GraphPad) gave an estimated pA_2 of 8.150, mean slope of 1.56 (i.e., greater than unity), and a low correction coefficient (r^2) of 0.629 for linearity. The Schild plot was clearly biphasic.

Table 5.1

Activity indices of Ip_nI compounds at $rP2X_{1-4}$ receptors

Compounds	$P2X_1$	$P2X_2$	$P2X_3$	$P2X_4$
	IC_{50} value		*IC_{50} value*	*EC_{50} value*
Ip_5I	0.0031±0.0004 μM	inactive	2.8±0.7 μM	0.0020±0.0004 μM
	(n_H, -0.96±0.08)	(0.1-30 μM)	(n_H, -1.02±0.14)	(n_H, 1.33±0.09)
Ip_4I	0.560±0.080 μM	inactive	1.0±0.3 μM	1.69±0.40 μM
	(n_H, -1.47±0.20)	(0.1-30 μM)	(n_H, -1.16±0.10)	(n_H, 2.29±0.17)
Ip_3I	>30 μM	inactive	>30 μM	inactive
		(0.1-30 μM)		(0.3-30 μM)

IC_{50} and EC_{50} values for the inhibitory and facilitatory effects of Ip_nI compounds on ATP-responses at recombinant $P2X_{1-4}$ receptors. Slopes of inhibition and facilitation curves are given in brackets. Data: mean±s.e.mean (n=5).

Table 5.2

Schild analysis of Ip_5I displacement of ATP C/R curves at $rP2X_1$ receptors

Ip_5I (nM)	EC_{50} values and Hill coefficients		pA_2	n
	Control	*+ Ip_5I*		
10	0.38±0.07 μM	1.89±0.87 μM	8.43±0.29	4
	(n_H, 0.96±0.06)	(n_H, 0.89±0.08)	(range, 7.99-8.89)	
30	0.42±0.06 μM	3.56±2.05 μM	8.28±0.37	4
	(n_H, 0.99±0.14)	(n_H, 0.82±0.09)	(range, 7.70-8.86)	
100	0.70±0.15 μM	92.1±15.7 μM	8.99±0.19	4
	(n_H, 1.02±0.15)	(n_H, 0.86±0.16)	(range, 8.49-9.23)	

EC_{50} values and Hill coefficients (n_H) for the ATP C/R relationship in the absence (control) and presence (+Ip_5I) of diinosine pentaphosphate (10-100 nM). Data: mean±s.e.mean of paired sets of observations.

5.5 DISCUSSION

The present results showed that Ip_5I was an effective antagonist of ATP-responses at Group 1 P2X receptors ($P2X_1$ and $P2X_3$), being selective for $rP2X_1$ receptors at low (≤100 nM) concentrations. This pentaphosphate was 900-fold less potent at $P2X_3$ receptors (comparing IC_{50} values), at which micromolar concentrations were required to block ATP-activated inward currents. The blocking actions of Ip_5I at $P2X_1$ receptors at nanomolar concentrations initially seemed consistent with a competitive antagonism. However, several features suggested that the mechanism of Ip_5I blockade was more complex than originally thought. First, micromolar concentrations of Ip_5I caused a nonsurmountable inhibition of ATP-responses (Fig. 5.8A). Second, determinations of the pA_2 value were dependent on the Ip_5I concentration used (see Table 5.2). Third, a Schild plot of combined Ip_5I data (10, 30 and 100 nM) was non-linear and the slope (by regression analysis) was greater than unity (Fig. 5.8B). Taken together, these features suggested that Ip_5I is not a simple competitive antagonist at $rP2X_1$ receptors. Also, the parent compound Ap_5A is a partial agonist at $rP2X_1$ receptors (Wildman *et al.*, 1999a), suggesting that the binding site for the pentaphosphate (as either Ap_5A or Ip_5I) might not be the same position as the ATP docking site.

For $P2X_1$ receptors, the blocking activity of the Ip_nI series decreased as the phosphate chain was reduced in length. Thus, Ip_5I was 180-fold more potent than Ip_4I and greater than 10,000-fold more potent than Ip_3I. This potency order for antagonism clearly contrasted with the agonist potency order of their parent compounds at $rP2X_1$ receptors, where Ap_4A (7.4) >Ap_5A (6.0) >Ap_3A (>4) (pEC_{50} values) (Wildman *et al.*, 1999a). Like Ip_5I, Ip_4I was a non-competitive antagonist at $rP2X_1$ receptors and significantly reduced

the maximum ATP effect. Its parent compound, Ap_4A, is a partial agonist at $rP2X_1$ receptors, suggesting the binding site for the tetraphosphate (as either Ap_4A or Ip_4I) might again differ from the ATP docking site. Interestingly, neither dinucleotide triphosphate (Ip_3I and Ap_3A) interacted well with $rP2X_1$ receptors in terms of antagonist and agonist activities, yet $rP2X_1$ receptor is activated by a number of mononucleoside triphosphates.

The $rP2X_3$ receptor showed a slight preference for Ip_4I over Ip_5I, their pA_2 values being 6.75 and 6.27, respectively. Both diinosine compounds appeared to act as competitive antagonists, causing a parallel rightwards shift of the ATP C/R curve without reducing the maximum ATP effect (Fig. 5.6B, 5.7B). Their parent compounds, Ap_4A and Ap_5A, are both full agonists at $rP2X_3$ receptors, at which the tetraphosphate (pEC_{50}, 6.10) is slightly more potent than the pentaphosphate (pEC_{50}, 5.88) (Wildman *et al.*, 1999a). As far as the triphosphate is concerned, Ip_3I is a weak antagonist and Ap_3A a partial agonist at $rP2X_3$ receptors.

The Ip_nI series lacked activity at $rP2X_2$ receptors at which the parent Ap_nA compounds otherwise showed interesting effects. Ap_4A is a full agonist at $rP2X_2$ receptors, although 4-fold less potent than ATP, while nanomolar Ap_5A is a potent potentiator of ATP-responses (Pintor *et al.*, 1996). Thus, the inability of Ip_4I and Ip_5I to interact with $rP2X_2$ receptors contrasted sharply with earlier results with their parent compounds (Ap_4A and Ap_5A), although the lack of activity of the diinosine polyphosphates at least reflects the efficiency of the enzyme degradation process to make Ip_nI compounds. Ip_5I was reported to be inactive against ATP-responses at $P2X_2$-like receptors in neonatal rat cerebellar Purkinje neurons, although Ip_3I and Ip_4I have not yet been tested in this model (Garcia-Lecea *et al.*, 1999).

For rP2X$_4$ receptors, the potentiating effects of Ip$_5$I and Ip$_4$I struck a chord with similar potentiating effects of other compounds (*e.g.* suramin, PPADS and Reactive blue 2) tested as P2 receptor antagonists at the rat P2X$_4$ receptor (Bo *et al.*, 1995) and mouse P2X$_4$ receptor (Townsend-Nicholson *et al.*, 1999). The Ip$_n$I compounds proved just as ineffective as antagonists at rP2X$_4$ receptors as other compounds tested so far. The parent dinucleotide of Ip$_4$I is a partial agonist at rP2X$_4$ receptors (pEC$_{50}$, 5.5) (Wildman *et al.*, 1999a). However, the potentiating effect of Ip$_4$I was not accompanied by any change in holding currents and was not believed to be due to Ap$_4$A contamination.

Of the Ip$_n$I series, only Ip$_5$I has been tested at native P2X receptors. Ip$_5$I is considerably less potent as an antagonist at the P2X$_1$-like receptor in guinea pig vas deferens (pA$_2$, 6.5±0.1) (Hoyle *et al.*, 1997), compared to its blocking activity at the recombinant rP2X$_1$ receptor in the present study (pA$_2$ range, 7.70-9.23). This reduction in activity may be due to Ip$_5$I breakdown by ecto-nucleotidases in guinea-pig vas deferens, or perhaps due to a difference between guinea-pig and rat P2X$_1$ receptors. The guinea-pig P2X$_1$ receptor has not yet been cloned and, furthermore, Ip$_5$I has not yet been tested on the human P2X$_1$ receptor. Thus far, the blocking activity of Ip$_5$I has only been characterised at the rat P2X$_1$ receptor. Potential species differences in blocking activity notwithstanding, the observed Ip$_5$I activity at native P2X$_1$ receptors in guinea-pig still compares favourably with the non-competitive blocking activity of suramin (pK_b, 5.3±0.2), iso-PPADS (pK_b, 6.6±0.2) and Reactive blue 2 (pK_b, 5.8±0.2) at P2X$_1$-like receptors in rat vas deferens (Khakh *et al.*, 1994). To this end, Ip$_5$I may yet prove to be a useful pharmacological tool in bioassays of naturally-occurring P2X$_1$ receptors, for example human HL60 cells (Buell *et al.*, 1996c) and murine thymocytes (Chvatcho *et al.*,

1996) as well as $P2X_1$-like receptors in vas deferens and vascular smooth muscle of various mammalian species (Humphrey *et al.*, 1998a).

SECTION B

CHAPTER 6

DIINOSINE PENTAPHOSPHATE: AN ANTAGONIST WHICH DISCRIMINATES BETWEEN RECOMBINANT $P2X_3$ AND $P2X_{2/3}$ RECEPTORS AND BETWEEN TWO P2X RECEPTORS IN RAT SENSORY NEURONS

6.1 ABSTRACT

The antagonist activity of trinitrophenyl-ATP (TNP-ATP) and diinosine pentaphosphate (Ip_5I) have been compared at recombinant P2X receptors expressed in *Xenopus* oocytes and at native P2X receptors in sensory neurons from dorsal root and nodose ganglia. Slowly-desensitizing responses to α,β-methylene ATP (α,β-meATP) recorded from oocytes expressing $P2X_{2/3}$ receptors were inhibited by TNP-ATP at sub-micromolar concentrations. However, Ip_5I at concentrations up to 30 μM was without effect. Nodose ganglion neurons responded to α,β-meATP with slowly-desensitizing inward currents. These were inhibited by TNP-ATP (IC_{50}, 20 nM), but not by Ip_5I at concentrations up to 30 μM. In DRG neurons that responded to ATP with a rapidly-desensitizing inward current, the response was inhibited by TNP-ATP with an IC_{50} of 0.8 nM. These responses were also inhibited by Ip_5I with an IC_{50} of 0.1 μM. Both antagonists are known to inhibit homomeric $P2X_3$ receptors. Some DRG neurons responded to α,β-meATP with a biphasic inward current, consisting of transient and sustained components. While the transient current was abolished by 1 μM Ip_5I, the sustained component remained unaffected. In conclusion, Ip_5I is an antagonist at homomeric $P2X_3$ receptors but not at heteromeric $P2X_{2/3}$ receptors, and therefore should be useful tool for elucidating the subunit composition of native P2X receptors.

6.2 INTRODUCTION

ATP can excite sensory neurons from dorsal root and nodose ganglia as well as neurons in the dorsal horn of the spinal cord (Jahr & Jessel, 1983; Krishtal *et al.*, 1983). Furthermore, when ATP is injected intradermally, it causes intense pain, this effect suggesting a role for ATP in nociception and primary afferent neurotransmission (for review see Burnstock & Wood, 1996; Burnstock, 2000). Immunohistochemical and *in situ* hybridization studies indicated that of the seven P2X subunits cloned, $P2X_1$-$P2X_6$ are expressed in sensory ganglia (Chen *et al.*, 1995; Collo *et al.*, 1996; Vulchanova *et al.*, 1997; Xiang *et al.*, 1998). Functional studies *in vitro* so far suggested that it is the $P2X_2$ and $P2X_3$ subunits that are important for the activation of primary afferents by ATP (Lewis *et al.*, 1995; Robertson *et al.*, 1996; Cook *et al.*, 1997). However, the lack of suitable pharmacological tools has so far prevented the characterizaion of P2X receptors on sensory neurons *in vivo*.

P2X receptors are oligomeric complexes of uncertain stoichiometry, but possibly composed of either three subunits (Nicke *et al.*, 1998; Stoop *et al.*, 1999) or four subunits (Kim *et al.*, 1997; Ding & Sachs, 2000). In sensory neurons, the expression of $P2X_2$ and $P2X_3$ receptors can give rise to the formation of both homomeric $P2X_2$ and $P2X_3$ receptors as well as at least one population of heteromeric $P2X_{2/3}$ receptors (Lewis *et al.*, 1995; Thomas *et al.*, 1998; Grubb & Evans, 1999). The development of more selective agonist and antagonists will help to clarify the involvement of different subunits in the formation of native P2X receptors and the role of these different receptors in physiological and pathophysiological processes.

Diinosine pentaphosphate (Ip_5I) is a very potent antagonist at recombinant $P2X_1$ receptors (pA_2, 8.2) and is quite active at $P2X_3$ receptors with a pA_2 of 6.3 (King *et al.*, 1999). In this study, I have investigated the antagonist activity of Ip_5I at the

recombinant heteromeric $P2X_{2/3}$ receptor. I have then compared it with trinitrophenyl-ATP (TNP-ATP) as an antagonist at the rapidly-desensitizing P2X receptors present on dorsal root ganglion (DRG) neurons and the slowly-desensitizing α,β-meATP sensitive receptor present on nodose ganglion neurons. While the former are believed to be homomeric $P2X_3$ (Robertson *et al.*, 1996; Cook *et al.*, 1997), the latter are thought to be heteromeric $P2X_{2/3}$ receptors (Lewis *et al.*, 1995).

6.3 METHODS

Native receptors in sensory neurons

Dissociated sensory neurons were prepared following a protocol used previously for isolation of autonomic ganglion neurons (Zhong *et al.*, 1998). Rat pups (0-2-days-old) were killed by cervical dislocation followed by decapitation. Dorsal root or nodose ganglia were removed and placed in Ca^{2+}/Mg^{2+} free Hanks' Balanced Salt Solution containing 10 mM HEPES (pH 7.3) buffer (HBSS). Ganglia were treated with collagenase 1.5 mg ml^{-1} (CLS II; Worthington Biochemical Corporation, Reading, U.K.) for 40 min, followed by trypsin 1 mg ml^{-1} (Sigma) for 15 min. The ganglia were then suspended in growth medium comprising of L-15 medium (Gibco) supplemented with 10% bovine serum albumin, 2 mg ml^{-1} $NaHCO_3$, 5.5 mg ml^{-1} glucose, 200 IU ml^{-1} penicillin and 200 μg ml^{-1} streptomycin. Following gentle trituration with a fire-polished Pasteur pipette single cells were harvested by centrifugation at 900 r.p.m. for 5 min. The resultant pellet was re-suspended in growth medium and plated onto 35 mm culture dishes that had been pre-treated with 10 μg ml^{-1} laminin (Sigma). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2, and used between 2 and 10 h after plating.

Whole cell voltage-clamp recording was carried out at room temperature using an Axopatch 200B amplifer (Axon Instruments, Foster City, U.S.A.) Membrane

potential was held at -70 mV. External solution contained (mM): NaCl 154, KCl 4.7, $MgCl_2$ 1.2, $CaCl_2$ 2.5, HEPES 10, Glucose 5.6, the pH was adjusted to 7.4 using NaOH. Recording electrodes (resistance 2-4 MΩ) were filled with internal solution that contained (mM): CsCl 120, HEPES 10, tripotassium citrate 10, the pH was adjusted to 7.2 using CsOH. Data were acquired using pCLAMP software (Axon Instruments). Signals were filtered at 2 kHz (-3 dB frequency, Bessel filter, 80 dB/decade).

Drugs were applied rapidly through a 4-barrel manifold comprising fused glass capillaries inserted into a common outlet tube (tip diameter of ~200 μm) which was placed about 200 μm from the cell (Dunn *et al.*, 1996). Solutions were delivered by gravity flow from independent reservoirs with solution flow controlled by computer driven solenoid valves. One barrel was used to apply drug free solution to enable rapid termination of drug application. Solution exchange measured by the change in open tip current on switching from 150 mM NaCl to 150 mM KCl solution was complete in 20 ms; however, complete change of solution around an intact cells was considerably slower (≤100 ms). Traces were acquired using Fetchex (pCLAMP software) and plotted using Origin (Microcal, Northampton, MA, U.S.A.).

Recombinant receptors expressed in *Xenopus* oocytes

Applications of agonists were separated by a 20 min interval unless otherwise stated. The P2 receptor antagonists TNP-ATP (3-1000 nM) and Ip_5I (0.03-100 μM) were applied for 1 min prior to and during agonist application. For concentration-inhibition curves, data were normalized with respect to the response evoked in the absence of antagonist.

Data analysis

All data are expressed as the mean ±s.e.mean. Statistical analysis (Student's *t*-test) was performed using Origin 5 (Microcal, Northampton, MA, U.S.A.); p values < 0.05 were considered significant. Concentration-responses data were fitted with the Hill equation: $Y = A/[1+(K/X)^n]$, where: A is the maximum effect, K is the EC_{50}, and n is the Hill coefficient, using Origin 5. The combined data from the given number of cells were fitted, and the results are presented as values±s.e.mean, determined by the fitting routine.

6.4 RESULTS

Recombinant receptors expressed on *Xenopus* oocytes

Oocytes injected with $P2X_3$ cRNA responded to ATP (3 μM) with a rapidly-desensitizing inward current (Fig. 6.1A). Micromolar concentrations of Ip_5I produced a concentration dependent inhibition of this response (Fig. 6.1A, B)

. In contrast, oocytes injected with $P2X_2$ cRNA gave a slowly-desensitizing response to ATP that was not affected by Ip_5I at concentrations up to 30 μM (Fig. 6.1A, B). In oocytes co-injected with $P2X_2$ and $P2X_3$ cRNA, it was necessary to use α,β-meATP as the agonist, to prevent activation of homomeric $P2X_2$ receptors. Nevertheless, α,β-meATP evoked a biphasic response (see Fig. 6.2) that I attribute to the activation of homomeric $P2X_3$ and heteromeric $P2X_{2/3}$ receptors (n=8). By using a pre-application of 10 μM α,β-meATP to produce selective desensitization of the $P2X_3$ receptor, the heteromeric receptor could be studied in isolation. Ip_5I at concentrations up to 30 μM produced no inhibition of the slowly-desensitizing response to 1 μM α,β-meATP (Fig. 6.1A, B). In contrast, sub-micromolar concentrations of TNP-ATP (0.01-0.1 μM) produced a

concentration-dependent reduction in the response to α,β-meATP (Fig. 6.1C), consistent with the previously published IC_{50} of 7 nM (Virginio *et al.*, 1998). Similar results were obtained in all four oocytes tested. I next investigated the effect of these two antagonists on co-injected oocytes, without prior desensitization of the $P2X_3$ receptor. Under these conditions, α,β-meATP evoked a biphasic response composed of transient and sustained inward currents. TNP-ATP produced a concentration dependent reduction in both the transient and sustained parts of the response (Fig. 6.2A). In contrast, Ip_5I selectively abolished the transient component of the response while leaving the sustained current unaffected (Fig. 6.2B).

Native receptors in nodose ganglion neurons

In agreement with the observations of Khakh and colleagues (1995), all neurons dissociated from neonatal rat nodose ganglia responded to 10 μM α,β-meATP with a rapidly-activating and slowly-desensitizing response (Fig. 6.3A) attributed to the activation of heteromeric $P2X_{2/3}$ receptors. These neuron posses both homomeric $P2X_2$ and heteromeric $P2X_{2/3}$ receptors (Thomas *et al.*, 1998), so α,β-meATP was used as the agonist to avoid activation of homomeric $P2X_2$ receptors. Transient responses to α,β-meATP are not evoked in these neurons, indicating the absence of homomeric $P2X_3$ receptors. Consequently, the desensitizing pre-pulse used in the oocytes experiments was not necessary. TNP-ATP produced a concentration dependent inhibition of this response with an IC_{50} of 20.5±2.8 nM (Fig. 6.3C). I investigated further the antagonism produced by TNP-ATP, by looking at its action on the α,β-meATP log-concentration-response curve. In the presence of 0.1 μM TNP-ATP, a concentration that I had found to reduce the response to 10 μM α,β-meATP by about 75%, there was a rightward shift in the α,β-meATP log-concentration-response curve, increasing the

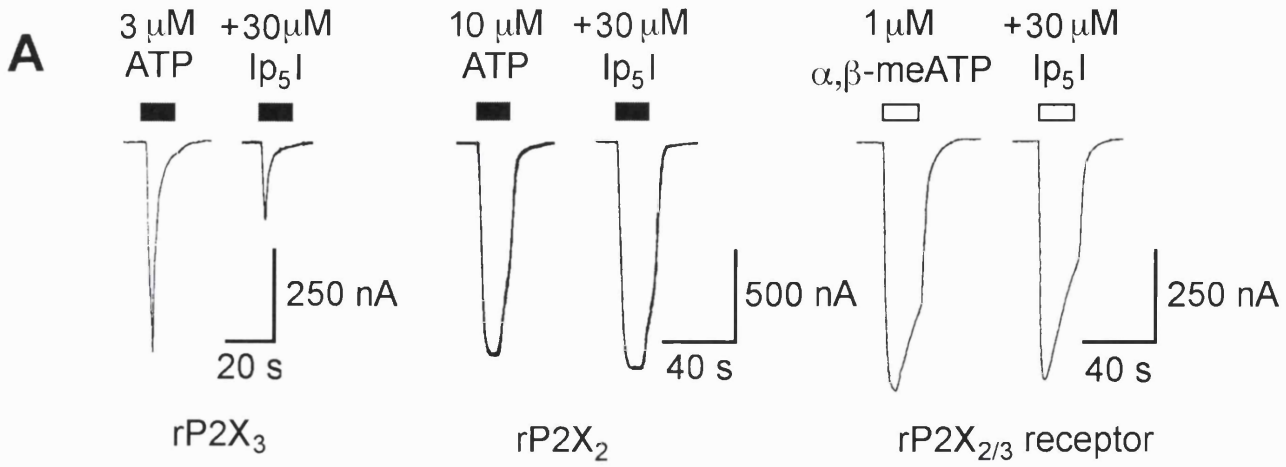
EC_{50} from 39-167 μM (Fig. 6.4A). However, there was also a marked reduction in the slope of the curve, suggesting that TNP-ATP does not act as a simple competitive antagonist. In contrast, Ip_5I at concentrations up to 30 μM failed to produce any antagonism of the α,β-meATP response in nodose ganglion neurons (Fig. 6.3D).

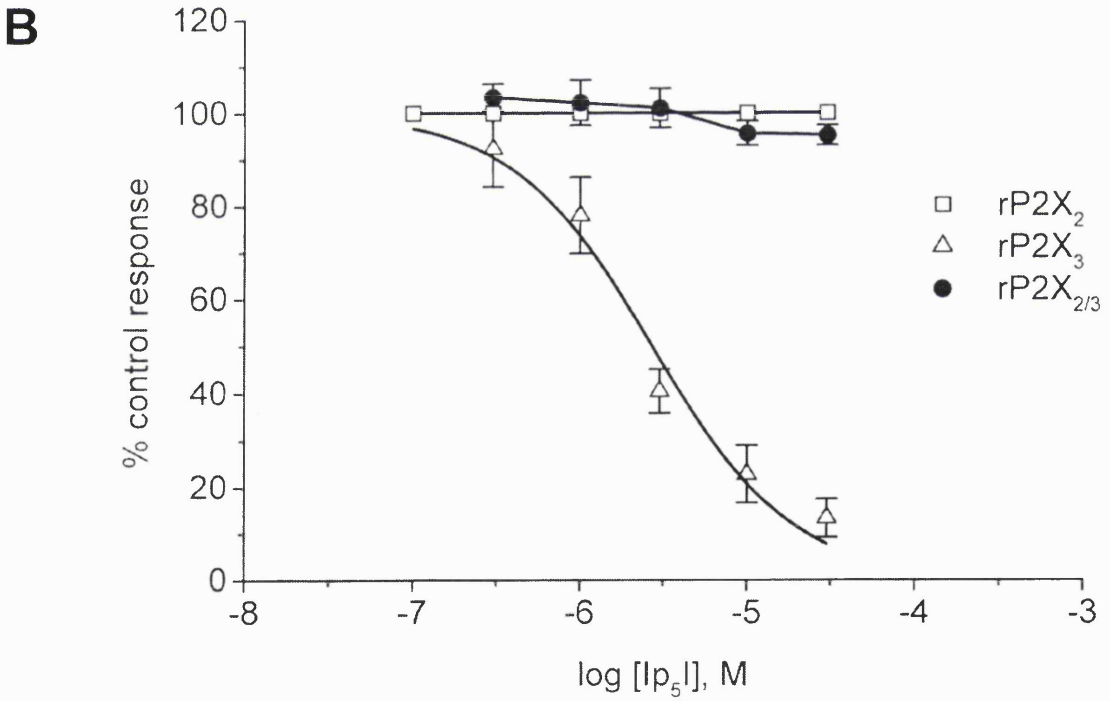
Native receptors in DRG neurons

DRG neurons can respond to ATP with either rapidly-desensitizing, sustained, or biphasic responses (Robertson *et al.*, 1996; Burgard *et al.*, 1999; Grubb & Evans, 1999). In these experiments, approximately 50% (105/209) of dissociated DRG neurons, responded to 10 μM ATP with only a fast activating and rapidly-desensitizing inward current. This response declined to less than 10% of the peak by the end of a 1 s agonist application and is believed to be mediated by homomeric $P2X_3$ receptors (Fig. 6.3B). TNP-ATP produced a rapid and reversible inhibition of this response. The effect of TNP-ATP was concentration dependent with an IC_{50} of 0.77±0.12 nM (Fig. 6.3C). Ip_5I also produced a reversible, concentration dependent inhibition of the transient ATP response in DRG neurons, with an IC_{50} of 0.12±0.03 μM (Fig. 6.3D). I investigated the nature of the antagonism produced by Ip_5I, by examining its effect on the ATP log-concentration response curve. In the presence of 0.3 μM Ip_5I, a concentration predicted to give approximately 75% inhibition, the maximum response to ATP was greatly reduced, while the EC_{50} was almost unchanged (2.3 μM compared with 2.1 μM; Fig. 6.4B).

A small percentage of DRG neurons responded to α,β-meATP with a clearly biphasic inward current, consisting of both transient and sustained components, similar to those observed in co-injected oocytes. In four such cells where the current at 1 s was

36±6% of the peak current, 1 μM Ip_5I, abolished the transient component, while leaving the sustained current unaffected (Fig. 6.5).


A
3 μM ATP
+30μM Ip5I
250 nA
20 s
rP2X3
10 μM ATP
+30 μM Ip5I
500 nA
40 s
rP2X2
1μM α,β-meATP
+30 μM Ip5I
250 nA
40 s
rP2X2/3 receptor


B
% control response
120
100
80
60
40
20
0
-8
-7
-6
-5
-4
-3
log [Ip5I], M
rP2X2
rP2X3
rP2X2/3

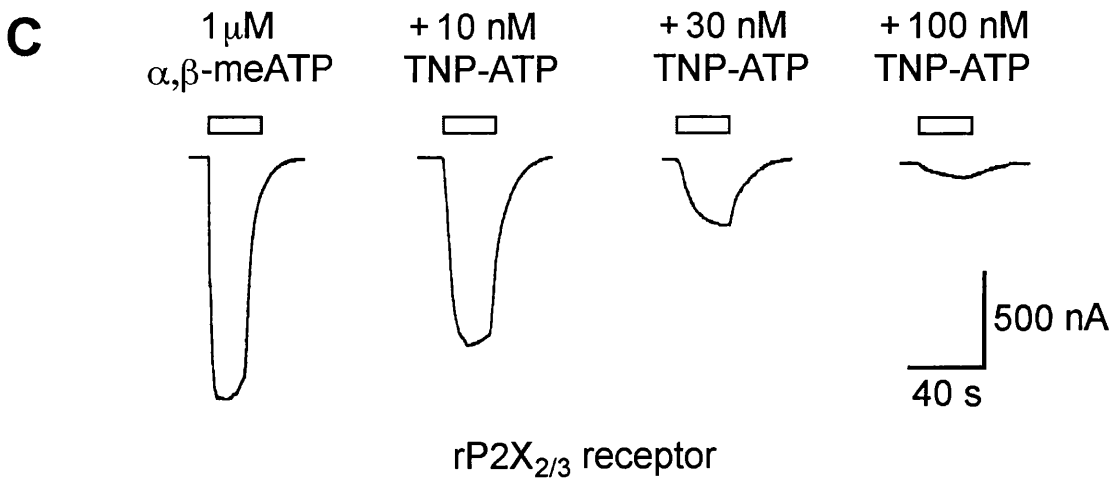


Figure 6.1 Comparison of the action of TNP-ATP and Ip_5I on recombinant $P2X_2$, $P2X_3$ and $P2X_{2/3}$ receptors. (A) Responses of three different *Xenopus* oocytes expressing $P2X_3$, $P2X_2$ and $P2X_{2/3}$ receptors, to ATP or α,β-meATP alone, and in the presence of 30 μM diinosine pentaphosphate (Ip_5I). (B) Concentration-effect curves for the inhibition of $P2X_3$, $P2X_2$ and $P2X_{2/3}$ receptors by Ip_5I. ATP was used as the agonist at 3 μM and 10 μM for $P2X_3$ and $P2X_2$ receptors, respectively, while 1 μM α,β-meATP was used in experiments on the heteromeric receptor. Data: mean±s.e.mean, n=5. Antagonists were given for 60 s before and during the agonist application. (C) Responses to α,β-meATP recorded from an oocyte expressing $P2X_{2/3}$ receptors in the presence of increasing concentrations of TNP-ATP. V_H: -50 mV. Antagonists were present for 60 s before and during the agonist applications. In oocytes co-injected with $P2X_2$ and $P2X_3$ transcripts, the transient current due to activation of homomeric $P2X_3$ receptors were abolished by a condition application of α,β-meATP (10 μM, 20s) given 60 s before the test response, leaving only the sustained response mediated by $P2X_{2/3}$ receptors.

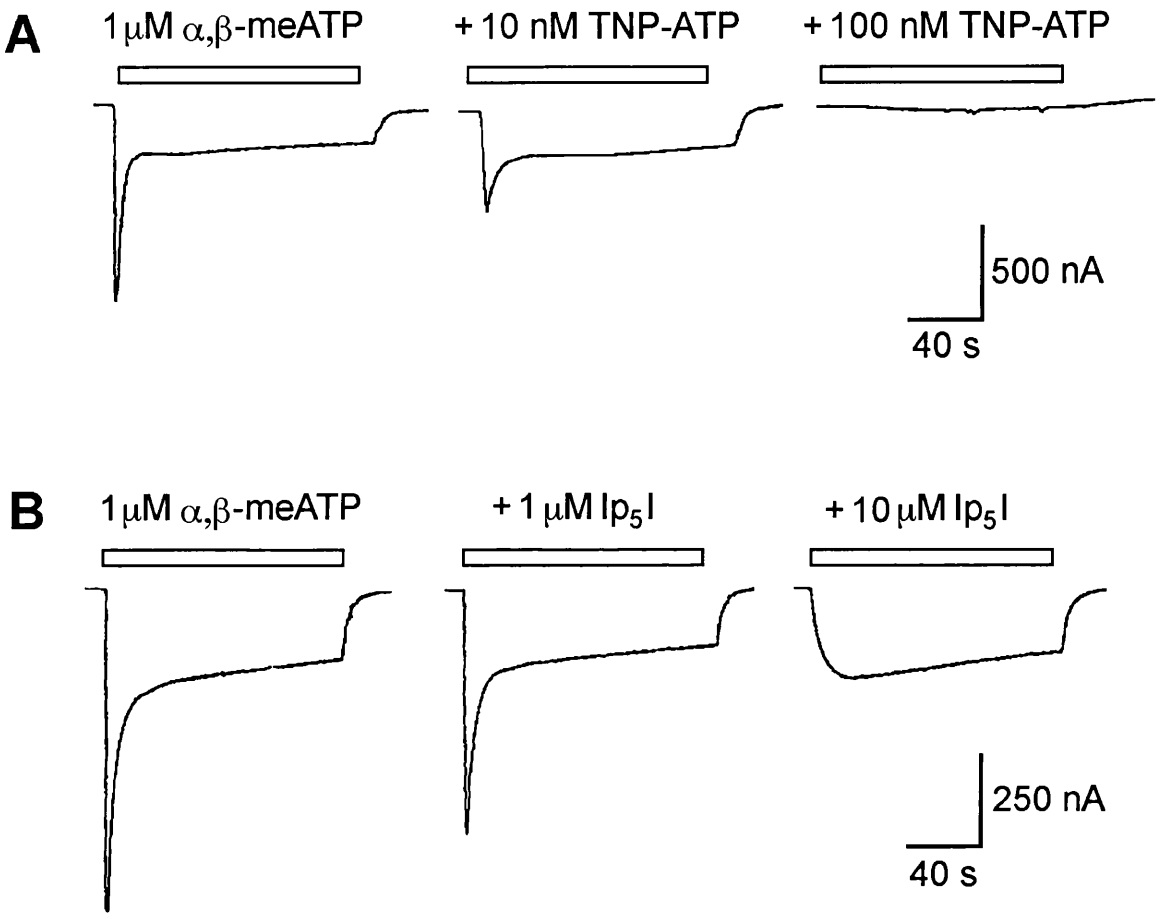
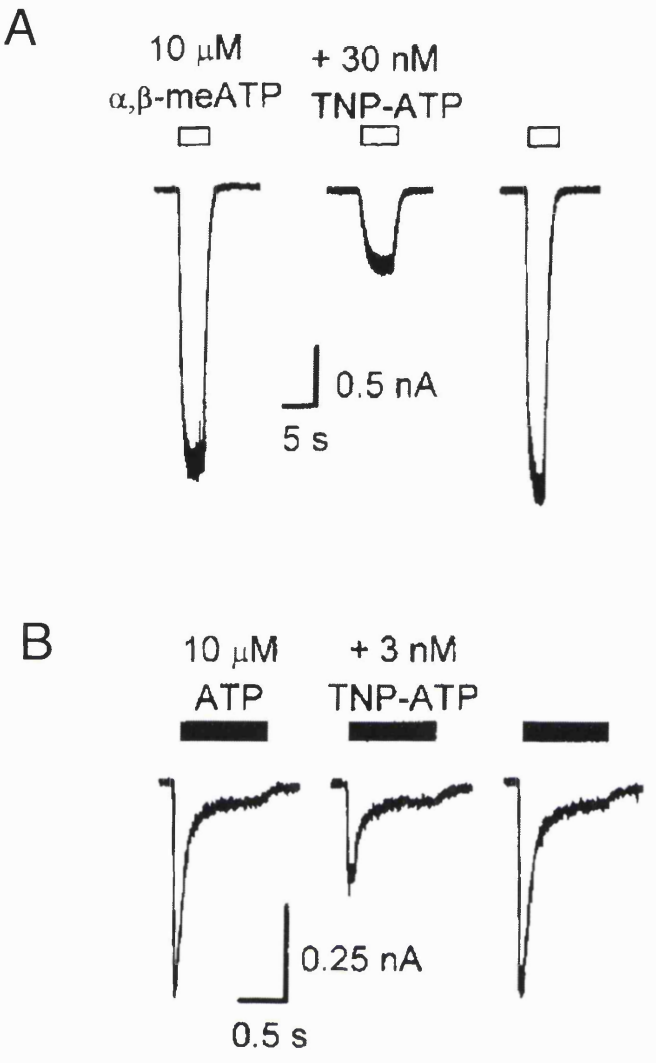


Figure 6.2 The effect of Ip_5I and TNP-ATP on composite responses at recombinant P2X receptors. (A) Response of an oocyte, co-injected with $rP2X_2$ and $rP2X_3$ transcripts and expressing both $P2X_3$ and $P2X_{2/3}$ receptors, to 1 μM α,β-meATP, alone and in the presence of 10 and 100 nM TNP-ATP. (B) Response of another oocyte to 1 μM α,β-meATP alone, and in the presence of 1 and 10 μM Ip_5I. V_H = -50 mV. Antagonists were present for 60 s before and during the agonist application.


A
10 μM
α,β-meATP
+ 30 nM
TNP-ATP
0.5 nA
5 s
B
10 μM
ATP
+ 3 nM
TNP-ATP
0.25 nA
0.5 s
C
% control response
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
log [TNP-ATP], M
DRG
nodose

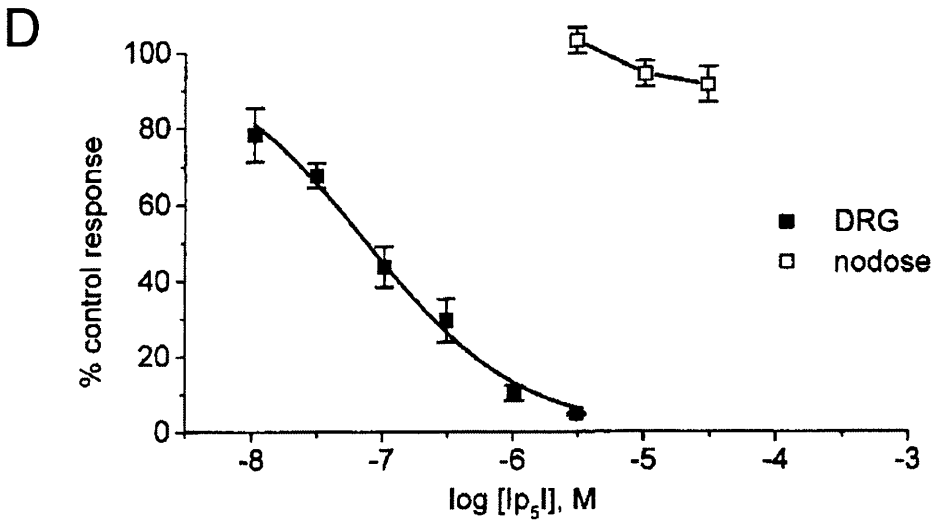


Figure 6.3 Inhibition of P2X receptors in sensory neurons by TNP-ATP. (A) Responses of a nodose ganglion neuron to 10 μM α,β-meATP before, during and following washout of 30 nM TNP-ATP. Responses were recorded at 3.5 min intervals and antagonist was present for 3 min before, and during the second agonist application. (C) Concentration-dependent inhibition of I_{ATP} (ATP, 10 μM) of DRG neurons and $I_{\alpha,\beta\text{-meATP}}$ (α,β-meATP, 10 μM) of nodose neurons by TNP-ATP. Data: mean±s.e.mean, n = 3-12. IC_{50} values are 0.77±0.12 μM and 20.6±2.8 nM and Hill coefficients 0.6 and 1.3 for DRG and nodose ganglion neurons, respectively. (D) Concentration-effect curves for the inhibition by Ip_5I of the response of DRG and nodose ganglion neurons to 10 μM ATP and 10 μM α,β-meATP, respectively. Data: mean±s.e.mean, n = 3-6. IC_{50} value is 0.07±0.007 μM and Hill coefficient 0.73 for DRG neurons. Responses of nodose ganglion neurons in the presence of Ip_5I at concentrations up to 30 μM were not significantly different from control responses ($p > 0.05$). V_H: -60 mV in A-D.

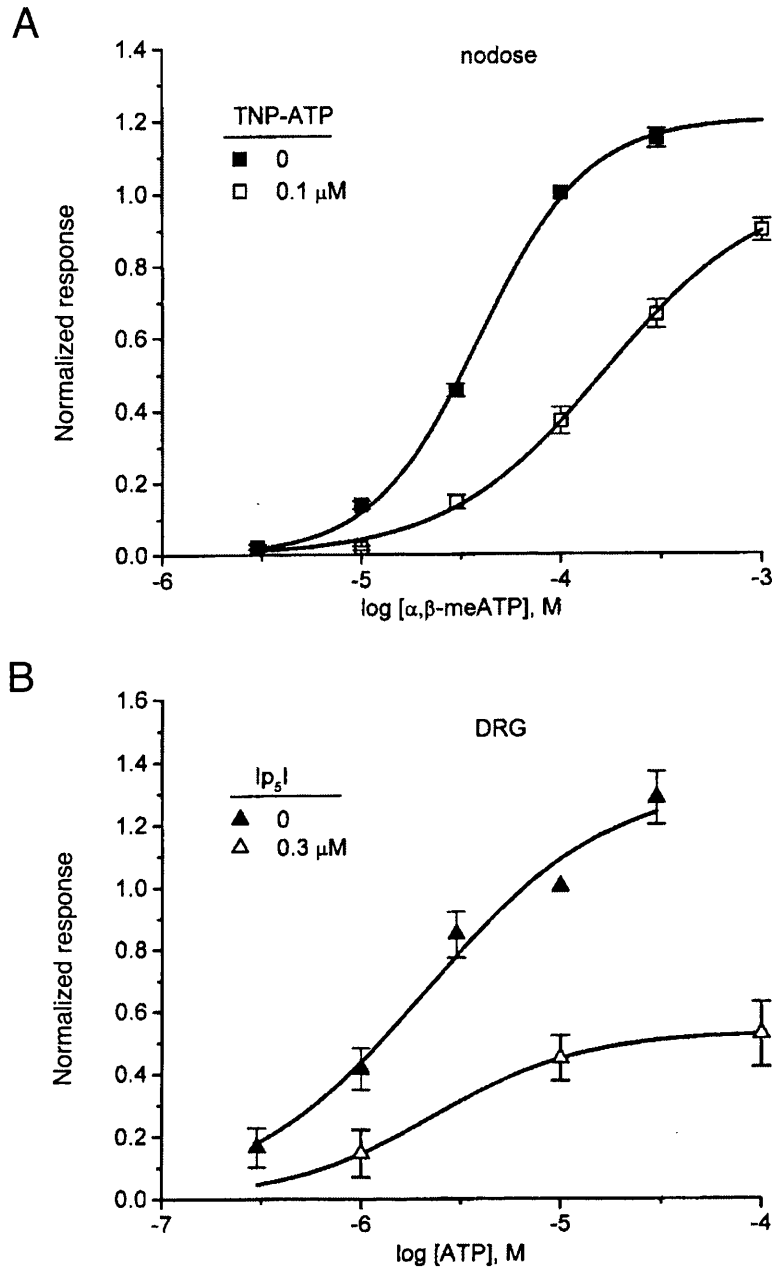


Figure 6.4 The nature of antagonism produced by TNP-ATP and Ip_5I. (a) Concentration-response curves for α,β-meATP evoked currents recorded from nodose ganglion neurons in the absence and presence of 0.1 μM TNP-ATP. Responses were normalized to that produced by 100 μM α,β-meATP in the absence of antagonist in the same cell. Data:mean±s.e.mean, n=3-6. EC_{50} values are 39.2±2.5 μM and 167±22 μM and Hill coefficients of 1.6 and 1.1 in the absence and presence of antagonist, respectively. (B) Concentration-response curves for the rapidly-desensitizing inward currents produced by ATP in DRG neurons in the absence and presence of 0.3 μM Ip_5I. Data: mean±s.e.mean, n=4-6. Responses were normalized to that produced by 10 μM ATP in the same cell, in the absence of antagonist. V_H: -60 mV.

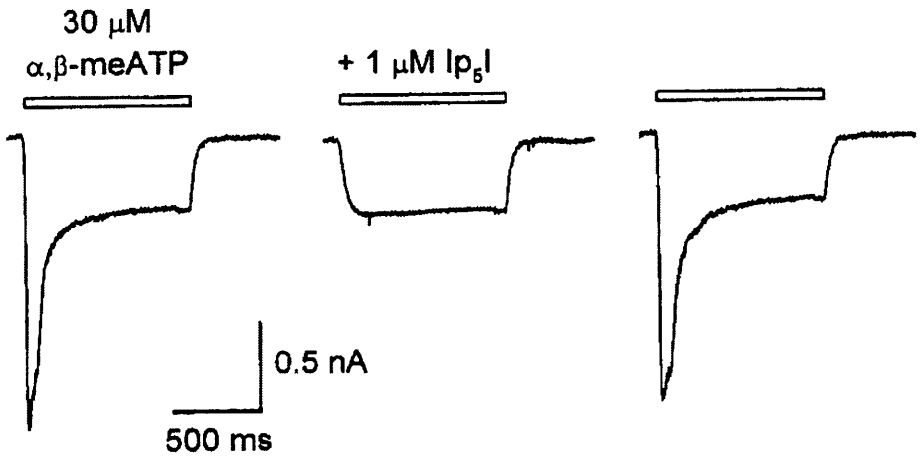


Figure 6.5 Effect of Ip_5I on mixed responses in dorsal root ganglion neurons. The traces show membrane currents evoked by 30 μM α,β-meATP in a DRG neuron voltage-clamped at -60 mV, alone and in the presence of 1 μM Ip_5I. While the transient component of the response was abolished, the sustained component was unaffected by the antagonist. Similar results were observed in a total of four neurons.

Table 6.1

Comparison of the antagonist activity of diinosine pentaphosphate (Ip_5I) and trinitrophenyl-ATP (TNP-ATP) at recombinant and native P2X receptors.

	IC_{50} (nM)	
Receptor	Ip_5I	TNP-ATP
$P2X_1$	3.1±0.4[a]	6±3[b]
$P2X_2$	ND	2000±200[b]
$P2X_3$	2800±700	0.9±0.2[b]
$P2X_{2/3}$	ND	7±3[b]
Nodose neuron	ND	21±3
DRG neuron (fast)	70±7	0.8+0.1

The IC_{50} values determined at recombinant P2X receptors expressed in *Xenopus* oocytes or HEK293 cells are presented along with those determined at native receptors in sensory neurons. ND, no inhibition detected at concentrations up to 30 μM. [a] data from King *et al.*, (1999); [b] data from Virginio *et al.*, (1998).

I would like to acknowledge the help of Dr. P.M. Dunn, who provided Figures 6.3-6.5 and the DRG data in Table 6.1, as part of a collaborative study of Ip_5I antagonism at recombinant $P2X_{2/3}$ receptors and native $P2X_{2/3}$-like receptors.

6.5 DISCUSSION

Ip_5I is an antagonist at recombinant $P2X_1$ and $P2X_3$ receptors, but is ineffective at $P2X_2$ receptors (King *et al.*, 1999). I have now extended these studies, and found that this compound is also ineffective as an antagonist at the heteromeric $P2X_{2/3}$ receptor. It thus shows at least 100 fold selectivity for $P2X_3$ over $P2X_{2/3}$ receptors. In contrast, although TNP-ATP is much more potent than Ip_5I as an antagonist at $P2X_3$ receptors, it is also very active at heteromeric $P2X_{2/3}$ receptors, with at best a 10 fold selectivity between these receptors sub-types (Virginio *et al.*, 1998).

It has been suggested that the rapidly-desensitizing ATP responses in sensory neurons are due to the activation of homomeric $P2X_3$ receptors (Robertson *et al.*, 1996; Cook *et al.*, 1997). In contrast, the slowly-desensitizing α,β-meATP sensitive receptor found in nodose ganglion cells results from the activation of heteromeric $P2X_{2/3}$ receptors (Lewis *et al.*, 1995). In keeping with this view, I have not only confirmed that the rapidly-desensitizing receptor is blocked by nanomolar concentrations of TNP-ATP, but have also demonstrated its sensitivity to Ip_5I. In contrast, the slowly-desensitizing response to α,β-meATP in nodose ganglion neurons is not affected by Ip_5I at concentrations up to 30 μM (the highest concentration tested). This is in keeping with my observations that the recombinant $P2X_{2/3}$ receptor is resistant to Ip_5I.

In dissociated neurons, responses mediated by homomeric $P2X_3$ receptors can readily be distinguished from those of heteromeric $P2X_{2/3}$ receptors by their time course. However, in multicellular preparations and *in vivo*, this distinction may be less clear (see Kirkup *et al.*, 1999; Rong *et al.*, 2000). In such situations, an antagonist like Ip_5I should be very useful in identifying the type of P2X receptor involved.

The nature of the antagonism produced by TNP-ATP and Ip_5I appear to be complex. Thus, at the recombinant $P2X_3$ receptor, TNP-ATP produces a non-surmountable antagonism (Virginio *et al.*, 1998). Such behaviour may in part results

from the very rapid desensitization of this receptor, which will prevent equilibrium being established between the receptor, agonist and antagonist. The antagonism at the $P2X_{2/3}$ receptor in nodose ganglion neurons produced by 0.1 μM TNP-ATP appeared to be surmountable, although there was a reduction in the slope of the log-concentration-response curve indicating a non-competitive interaction. At recombinant $P2X_1$ and $P2X_3$ receptors low concentrations of Ip_5I produce parallel shifts in the agonist log-concentration response curves, but the antagonism was found to be non-competitive (King *et al.*, 1999). These experiments investigating the antagonism of the native P2X receptor in DRG neurons by Ip_5I showed a non-surmountable, non-competitive antagonism. However, the very rapid desensitization of this response prevents true equilibrium being established and may again complicate interpretation of the results. The rapid desensitization of $P2X_3$ and $P2X_1$ receptors is determined by the intracellular domains (Werner *et al.*, 1996; King *et al.*, 1997a; Smith *et al.*, 1999). It would therefore be interesting to see how TNP-ATP and Ip_5I interact with chimeric receptors possessing the extracellular loop of the $P2X_3$ receptor and the cytoplasmic and trans-membrane segments of the $P2X_2$ receptor. Such receptors should retain the pharmacology of the $P2X_3$ receptor, but with the slow desensitization of the $P2X_2$ receptor.

My results on the action of TNP-ATP and Ip_5I at recombinant P2X receptors expressed in *Xenopus* oocytes, are in broad agreement with the results on the native receptors found in sensory neurons (Table 6.1). However, the results are not identical. In particular, Ip_5I produces a non-surmountable antagonism of the rapidly-desensitizing receptor in DRG neurons and it is considerably more potent than at the recombinant $P2X_3$ receptor. The reason for this is at present unclear, but receptors expressed in *Xenopus* oocytes do show some differences from those expressed in mammalian cells (Evans *et al.*, 1995; Lewis *et al.*, 1997). An alternative, though perhaps less likely

explanation is that the rapidly-desensitizing receptor present on DRG neurons is not in fact a homomeric $P2X_3$ receptor, but incorporates some additional subunit(s). However in general, my results thus support the notion that the slowly-desensitizing α,β-meATP response in nodose ganglion neurons is mediated by heteromeric $P2X_{2/3}$ receptors, while the rapidly-desensitizing response seen in DRG neurons is mediated by homomeric $P2X_3$ receptors.

In conclusion, I have demonstrated that Ip_5I is an effective antagonist of the homomeric $P2X_3$ with an IC_{50} of 3 μM, but is inactive at the heteromeric $P2X_{2/3}$ receptor. The ability of Ip_5I to discriminate between the rapidly-desensitizing responses in DRG neurons and the slowly-desensitizing α,β-meATP response in nodose ganglion neurons supports the notion that these responses are mediated by $P2X_3$ and $P2X_{2/3}$ receptors, respectively. Ip_5I should be useful tool for further elucidating the subunit composition of native P2X receptors.

SECTION B

CHAPTER 7

RAT CHROMAFFIN CELLS LACK P2X RECEPTORS WHILE THOSE OF THE GUINEA-PIG EXPRESS A P2X RECEPTOR WITH NOVEL PHARMACOLOGY

7.1 ABSTRACT

Whole-cell patch-clamp recording was used to determine the functional expression and pharmacological properties of P2X receptors in chromaffin cells dissociated from adrenal medullae of rats and guinea-pigs. In rat chromaffin cells maintained in culture for 1 - 7 days, ATP and UTP failed to evoke any detectable response. Guinea-pig chromaffin cells responded to ATP (100 μM) with a rapidly activating inward current. The amplitude of the response to ATP increased over the period cells were maintained in culture and so did the number of cells giving a detectable response, with 69% of cells responding after ≥ 4 days of culture. The response to ATP desensitized slowly, and had a reversal potential of 2.5 mV. The EC_{50} for ATP was 43 μM. The potency order for ATP analogues was 2-MeSATP>ATP>ADP. Adenosine, UTP and αβ-meATP were inactive. Suramin (100 μM) and Cibacron blue (50 μM) inhibited the ATP (100 μM)-activated current by 51 % and 47 %, respectively. PPADS antagonized the response to ATP (100 μM) with an IC_{50} of 3.2 μM. The ATP concentration-response curve shifted to the left at pH 6.8 (EC_{50}: 19 μM) and right at pH 8.0 (EC_{50}: 96 μM), without changing the maximal response. Zn^{2+} inhibited the response to ATP (100 μM) with an IC_{50} of 48 μM. This study indicates that expression of ATP-gated cation channels in chromaffin cells is species dependent. The P2X receptors in guinea-pig chromaffin cells show many properties characteristic of the $P2X_2$ receptor subtype.

7.2 INTRODUCTION

Adenosine 5'-triphosphate (ATP) is released from nerve terminals and acts as a neurotransmitter in the nervous system (see reviews: Burnstock, 1996b). In addition, ATP has been suggested as an intercellular signalling molecule in a number of systems such as regulation of smooth muscle contraction and neuroendocrine secretion via ionotropic (P2X) and metabotropic (P2Y) receptors (see reviews: Abbracchio & Burnstock, 1994; Ralevic & Burnstock, 1998).

Chromaffin cells of the adrenal medulla are exposed to ATP from two distinct sources: splanchnic nerve terminals (Parker *et al.*, 1990), where it is co-released with acetylcholine (Burnstock, 1981; Vizi *et al.*, 1997) and from chromaffin cells themselves. ATP is found to be co-stored with catecholamines in the granules of adrenal chromaffin cells at an ATP: catecholamines ratio of about 1: 4 and co-released with catecholamines (Todorov *et al.*, 1996). The occurrence of P2X receptors in rat adrenal gland has been suggested on the basis of Northern blot analysis and immunohistochemical studies (Bo *et al.*, 1995; Vulchanova *et al.*, 1996), indicating a possible role for ATP in the regulation of catecholamine secretion.

Extracellular ATP stimulates catecholamine secretion from perfused guinea-pig and bovine adrenal glands, cultured chromaffin cells (Asano *et al.*, 1995; Lin *et al.*, 1995) and from rat pheochromocytoma (PC12) cells (Inoue *et al.*, 1989; Sela *et al.*, 1991). This effect may be accomplished by increasing the influx of extracellular Ca^{2+} (Castro *et al.*, 1995; Reichsman *et al.*, 1995) via ATP-gated ion channels (Inoue *et al.*, 1989; Nakazawa & Inoue 1992). An ATP-activated inward current was observed in a subset of chromaffin cells from guinea-pig (Otsuguro *et al.*, 1995). In contrast, ATP failed to produce any inward current in rat adrenal chromaffin cells (Hollins & Ikeda, 1997). ATP-activated membrane currents were reported in a subpopulation of bovine

chromaffin cells (Diverse-Pierluissi *et al.*, 1991), but were not observed in the studies by Currie & Fox (1996).

The relative proportion of adrenaline- and noradrenaline-containing cells varies in different species of animals with e.g. about 91% adrenaline-containing cells in the rat, 71% in cattle and 98% in the guinea-pig (Holzbauer & Sharman, 1972). Thus, dissociated chromaffin cells from both rats and guinea-pigs should provide a relatively homogeneous population of adrenaline-secreting cells. In the present study, I investigated the expression of ATP-activated currents in cultured chromaffin cells dissociated from rats and guinea-pigs and characterized the pharmacological properties of the P2X receptors present on guinea-pig cells.

7.3 METHODS

Chromaffin cell preparation

See Chapter 2.

Whole-cell recordings from chromaffin cell

See Chapter 2.

Data analysis

The pooled data for concentration-effect curves were fitted with the Hill equation as defined by Prism v2.0, (GraphPad). The values presented are the fitted value ± s.e. mean. All responses to agonists were normalized to that evoked by ATP (100 μM) in the same cell (pH 7.4). All other data are expressed as the mean ± s.e. mean. In this study, because agonist concentrations exceeding 300 μM were not used, the concentration-response curves did not always reach a maximum and, where necessary,

the maximum has been estimated by the curve fitting routine. Statistical significance of results was assessed using Student's *t*-test, with a probability level of $P < 0.05$ taken to be statistically significant; *n* refers to the number of cells tested.

7.4 RESULTS

Chromaffin cells were identified using a combination of morphological and functional criteria. Recordings were only made from phase bright round cells having non-granular cytoplasm. Chromaffin cell plasma membrane is endowed with cholinergic nicotinic receptors. All cells tested were subjected to a standard brief test pulse of 10 μM dimethylphenylpiperazinium iodide (DMPP, an agonist at nACh receptors), and only those which responded with a pronounced inward current were studied further.

Responses to ATP

In agreement with the observation of Hollins & Ikeda (1997), no detectable inward current was evoked by ATP (100 μM) in chromaffin cells dissociated from adrenal medullae of adult rats, despite a robust response to 10 μM DMPP (Fig. 7.1A). Although the responsiveness of guinea-pig chromaffin cells changed with time in culture (see below), rat cells cultured for 1 to 7 days failed to respond to ATP (100-300 μM). The absence or presence of nerve growth factor in the culture medium, or the use of different media (DMEM or Leibovitz's L-15) failed to induce any ATP sensitivity.

In contrast, ATP (100 μM) did elicit an inward current in guinea-pig chromaffin cells (Fig. 7.1B). The current activated rapidly, and on reaching its maximal amplitude, decreased slowly whilst in the continued presence of agonist. When compared with a current of similar amplitude produced by 10 μM DMPP, the half-decay time of the ATP-activated current (17.8 ± 3.2 s, n=4) was significantly longer ($P < 0.05$) than that of DMPP-evoked current (6.7 ± 1.4 s, n=3). Current-voltage relations were determined

from membrane potential ramps (-90 to +30 mV, 200 ms duration), before and during ATP (100 μM) application. An example of a subtracted current-voltage relationship is illustrated in Fig. 7.1C. The ATP-evoked response had a mean zero current potential of 2.5 ± 2.7 mV (*n*=5), and demonstrated profound inward rectification.

Effect of time in culture on the response to ATP

During initial experiments on the ATP-activated currents, I found considerable variation in responses from one experiment to another. Much of this variability was found to result from the time that the cells were maintained in culture as shown in Table 7.1. Data on the response to 10 μM DMPP recorded from the same cells are shown for comparison. The response to ATP (100 μM) was observed only in a minor fraction (22%) of the cells cultured for 1-3 days and had small amplitude 81.4 ± 18.7 pA (*n*=7). However, 51 out of 57 cells cultured for 6-7 days gave responses to 100 μM ATP with a mean amplitude of 255.2 ± 31.4 pA. With increasing time in culture, the amplitude of the peak current, which varied considerably from cell to cell, increased further. Detailed analysis of the frequency distributions of the ATP-activated current is showed in Fig. 7.2. In contrast to the increasing percentage of responding cells and amplitude of response to ATP, the currents evoked by 10 μM DMPP decreased during the time in culture (Table 7.1). The mean DMPP-evoked currents produced by cells in culture for 7 days (115.2 ± 10.1 pA, *n*=30) was 59% of that produced by cells cultured for 1-3 days (213.1 ± 34.3 pA; *n*=32).

The membrane capacitance remained relatively constant throughout the time the cells were kept in culture (Table 7.1). Similarly, the mean value of membrane capacitance (7.8 ± 0.2 pF, *n*= 154) obtained from the cells which produced responses to 100 μM ATP was not significantly different from that (7.6 ± 0.3 pF, n= 92, $p > 0.05$)

for cells which produced no detectable response to same concentration ATP. The surface area of the chromaffin cells estimated from the cell capacitance (assuming specific membrane capacitance of 1 $\mu F/cm^2$) was 7.7 ± 0.3 $\times 10^{-6}$ cm^2 (n=246).

Agonist profile

Because the ATP response increased during the time in culture, all subsequent results were obtained from guinea-pig chromaffin cells cultured for 4-7 days. ATP induced a fast inward current in 69% (147/214) of these cells, with a mean amplitude of 196.1 ± 22.9 pA (*n*=147) at a holding potential of -70 mV. Neither adenosine nor AMP produced any detectable response at concentration up to 1 mM (*n*=4). ADP did not evoke any current at 100 μM (n=4), but at 1 mM induced a response 34.0 ± 2.6% (*n*=7) of that activated by 100 μM ATP. Membrane currents recorded from a guinea-pig chromaffin cell in response to increasing concentrations of ATP are shown in Fig. 7.3A. Concentration-response relationships obtained for ATP and three analogues are shown in Fig. 7.3B. Maximal currents were observed at a concentration of 300 μM for ATP and 2-MeSATP with EC_{50} values of 43 ± 4 μM (*n*=12) and 34 ± 4 μM (*n*=6) and Hill coefficients of 1.5 ± 0.1 and 1.5 ± 0.4, respectively. α,β-meATP (100-300 μM, *n*=7) and UTP (1 mM, *n*=4) failed to evoke any detectable response.

Effects of P2X receptor antagonists

Figure 7.4A shows the effects of suramin (100 μM), Cibacron blue (50 μM), and PPADS (10 μM) on the currents activated by a submaximal concentration ATP (100 μM). On average, suramin (100 μM) reduced the response to 100 μM ATP by 51.7 ± 6.0% (*n*=6). The inhibition by suramin reversed rapidly on washout, with the ATP-activated currents being 83.4 ± 13.3% (*n*=6) of the control amplitude 4 min after returning to suramin free solution. Cibacron blue (10 μM) produced a small though not

statistical significant increase of 9.5 ± 7.2% (n=5) on ATP-activated currents, but at 50 μM there was significant inhibition of 47.2 ± 5.8% (n=7, $p < 0.01$). PPADS (10 μM) attenuated the response to 100 μM ATP by 90.0 ± 4.1% (n=8). The recovery from this inhibition was slow and incomplete. In guinea-pig chromaffin cells, I found that PPADS (0.3-30 μM) reduced the amplitude of the ATP-activated current in a concentration-dependent manner (Fig. 7.4B). The concentration of PPADS required to produce half-maximal inhibition of the inward current was 3.2 ± 0.5 μM (n=8).

Effect of extracellular pH

No detectable current was observed when extracellular solution at pH 6.8 or pH 8.0 were applied to guinea-pig chromaffin cells (n=5). The records in Figure 7.5A show currents activated when 100 μM ATP was applied to the same cell in solutions at pH 6.8, 7.4, and 8.0. Lowering pH to 6.8 produced a significant increase of the amplitude of the current activated by 30 μM to 207.4 ± 16.5% (n=8, $p < 0.01$) while that to 100 μM was not significantly changed being 113.1 ± 15 % (n=8, $p > 0.05$) of the response evoked at pH 7.4. Elevating pH to 8.0 decreased the amplitude of the current activated by 30 and 100 μM ATP to 37.6 ± 3.4% (n=8) and 66.1 ± 5.3% (n=8) of that evoked at pH 7.4, respectively. Figure 7.5B shows concentration-response curves for the ATP-activated current at pH 6.8, and 8.0, compared with that obtained at pH 7.4. The EC_{50} values determined for these ATP concentration-response curves were 19 ± 3 μM (n=8) at pH 6.8, and 96 ± 15 μM (n=8) at pH 8.0. In contrast, neither the slopes nor maximal responses were significantly affected by the alteration of pH (P>0.05). The Hill coefficients of the ATP concentration-response curves were 1.4 ± 0.2, 1.5 ± 0.1 and 1.5 ± 0.3 at pH 6.8, 7.4 and 8.0, respectively. The relative maximal responses (normalized to responses to 100 μM ATP at pH 7.4) were 134 ± 13 %, 126 ± 7 % and 113 ± 18% at pH 6.8, 7.4 and 8.0, respectively.

Inhibitory effect of Zn^{2+}

Micromolar concentrations of Zn^{2+} potentiate responses to ATP in rat autonomic neurons (Cloues 1995; Zhong *et al.*, 1998). However I observed no potentiation, but a concentration dependent inhibition (Fig. 7.6). At a concentration of 100 μM, co-application of Zn^{2+} with 100 μM ATP inhibited the response by 63.2 ± 9.1% (n=6). This inhibition by Zn^{2+} was reversed following a 2 min washout. The concentration dependence of this effect is shown in Fig. 7.6C. Fitting the Hill equation to the data yielded an IC_{50} of 48 ± 7 μM (n=6).

Table 7.1

Effects of time in culture on responsiveness of cells to ATP.

Time in culture (day)	**Capacitance** (pF)	**DMPP** (pA)	**ATP** (pA)	**% Responding to ATP**
1-3	7.5 ± 0.4 (32)	213.1 ± 34.3 (32)	81.4 ± 18.7 (7)	22
4	7.5 ± 0.3 (82)	204.5 ± 22.5 (82)	175.3 ± 18.6 (43 **)	52
5	8.0 ± 0.3 (75)	146.6 ± 11.2 (75 *)	181.9 ± 21.3 (53 **)	71
6-7	7.9 ± 0.6 (57)	128.6 ± 15.8 (57 **)	255.2 ± 31.4 (51 **)	89

All data are given as means ± s.e. mean. Number of cells is given in parentheses. DMPP (10 μM) and ATP (100 μM) were applied in a sequence separated by a 2 min interval to the same cells. For estimation of percentage of cells responding to ATP, a minimum detection threshold of 20 pA was used. Statistical significance (* $p < 0.05$, ** $p < 0.01$) was determined by Student's *t*-test by comparing with the values at 1-3 days.

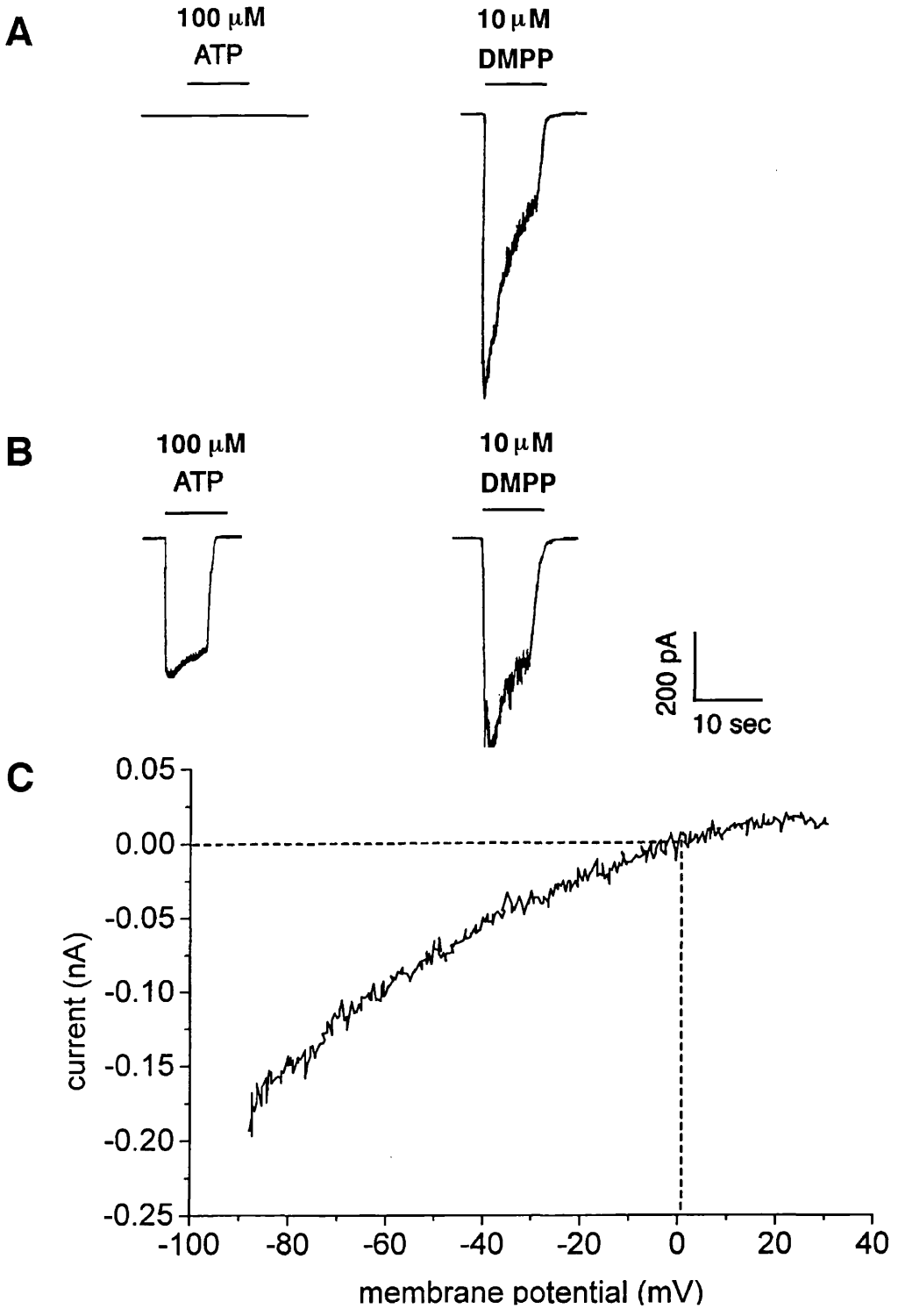


Figure 7.1 A comparison of inward currents evoked by extracellular application of ATP (100 μM) and DMPP (10 μM). Chromaffin cells dissociated from adrenal medulla of rat (A) and guinea-pig (B) were voltage clamped at a holding potential at –70 mV. Agonists were applied for the duration of the bar and with a 2 min interval between successive responses. (C) Example of the current-voltage relationship for the ATP-activated current in a guinea-pig chromaffin cell. The mean zero current potential was 2.5 ± 2.7 mV (n=5). Current-voltage relationships were obtained by subtracting the current evoked by a membrane potential ramp (-90 to +30, 200 ms) in the absence of agonist from that recorded in the presence of 100 μM ATP.

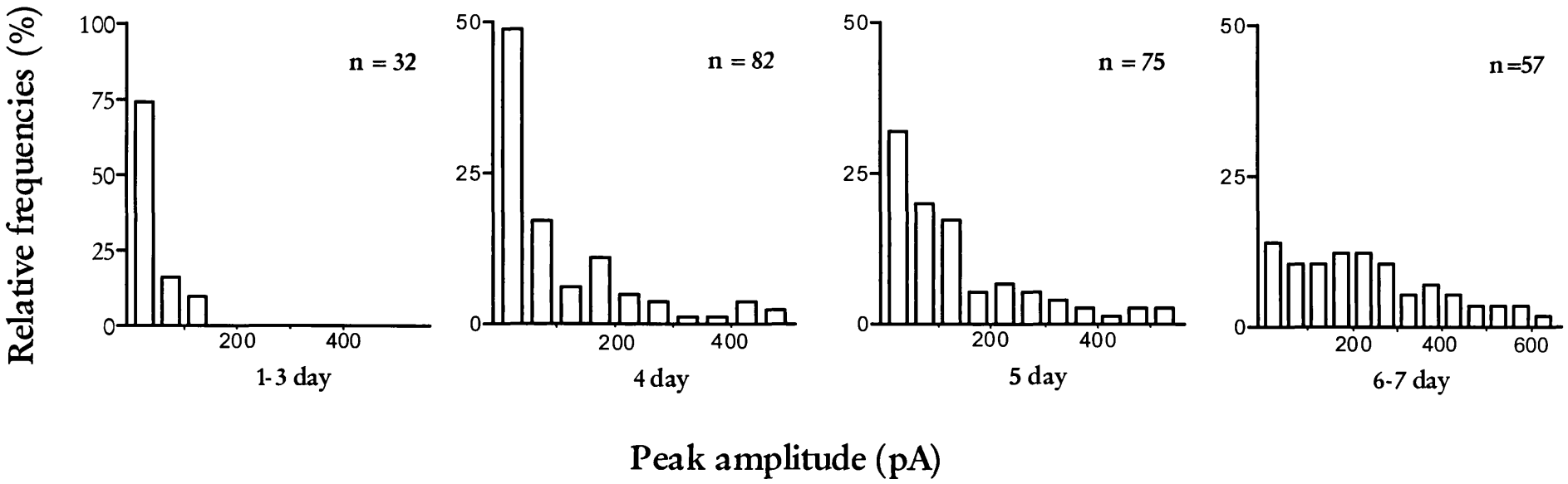


Figure 7.2 Relative frequency distribution for the amplitude of ATP (100 μM) -activated current in guinea-pig chromaffin cells cultured for varying periods. Relative frequencies were calculated as percent of total responsive cells (*n*) within each distribution.

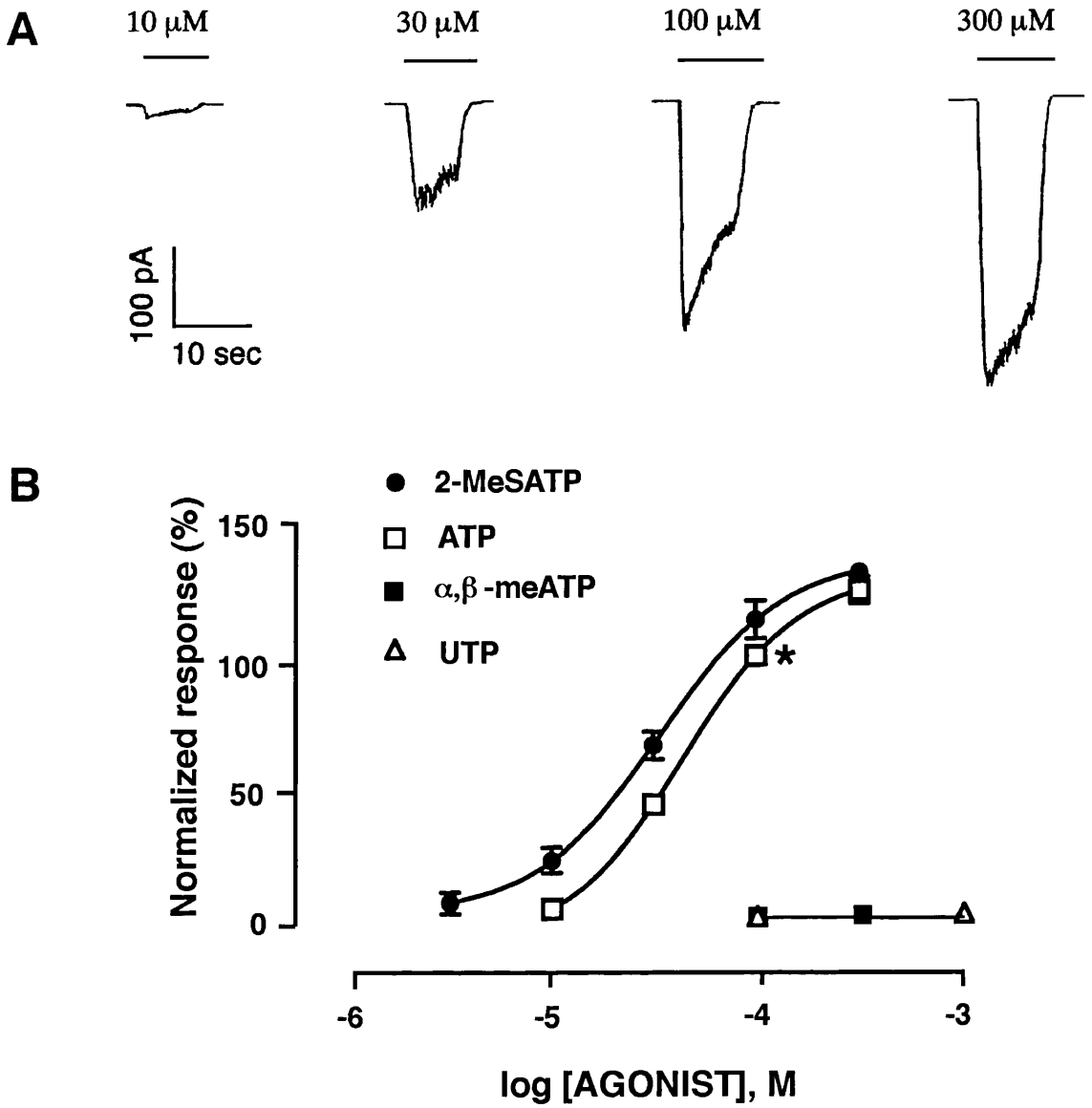


Figure 7.3 (A) Currents evoked by sequential application of ATP (10, 30, 100 and 300 μM) at a holding potential of –70 mV in guinea-pig chromaffin cells. Agonists were applied at 2 min intervals. (B) Concentration-response curves for 2-MeSATP (filled circles), ATP (open squares), α,β-meATP (filled squares) and UTP (open triangles). Peak amplitude of agonist-activated current was normalized to the response induced by 100 μM ATP (*) in the same cell. Each point represents the mean ± s.e. mean from 6-12 cells.

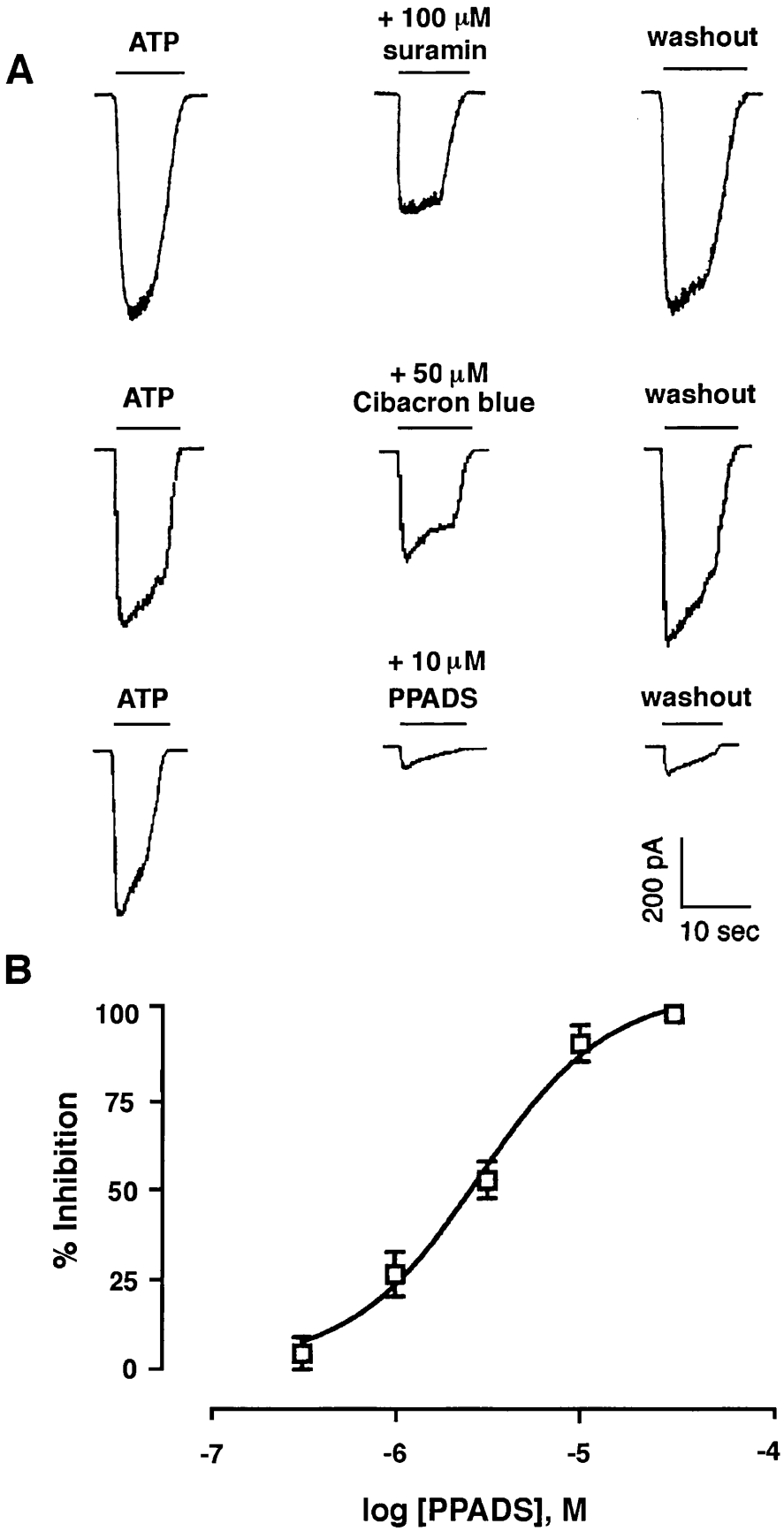

A
ATP
+ 100 μM
suramin
washout
ATP
+ 50 μM
Cibacron blue
washout
ATP
+ 10 μM
PPADS
washout
200 pA
10 sec
B
100
75
50
25
0
% Inhibition
-7
-6
-5
-4
log [PPADS], M

Figure 7.4 Blockade of ATP responses by P2 receptor antagonists. (A) Each row shows three consecutive currents evoked from the same chromaffin cell by 100 μm ATP at 2 min intervals before, in the presence of and 2 min after washing out the antagonist. Antagonists were present for 2 min before and during the second application of ATP. The cells were voltage-clamped at -70 mV. (B) Concentration-effect relationship for inhibition of ATP-activated currents by PPADS. Fitting the Hill equation to the data gave an IC_{50} value for PPADS of 3.2 ± 0.5 μM. Each point represents the mean ± s.e.mean from 4-6 cells.

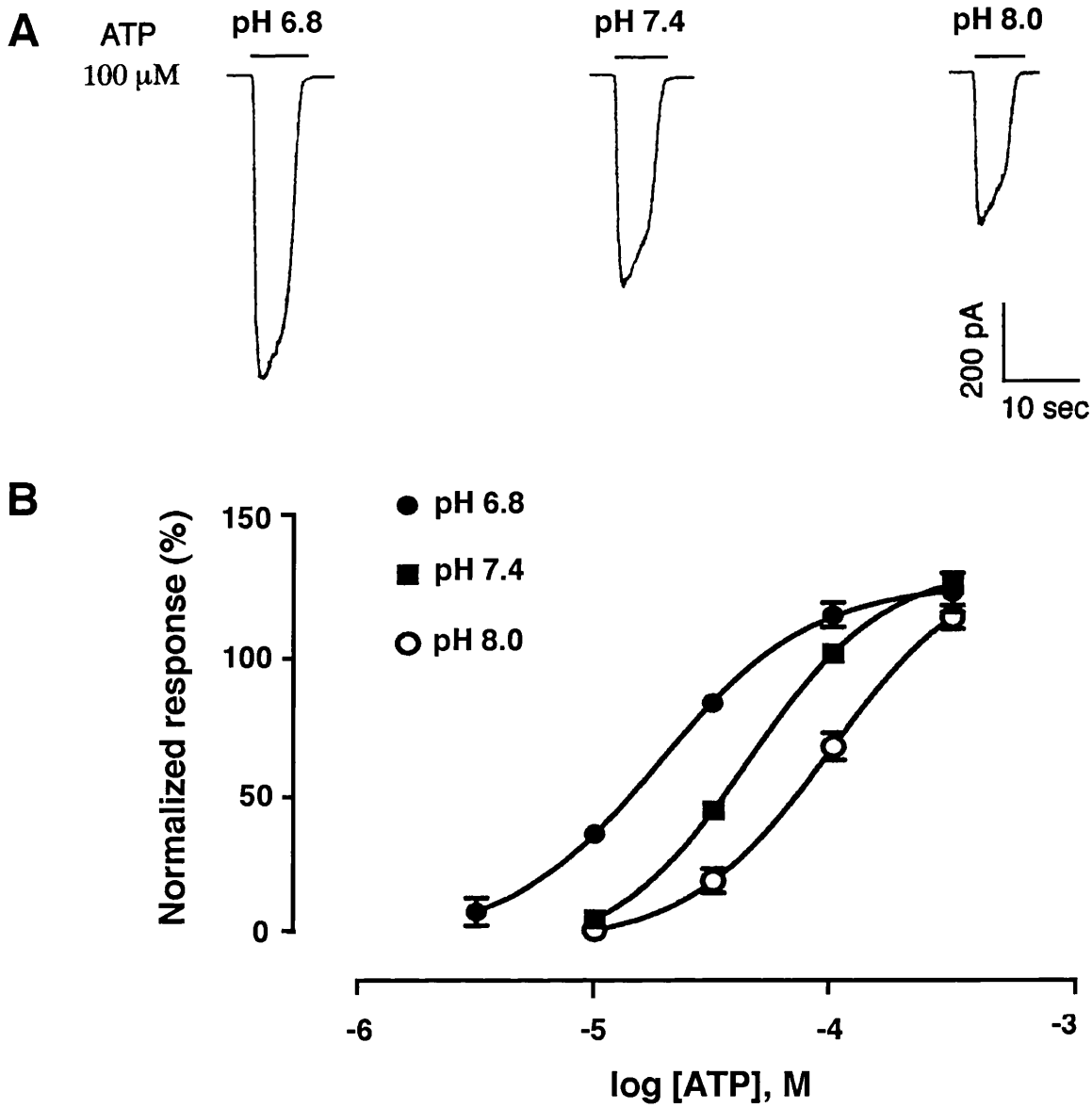


Figure 7.5 Effect of pH on ATP-activated currents. (A) Traces showing currents activated by successive applications of 100 μM ATP at pH 6.8, 7.4 and, 8.0. A 2 min interval separated each agonist application. (B) Concentration-response curves for ATP at pH 6.8 (filled circles) and pH 8.0 (open circles). All responses were normalized with respect to that produced by 100 μM ATP at pH 7.4 on the same cell. The concentration-response curve obtained at pH 7.4 is reproduced from Figure 7.3 for comparison. Each curve was generated from a separated sample of cells. Changing the pH altered the EC_{50} for ATP from 43 μM at pH 7.4 to 19 ± 3 μM (n=8), and 96 ± 15 μM (n=8) at pH 6.8 and 8.0, respectively.

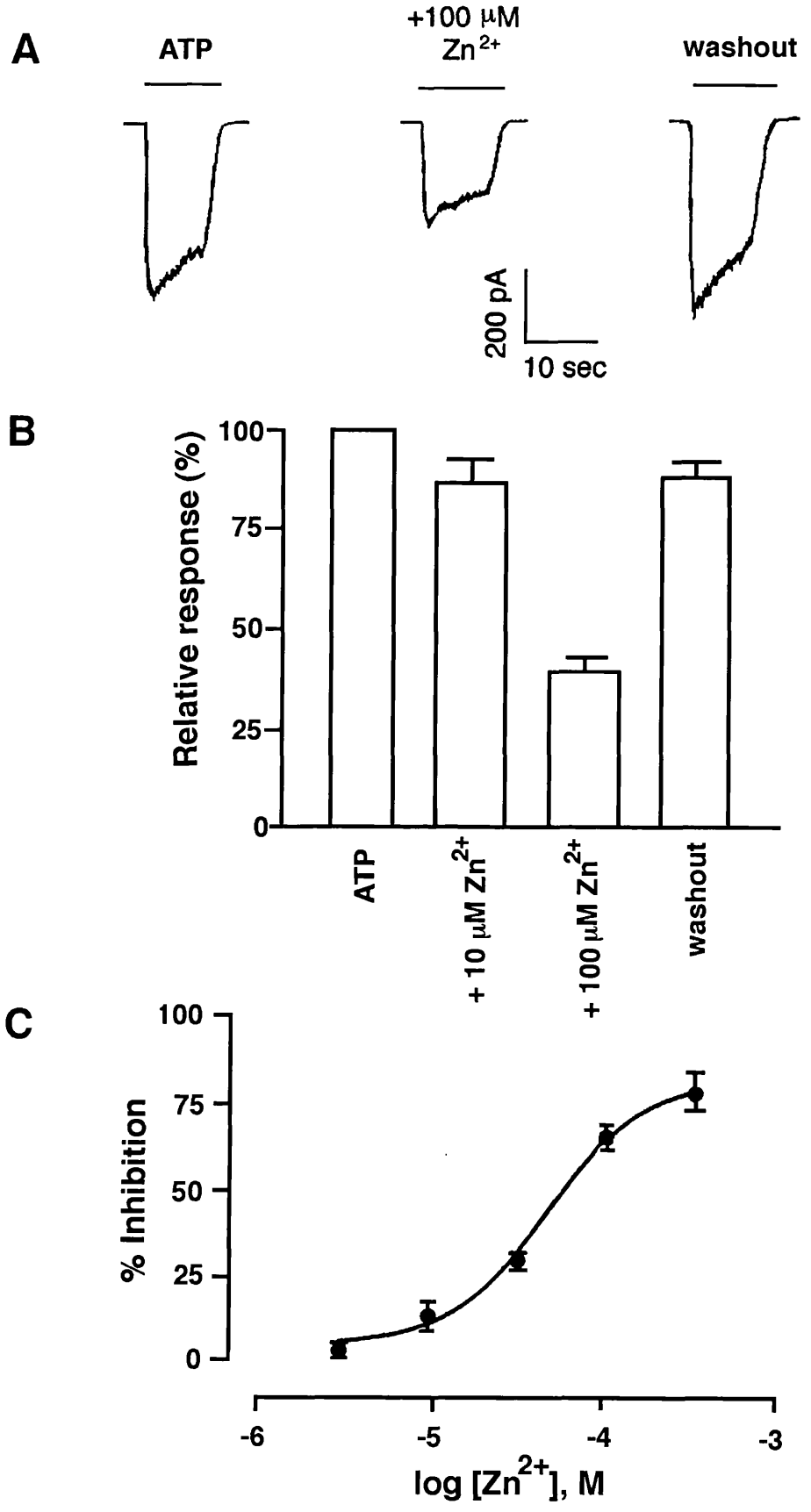

A
ATP
+100 μM
Zn2+
washout
200 pA
10 sec
B
Relative response (%)
100
75
50
25
0
ATP
+ 10 μM Zn2+
+ 100 μM Zn2+
washout
C
% Inhibition
100
75
50
25
0
-6
-5
-4
-3
log [Zn2+], M

Figure 7.6 Effect of Zn^{2+} on ATP evoked responses. (A) Traces showing the inhibition by 100 μM Zn^{2+} of currents activated by ATP (100 μM). Responses were recorded at 2 min intervals, and Zn^{2+} was applied simultaneously with ATP without any pre-equilibration. (B) Bar graph illustrating the inhibition of ATP-activated current by 10 μM and 100 μM Zn^{2+} and recovery after washout for 2 min (*n*=6). (C) Concentration-response relationship for inhibition of ATP (100 μM)-activated currents by Zn^{2+}. The curve is drawn according to the Hill equation with an IC_{50} value for Zn^{2+} of 48 ± 7 μM and a maximum inhibition of 79 ± 9 %. Each point represents the mean ± s.e.mean for 6 cell.

7.5 DISCUSSION

Species Dependence

P2X receptors are present on rat autonomic neurons (see, Evans & Surprenant, 1996) and rat phaeochromocytoma (PC12) cells (Inoue *et al.*, 1989). One might therefore expect to find them on adrenomedullary cells which are of the same embryological origin. I was consistently unable to detect any ATP-evoked currents in cultured chromaffin cells dissociated from adrenal medulla of adult *rats*. This observation is in agreement with the findings of Hollins & Ikeda (1997). In contrast, I was able to observe ATP-evoked responses in chromaffin cells dissociated from *guinea-pig* adrenal glands. On average, 69 % guinea-pig chromaffin cells exhibited an inward current on application of ATP when they had been maintained in culture for 4-7 days. In a study on bovine chromaffin cells only a small fraction were responsive to ATP, showing an inward cationic current or calcium influx, when cultured for 7-14 days before use (Diverse-Pierluissi *et al.*, 1991; Nuñez *et al.*, 1995). These differences between chromaffin cells dissociated from adrenal medullae of rats, guinea-pigs or cattle are unlikely to result from the culture conditions because the chromaffin cells from rats or guinea-pigs used in this study were dissociated and cultured under the identical conditions. Thus, the expression of P2X receptors in chromaffin cells appears to be dependent on species.

Both $P2X_1$ and $P2X_2$ immunoreactivity has been described in chromaffin cells of the rat adrenal medulla and in PC12 cells (Vulchanova *et al.*, 1996). Furthermore, RNA for $P2X_2$ and $P2X_4$ receptors has been found in the rat adrenal gland (Bo *et al.*, 1995; Collo *et al.*, 1996). However, in another study, neither $P2X_1$, $P2X_2$, nor $P2X_4$ receptors were detected on rat adrenal medullary cells using specific polyclonal antibodies (M. Afework, personal communication). Despite this

conflicting biochemical evidence, while PC12 cells express functional P2X receptors, rat adrenomedullary cells do not, according to the present study.

Is the response to ATP an in vitro phenomenon?

The growing percentage of responding cells and increasing amplitude of the ATP-activated current during time in culture raises an important question: is the response to ATP physiologically significant or is it an *in vitro* phenomenon caused by the conditions of cell culture? A time-related increase of catecholamine secretion induced by extracellular ATP was observed with cultured bovine chromaffin cells (Lin *et al.*, 1995). However, these authors were able to demonstrate ATP evoked catecholamine release from intact adrenal glands. Thus the increasing response to ATP with time in culture might indicate the replacement of receptors "lost" during enzyme treatment rather than hyperexpression *per se*.

P2X receptor mediated agonist-activated current

The inward current on guinea-pig chromaffin cells appeared to be due to activation of P2X receptors for the following reasons: rapid activation and deactivation; reversal potential (close to 0 mV) expected for a non-selective cationic current; ADP is far less potent than ATP; neither UTP nor adenosine induced any obvious current.

What P2X subtype?

To date, seven P2X subunits have been cloned (see, Ralevic & Burnstock, 1998). In addition, some exist as multiple spliced variants, and some can combine to form heteromultimeric receptors with unique properties (Brändle *et al.*, 1997; Lewis *et al.*, 1995; Parker *et al.*, 1998). The ATP-gated cation channel in guinea-pig chromaffin cells shares a number of pharmacological properties with autonomic neurons, myenteric neurons and PC12 cells from which the rat $P2X_2$ receptor was originally cloned (Brake *et al.*, 1994). For examples, α,β-meATP-insensitive, non-

desensitising inward currents are the characteristics of responses in PC12 cells (Nakazawa *et al.*, 1990), superior cervical neurons (Khakh *et al.*, 1995), rat cardiac parasympathetic ganglia (Fieber & Adams, 1991), myenteric neurons of small intestine (Zhou & Galligan, 1996) and rat pelvic ganglion neurons (Zhong *et al.*, 1998).

A distinct feature of the P2X receptor in guinea-pig chromaffin cells is the effect of Cibacron blue on ATP-activated currents. A low concentration (10 μM) had little effect, whereas at a high concentration (50 μM) inhibited the ATP response. Although the lack of inhibitory effect at the low concentration might be due to blockade of ecto-ATPase by Cibacron blue, this seems unlikely since the agonist was applied in a rapidly flowing solution. Furthermore, Cibacron blue has been shown to block ATP-activated currents in guinea-pig coeliac neurons (Silinsky & Gerzanich, 1993), rat parasympathetic cardiac neurons (Feiber & Adams, 1991), rat pelvic ganglion neurons (Zhong *et al.*, 1998), and inhibits ATP-inward current and dopamine secretion in PC12 cells (Inoue *et al.*, 1991). Interestingly, this antagonist appears to be less potent at guinea-pig receptors than it is on rat neurons and potentiated responses to ATP were observed in guinea-pig myenteric neurons (Barajas-López *et al.*, 1996). Cibacron blue potently inhibited ATP-responses at recombinant $rP2X_2$ receptors without any sign of potentiation at low concentrations (King *et al.*, 1997b).

Acid pH enhances the sensitivity of native ATP-gated ion channels in rat nodose ganglion neurons (Li *et al.*, 1996a) bullfrog dorsal root ganglion neurons (Li *et al.*, 1997a), rat and guinea-pig autonomic neurons (Zhong *et al.*, 1998; Zhong *et al.*, 1999). Of the recombinant $P2X_{1-4}$ receptors so far tested, only $P2X_2$ receptors are made more sensitive to ATP by lowering pH (King *et al.*, 1996c; Stoop *et al.*, 1997), although the effects of pH remain to be determined on $P2X_5$ and $P2X_6$

receptors. The modulation of responses by pH observed in this study would be consistent with the receptor being of the $P2X_2$ subtype.

A striking pharmacological property of the ATP-activated current in guinea-pig chromaffin cell is its inhibition by Zn^{2+}. This is in marked contrast to the action of Zn^{2+} on rat autonomic (Cloues, 1995; Zhong *et al.*, 1998) and sensory (Li *et al.*, 1993) neurons, and recombinant $P2X_2$ receptors (Wildman *et al.*, 1998), where it potentiates the effect of ATP. However, inhibition of P2X receptor-mediated response by Zn^{2+} has been observed in dorsal root ganglion neurons from bullfrog (Li *et al.*, 1997b) and guinea-pig sympathetic neurons (Zhong *et al.*, 1999). Of the recombinant P2X subunits tested, $P2X_1$ (S.S. Wildman, personal communication) and $P2X_7$ (Virginio *et al.*, 1997) are inhibited by Zn^{2+}.

While some properties of the P2X receptor present on guinea-pig chromaffin cells: slow desensitization, insensitivity to α,β-meATP, potentiation by low pH and inhibition by suramin are consistent with those of the cloned $P2X_2$ receptors, other properties including inhibition by Zn^{2+} and low sensitivity to Cibacron blue are not. To date, three spliced variants of the guinea-pig $P2X_2$ receptor have been cloned (Parker *et al.*, 1998), although their functional properties and pharmacology have yet to be described. Thus, the pharmacological profile I have observed might be explained by differences between rat and guinea-pig receptors, the involvement of a novel spliced variant, or the presence of a novel heteromultimeric receptor.

In conclusion, this study has revealed three new observations regarding P2X receptors on chromaffin cells. Firstly, I found that the expression of functional P2X receptors is species-dependent. Secondly, cultured chromaffin cells gradually increased their response to ATP with time in culture. Most significantly, the pharmacological profile of the receptor present on guinea-pig chromaffin cells does not match that of any cloned receptors so far described.

CHAPTER 8

GENERAL DISCUSSION

In this thesis, four new and important findings related to native P2X receptors in sensory neurons have been documented. One is that coexpression of $P2X_2$ and $P2X_3$ receptor subunits in varying amounts gives rise to a spectrum of agonist responses comparable to that seen in sensory neurons. It is, therefore, speculated that variability in operational profiles of sensory P2X receptors may reflect different levels of expression of homomeric $P2X_2$, $P2X_3$ and heteromeric $P2X_{2/3}$ receptors, which are the major species of P2X receptor subtypes. The second finding is that, when VR1 and $P2X_3$ receptors are expressed in *Xenopus* oocyte, the activation of VR1 receptors reduces the sensitivity of $P2X_3$ receptors. It is a well-documented phenomenon that following or during excitation of VR1 receptors, capsaicin-sensitive sensory neurons become resistant to a subsequent vanilloid challenge, noxious heat and mechanical pressure. Data in this thesis imply that the sensitivity of $P2X_3$ receptors may be reduced following the activation of VR1 receptors in sensory neurons where $P2X_3$ and VR1 are frequently coexpressed. The third finding is that diinosine pentaphosphate (Ip_5I) is an effective antagonist at recombinant $P2X_1$ and $P2X_3$ receptors, but has no inhibitory effect at $P2X_2$, $P2X_4$ or heteromeric $P2X_{2/3}$ receptors. On the basis of its selectivity for $P2X_3$ over $P2X_{2/3}$ receptors, two dominant P2X subtypes in DRG neurons, Ip_5I may be a useful pharmacological tool in elucidating the P2X subunit composition in sensory neurons, particularly in intact tissue and *in vivo*. Finally, guinea-pig adrenal chromaffin cells functionally express an ATP-gated ionic channel with many characteristics of the recombinant $P2X_2$ receptor. Thus, the guinea-pig chromaffin cell may serve as a model for the study of the possible physiological function of P2X receptors in endocrine tissues.

8.1 A STUDY OF RECOMBINANT P2X RECEPTORS TO AID THE ELUCIDATION OF SUBUNIT COMPOSITION OF NATIVE P2X RECEPTORS IN SENSORY NEURONS

There have been various reports on the actions of ATP in sensory neurons (Bean, 1990; Bean *et al.*, 1990; Lewis *et al.*, 1995; Robertson *et al.*, 1996; Cook *et al.*, 1997). It is of physiological importance to elucidate P2X subunit composition in sensory neurons, because P2X receptors are implicated in transduction and transmission of nociception (Bland-Ward & Humphrey, 1997; Hamilton *et al.*, 1999b; Cockayne *et al.*, 2000; Souslova *et al.*, 2000). The lack of subtype-selective P2 antagonists has hampered the study of native P2X receptors in sensory neurons. However, as an alternative, the heterologous expression of homomeric and heteromeric P2X receptors has provided a template for further study of native P2X receptors in sensory neurons.

Heterologously expressed $P2X_3$ receptors are characterized by rapidly-desensitizing ion channels, potently activated by ATP and α,β-meATP (Chen *et al.*, 1995). This operational profile resembles rapidly-inactivating responses observed in 40-95% of neurons in rat DRGs (Bean, 1990; Robertson *et al.*, 1996; Grubb & Evans, 1999) and 28% of rat trigeminal ganglion neurons (Cook *et al.*, 1997). The recombinant $P2X_2$ receptor gives rise to a slowly-inactivating response and is not activated by α,β-meATP (Brake *et al.*, 1994; Evans *et al.*, 1995). So far, the $P2X_2$ receptor has not been found to be the dominant P2X receptor subtype in mammalian sensory neurons (Lewis *et al.*, 1995; Robertson *et al.*, 1996; Cook *et al.*, 1997). However, coexpression of $P2X_2$ and $P2X_3$ subunits yields a novel P2X receptor-$P2X_{2/3}$ that is activated by ATP and α,β-meATP to produce slowly-inactivating currents (Lewis *et al.*, 1995). Similar slow responses to both ATP and α,β-meATP have been observed in rat nodose ganglion neurons and a small subset

(4%) of rat DRG neurons and 55% rat trigeminal ganglion neurons (Khakh *et al.*, 1995; Lewis *et al.*, 1995; Cook *et al.*, 1997; Grubb & Evans, 1999). However, not all operational profiles of native P2X receptors in sensory neurons can be satisfactorily matched to either homomeric or heteromeric P2X receptors.

8.2 HETEROGENEITY OF P2X RECEPTORS IN SENSORY NEURONS

8.2.1 DRG neurons

Responses of sensory neurons to P2X receptor agonists vary considerably in peak amplitude and desensitizing kinetics from cell to cell in an individual DRG. Under voltage-clamp conditions, responses to ATP and α,β-meATP in bullfrog DRG neurons and a subset (4%) of rat DRG neurons desensitize slowly (Bean, 1990; Burgard *et al.*, 1999; Grubb & Evans, 1999). In contrast, for many rat DRG neurons, responses to these agonists desensitize rapidly (Robertson *et al.*, 1996; Burgard *et al.*, 1999; Grubb & Evans, 1999). Nevertheless, there is a small proportion (10%) of DRG neurons that have composite responses with both rapidly- and slowly-inactivating components (Burgard *et al.*, 1999; Grubb & Evans, 1999). Taken together, at least two populations of P2X receptors are present on rat adult DRG neurons, in all probability $P2X_3$ and $P2X_{2/3}$ receptors. These P2X receptors are expressed either separately or together in individual neurons to give rise to three distinctive waveforms to ATP or α,β-meATP. Rapidly-activating and rapidly-desensitizing currents have properties similar to that of the recombinant $P2X_3$ receptor (Chen *et al.*, 1995). Evidence also suggests that rapidly-inactivating currents are predominantly elicited in small-diameter, IB4-positive and capsaicin-sensitive DRG neurons (Petruska *et al.*, 2000). Moreover, ablation of the $P2X_3$ gene ($P2X_3$ null mutation) resulted in the loss of rapidly-inactivating ATP-activated current in rat DRG neurons (Cockayne *et al.*,

2000; Souslova *et al.*, 2000). Slowly-activating and slowly-desensitizing currents resemble those recorded from recombinant heteromeric $P2X_{2/3}$ receptors (Lewis *et al.*, 1995). A mixed response to P2X agonists can be accounted for by the expression of both $P2X_3$ and $P2X_{2/3}$ receptors.

So far, no evidence has shown that the homomeric $P2X_2$ receptor is the dominant P2X receptor subtype in sensory nerurons. However, a close inspection has revealed that the greater efficacy of ATP than that of α,β-meATP in a subset of DRG neurons, which could be accounted by the expression of homomeric $P2X_2$ receptors (Ueno *et al.*, 1999; Petruska *et al.*, 2000). The mean response to α,β-meATP in capsaicin-sensitive DRG neurons was 93% in relation to the mean response to ATP. These capsaicin-sensitive neurons predominantly expressed homomeric $P2X_3$ receptors. In contrast, it was 73% in capsaicin-insensitive neurons, which mainly expressed $P2X_{2/3}$ receptors. These data suggest that more than one P2X receptors may contribute to ATP-activated currents in capsaicin-insensitive neurons. In addition, of neurons responding to ATP with a slowly-inactivating current, 8 out of 17 were insensitive to α,β-meATP (Petruska *et al.*, 2000). Furthermore, the loss of expression of homomeric $P2X_3$ and heteromeric $P2X_{2/3}$ receptors in $P2X_3$-null mutants failed to completely abolish P2X responses in isolated DRG neurons (Souslova *et al.*, 2000). A small proportion of DRG neurons responded to ATP, but not α,β-meATP with a slowly-inactivating current. Taken together, these electrophysiological data suggest the occurrence of homomeric $P2X_2$ receptors in a subset of DRG neurons.

It has been reported that virtually all cultured neonatal rat DRG neurons respond to ATP and α,β-meATP with rapidly-inactivating inward currents (Robertson *et al.*, 1996; Rae *et al.*, 1998). The discrepancy could be explained by developmental differences in the expression of P2X receptor subunits. Indeed, it

has been found that the expression pattern of $P2X_1$, $P2X_2$ and $P2X_3$ receptors is altered during the different developmental stages (Kidd *et al.*, 1995; 1998). For example, $P2X_3$ receptor proteins were detected in restricted regions (e.g. mesencephalic trigeminal nucleus) of the embryonic and neonatal rat brain. In contrast, there was no detectable staining in any region of the adult rat brain (Kidd *et al.*, 1998). In addition, the P2X receptor subtype in sensory neurons appears to show some species differences. ATP-responses in DRG neurons from bullfrog and rat exhibit different kinetics (Bean, 1990; Bean *et al.,* 1990; Robertson *et al.*, 1996; Rae *et al.*, 1998). Bullfrog DRG neurons responded to ATP with slowly-desensitizing inward currents (Bean, 1990; Bean *et al.,* 1990), which are similar to responses at homomeric $P2X_2$ or heteromeric $P2X_{2/3}$ receptors (Brake *et al.*, 1994; Lewis *et al.*, 1995). In contrast, properties of the majority of rat DRG neurons resemble those of $P2X_3$ receptors (Chen *et al.*, 1995; Grubb & Evans, 1999). Species dependence has also been revealed by immunohistochemical results, which have shown different P2X receptor subunits expressed in central sensory terminals in the rat and monkey (Vulchanova *et al.*, 1998).

8.2.2 Nodose ganglion neurons

There are three lines of evidence indicating that at least two populations of receptors ($P2X_{2/3}$ and $P2X_2$) are expressed in rat nodose ganglion neurons. Firstly, experiments have established a greater efficacy of ATP than α,β-meATP, interpreted as the presence of homomeric $P2X_2$ receptors as well as heteromeric $P2X_{2/3}$ receptors (Li *et al.*, 1997a; Thomas *et al.*, 1998). It has also been observed that α,β-meATP evoked about 75% of the maximal current activated by ATP in bullfrog DRG cells and that the effectiveness of α,β-meATP to evoke currents relative to that of ATP was 50-60% in rat nodose ganglion cells. It has been argued

that a larger current evoked by ATP than α,β-meATP might reasonably indicate the presence of more than one population of P2X receptors in these sensory neurons because the alternative that α,β-meATP may be a partial agonist does not fit experimental data with homomeric P2X receptors. Secondly, while $P2X_3$ and $P2X_{2/3}$ receptors are blocked by nanomolar concentrations of TNP-ATP (Thomas *et al.*, 1998), it requires micromolar concentrations to block $P2X_2$ receptors (King *et al.*, 1997b; Thomas *et al.*, 1998). On the basis of a variable sensitivity of TNP-ATP, it has been suggested that the homomeric $P2X_2$ and heteromeric $P2X_{2/3}$ receptors are functionally expressed in nodose ganglion cells (Thomas *et al.*, 1998). Finally, nodose ganglion neurons of $P2X_3$-null mutant mice failed to respond to α,β-meATP, but gave rise to a slowly-inactivating ATP-activated current with a smaller amplitude than that observed for nodose neurons in wild-type mice (Cockayne *et al.*, 2000; Souslova *et al.*, 2000). Taken together, these data suggest that nodose ganglion neurons functionally express heteromeric $P2X_{2/3}$ and homomeric $P2X_2$ receptors.

8.2.3 Trigeminal and mesencephalic nucleus neurons

$P2X_3$ subunit immunoreactivity has been found in isolated trigeminal neurons and their peripheral endings, but not cell bodies in the mesencephalic nucleus of the fifth nerve (MNV) in the brainstem (Cook *et al.*, 1997; Eriksson *et al.*, 1998). Nociceptors in rat trigeminal ganglia, identified by retrograde fluorescent labelling, responded to ATP and α,β-meATP with one of the three waveforms: transient, persistent or mixed responses (Cook *et al.*, 1997). Rapidly-inactivating currents were present in 28% of nociceptors, which were attributed to the activation of homomeric $P2X_3$ receptors. Slowly-inactivating currents were present in 55% of nociceptors, which were believed to be $P2X_{2/3}$ heteromers. Mixed responses,

consisting of both transient and sustained components have been observed in 16% of nociceptors, suggesting the presence of both homomeric $P2X_3$ and heteromeric $P2X_{2/3}$ receptors. In contrast, all MNV neurons tested responded to ATP and α,β-meATP with slowly-inactivating currents. These agonist responses are unlikely to be mediated by homomeric $P2X_3$ or heteromeric $P2X_{2/3}$ receptors because the $P2X_3$ subunit immunoreactivity has not been found in the MNV (Cook *et al.*, 1997; Khakh *et al.*, 1997). It has been argued that the slowly-inactivating current might be accounted for by homomeric $P2X_5$ receptors since transcripts for $P2X_5$ are almost exclusively expressed in the MNV.

Apart from $P2X_3$ and $P2X_{2/3}$ receptors, functional homomeric $P2X_2$ receptors might also be expressed in these sensory neurons. Evidence has shown that both $P2X_2$ and $P2X_3$ mRNAs and proteins are concentrated in rat trigeminal ganglia (Collo *et al.*, 1996; Xiang *et al.*, 1998). In addition, a greater efficacy of ATP than α,β-meATP was demonstrated in nociceptors of rat trigeminal ganglia (Cook *et al.*, 1997). α,β-meATP activated current was 55% of that activated by ATP in these nociceptors. For similar reasons as discussed in section **8.2.2**, it appears possible that functional $P2X_2$ receptors are expressed in nociceptors in rat trigeminal ganglion neurons.

8.2.4 Are somal and nerve terminal P2X receptors identical?

P2X receptors, particular the $P2X_3$ subtype may function as important nociceptive receptors (Chen *et al.*, 1995; Cook *et al.*, 1997; Bland-Ward & Humphrey, 1997; Dowd *et al.*, 1998; Cockayne *et al.*, 2000; Souslova *et al.*, 2000). Nerve endings of unmyelinated primary afferents are not suitable for measuring action potentials and membrane currents because most nerve terminals are smaller than the tip of conventional patch-clamp recording electrodes. Thus, to date many of these

studies have been performed on somata of isolated sensory neurons (Bean, 1990; Lewis *et al.*, 1995; Robertson *et al.*, 1996; Cook *et al.*, 1997). Biochemical and physiological features of sensory neurons *in vivo* are well maintained in culture (Baccaglini & Hogan, 1983; Gavazzi *et al.*, 1999). For example, the relative proportions of the various subsets of DRG neurons (e.g. neuropeptide-containing and IB4-positive neurons) are represented in dissociated cultures. Electrophysiological studies have shown that ion channels expressing on nerve endings also appear in the plasma membrane of the cell body. For example, applications of ATP to neurites of nociceptors of trigeminal ganglia evoked action potentials, which could be detected at the soma and are similar to those elicited by somal applications of ATP (Cook *et al.*, 1997). It has been, therefore, suggested that subsets of sensory neurons in culture may be used to study cellular mechanisms of nociception (Baccaglini & Hogan, 1983; Vyklicky & Knotkova-Urbancova, 1996).

However, multiple P2X receptor subunits are expressed in sensory neurons (Collo *et al.*, 1996), which may give rise to construction of more than one P2X receptor subtype within a single cell. Indeed, at least two populations of P2X receptors have been suggested to be present in rat DRG neurons ($P2X_3$ and $P2X_{2/3}$, Burgard *et al.*, 1999; Grubb & Evans, 1999) and rat nodose ganglion neurons ($P2X_2$ and $P2X_{2/3}$, Thomas *et al.*, 1998). It is also possible that these P2X receptors may be expressed unevenly throughout the cell membrane. Immunohistochemical and electrophysiological studies have argued for differences in P2X receptor subtypes expressed on nerve terminals and somata (Vulchanova *et al.*, 1998; Hamilton *et al.*, 1999a). In contrast to a high degree of colocalization in neuron somata, $P2X_2$ and $P2X_3$ immunoreactivity were not colocalized in central processes of DRG neurons, indicating the different P2X subtype composition on

sensory neuron bodies and their central projections (Vulchanova *et al.*, 1998). Single unit recordings from *in vitro* skin-nerve preparations demonstrated an α,β-meATP-induced increase in nerve activity, which lasted for several minutes (Hamilton *et al.*, 1999a). When α,β-meATP was applied directly to the inflamed skin, half-maximal responses were obtained after 3-4 min. These results suggest the presence of heteromeric $P2X_{2/3}$ receptors on peripheral terminals of nociceptors. This prolonged response contrasted with the reported time course in isolated DRG neurons (Robertson *et al.*, 1996; Burgard *et al.*, 1999; Grubb & Evans, 1999). Therefore, P2X receptor subtypes on peripheral and central terminals may be different from those on somata of sensory neurons.

8.3 VARIABILITY IN OPERATIONAL PROFILES OF SENSORY P2X RECEPTORS MAY REFLECT DIFFERENT LEVELS OF EXPRESSION OF $P2X_2$, $P2X_3$ AND $P2X_{2/3}$ RECEPTORS.

In situ hybridization and immunohistochemical studies have shown that multiple P2X receptor subunits can be expressed in sensory neurons (Collo *et al.*, 1996; Vulchanova *et al.*, 1998; Xiang *et al.*, 1998). However, only the colocalization of $P2X_2$-like and $P2X_3$-like proteins have been fully demonstrated (Vulchanova *et al.*, 1998; Xiang *et al.*, 1998). For this reason, coexpression of $P2X_3$ and $P2X_2$ receptor subunits in varying amounts in *Xenopus* oocytes has been studied (Chapter 3). The resultant heterogeneous populations of P2X receptors evoke a spectrum of agonist responses that are comparable to ATP-responses seen in sensory neurons.

In *Xenopus* oocytes expressing $P2X_2$ and $P2X_3$ receptor subunits, ATP evoked complex responses consisting of transient and sustained currents. The shape of these responses could be transformed from a dominant transient current to a dominant sustained current by altering the ratio of $P2X_2$ and $P2X_3$ cRNA

injected. A similar spectrum of ATP responses has been observed in rat DRG neurons (Burgard *et al.*, 1999; Grubb & Evans, 1999). The transient component of the α,β-meATP response observed in co-injected oocytes is probably mediated by homomeric $P2X_3$ receptors. The kinetics of evoked responses and the EC_{50} for α,β-meATP are similar to those determined for oocytes injected with only $P2X_3$ cRNA, and those determined for rapidly-desensitizing ATP and α,β-meATP responses in DRG neurons (Robertson *et al.*, 1996, Rae *et al.*, 1998; Burgard *et al.*, 1999; Grubb & Evans, 1999; Petruska *et al.*, 2000). Fast agonist responses in DRG neurons are believed to be mediated by homomeric $P2X_3$ receptors. In co-injected oocytes, the ability of α,β-meATP to evoke a slowly-desensitizing response, potently antagonized by TNP-ATP is consistent with the presence of heteromeric $P2X_{2/3}$ receptors. Similar slow responses have been observed in a small proportion (4%) of DRG neurons, suggesting functional expression of $P2X_{2/3}$ receptors in these neurons (Grubb & Evans, 1999). Of the remain DRG neurons, approximately 10% showed a combination of fast and slow components. Similar mixed responses consisting of transient and sustained components in co-injected oocytes were attributed to the activation of homomeric $P2X_3$ and heteromeric $P2X_{2/3}$ receptors (Chapter 3).

Early electrophysiological evidence showed that the $P2X_{2/3}$ receptor was the dominant subtype in oocytes co-injected with cRNAs for $P2X_2$ and $P2X_3$ receptors (Lewis *et al.*, 1995). However, it may not be the only subtype that mediates slowly-inactivating ATP-activated currents in co-injected oocytes. Our data suggest that a sustained response to ATP may be mediated by two populations of P2X receptors: $P2X_{2/3}$ and $P2X_2$. The great efficacy of ATP and some of its analogues (e.g. 2-MeSATP and ATPγS) indicated the presence of an additional population of receptors other than heteromeric $P2X_{2/3}$ receptors. Results of cross-

desensitization experiments with ATP and α,β-meATP were consistent with the view that an additional population, presumably homomeric $P2X_2$ receptors are expressed in co-injected oocytes. Therefore, coexpression of $P2X_2$ and $P2X_3$ subunits in oocytes can give rise to the formation of three populations of receptors: homomeric $P2X_2$, $P2X_3$ and heteromeric $P2X_{2/3}$ receptors. The relative proportions of these receptors can be changed by altering the ratios of $P2X_2$ and $P2X_3$ cRNA injected. The factors that determine the assembly of these receptors are unclear at present. It is speculated that the variability in the operational profiles of native P2X receptors in sensory neurons may reflect different levels of expression of $P2X_2$, $P2X_3$, and $P2X_{2/3}$ receptors, which function as the major species of P2X receptors.

8.4 MODULATION OF P2X RECEPTORS

Studies have revealed that P2X receptors possess modulatory sites by which endogenous substances including protons and the divalent cation Zn^{2+} can regulate agonist responses. It has been reported that extracellular protons facilitate the function of P2X receptors in rat nodose ganglion neurons and bullfrog DRG neurons (Li *et al.*, 1996a; 1996b; 1997a), whilst Zn^{2+} potentiates ATP-activated currents in rat nodose ganglion neurons and a subset of spinal cord neurons (Li *et al.*, 1993).

8.4.1 Effects of protons on P2X receptors

Extracellular pH has a profound influence on the function of both the central and peripheral nervous systems (see review, Chesler, 1990). Regulation of ligand-gated ion channels by protons may be physiologically relevant in that acidification usually occurs during hypoxia, ischaemia and inflammation. Protons can produce or

exaggerate pain via activating or modulating nociceptive ion channels including acid-sensing ion channels and capsaicin-sensitive receptors (see review, McCleskey and Gold, 1999). It has also been shown that protons differentially modulate the activity of P2X receptors. For example, the affinity of $P2X_2$ receptors was enhanced 5-10-fold by acidifying the bathing solution, but was diminished 4-5-fold in an alkaline solution (pH 8.0) (King *et al.*, 1996c). In contrast, ATP-responses were decreased by acidification at recombinant $P2X_1$, $P2X_3$ and $P2X_4$ receptors (Stoop *et al.*, 1997; Wildman *et al.*, 1999b; 1999c). These authors also found that agonist responses at heteromeric $P2X_{2/3}$ were enhanced by low pH in bathing solutions. The finding in Chapter 3 is in general agreement with the previous report, though only a modest potentiating effect of protons was recorded at $P2X_{2/3}$ receptors.

A comparison of homomeric $P2X_{1-4}$ and heteromeric $P2X_{2/3}$ receptors has revealed that lowering the extracellular pH level only enhances sensitivity of $P2X_2$ and $P2X_{2/3}$ receptors. This fundamental difference between P2X receptors may be used to aid the elucidation of native P2X receptor subunits. For example, protons have been found to potentiate ATP-activated currents in rat nodose ganglion and bullfrog DRG neurons (Li *et al.*, 1996a; 1996b; 1997a), suggesting that either $P2X_2$, $P2X_{2/3}$ or both may be expressed in these sensory neurons. Rat nodose ganglion neurons are known to express varying proportions of $P2X_2$ and $P2X_{2/3}$ receptors as discussed in section **8.2.2**. On the basis of agonist profiles and kinetics of evoked responses, it has been suggested that bullfrog DRG neurons express either $P2X_2$, $P2X_{2/3}$, or both (Li *et al.*, 1997a). Thus, the potentiation of agonist responses by extracellular protons in these sensory neurons is consistent with the notion that $P2X_{2/3}$ and/or $P2X_2$ receptors are the dominant P2X subtypes in rat nodose ganglion and bullfrog DRG neurons. However, a small proportion of

rat nodose ganglion neurons (42 out of 188), which responded to ATP with slowly-inactivating currents, were not affected by changes in extracellular pH (Li *et al.*, 1996b). As six out of seven cloned P2X subunit mRNAs and proteins have been found in rat nodose ganglia (Collo *et al.*, 1996; Xiang *et al.*, 1998), it is possible that some P2X subtypes other than $P2X_{2/3}$ and $P2X_2$ may be expressed in a small number of nodose ganglion neurons. Nevertheless, the modulation of native P2X receptors by protons may play an important role in the function of sensory neurons (Li *et al.*, 1996a; 1996b; 1997a).

8.4.2 Effects of Zn^{2+} on P2X receptors

There are three pools for Zn^{2+}: vesicular, free and protein bound. Vesicular Zn^{2+} is found in presynaptic vesicles of a special class of Zn^{2+}-containing neurons, such as neurons of hippocampal mossy fibers (Frederickson, 1989). In these vesicles, the concentration of Zn^{2+} is estimated to be between 200-300 μM, which is high enough to modulate functions of excitatory (e.g. glutamate) and inhibitory (e.g. GABA) amino acid neurotransmitters in the CNS (see review, Choi & Koh, 1998). Histological studies have also shown that a subset of neurons in the dorsal horn of the spinal cord contain the divalent cation Zn^{2+}, suggesting a physiological role in sensory processing at the spinal cord level (Danscher, 1982). Indeed, intrathecal application of Zn^{2+} produced an antinociceptive and antihyperalgesic effect, though the mechanisms are not clear (Larson & Kitto, 1997). Very recently, Zn^{2+} was found to be concentrated and stored in vesicles of a population of primary sensory neurons (Velázquez *et al.*, 1999). Approximately 25% of the total small DRG neurons are Zn^{2+}-containing neurons, suggesting that endogenously occurring Zn^{2+} may play a role in modulating nociceptive transmission. Zn^{2+}-

containing terminals detected by immunohistochemistry and autometallography have also been found in the dorsal horn in the mouse spinal cord (Jo *et al.*, 2000).

Zn^{2+} is known to inhibit or facilitate a variety of ion channels including NMDA subclass of glutamate receptors (Peters *et al.*, 1987) and GABA receptors (Legendre & Westbrook, 1991). Studies have also demonstrated that Zn^{2+} has complex actions on P2X receptors. For example, Zn^{2+} enhanced ATP-mediated dopamine release from rat pheochromocytoma PC12 cells and potentiated ATP activity at P2X receptors in rat sympathetic and nodose ganglion neurons (Cloues *et al* 1993; Li *et al.*, 1993; Koizumi *et al.*, 1995). It appears that Zn^{2+} increases the opening frequency of single ATP-gated channels and mean burst duration of primary open conductance state (Cloues 1995; Wright & Li, 1995). In contrast, Zn^{2+} inhibited ATP-activated currents in bullfrog DRG neurons (Li *et al.*, 1997b).

Of the recombinant $P2X_{1-4}$ receptors, $P2X_2$ and $P2X_4$ receptors are sensitive to Zn^{2+} at micromolar concentrations (Garcia-Guzman *et al.*, 1997; Wildman *et al.*, 1998; 1999b), whilst $P2X_1$ and $P2X_3$ receptors are relatively insensitive to extracellular Zn^{2+} (Wildman *et al.*, 1999c). A modest potentiation of slowly-inactivating α,β-meATP-activated currents by Zn^{2+} was observed at heteromeric $P2X_{2/3}$ receptors (Chapter 3). This finding contrasted with the strong potentiation by Zn^{2+} of ATP responses at $P2X_2$ receptors, and its weak inhibitory action at $P2X_3$ receptors. Previous reports suggested that $P2X_2$ receptor was the only P2X subtype at which agonist responses were potentiated by both protons and Zn^{2+} at low micromolar concentrations (King *et al.*, 1996c; Stoop *et al.*, 1997; Wildman *et al.*, 1998). However, the results in Chapter 3 indicate that heteromeric $P2X_{2/3}$ receptors were also sensitive to both protons and Zn^{2+} at micromolar concentrations, though the potentiating effects of extracellular H^+ and Zn^{2+} were

modest at $P2X_{2/3}$ receptors compared to the reported facilitatory actions at $P2X_2$ receptors.

Behavioural studies suggest that Zn^{2+} released from the spinal cord may play a modulatory role in nociception (Larson & Kitto, 1997). It has been speculated that Zn^{2+} may exert an important inhibitory control over nociceptive transmission at the spinal level, possibly by modulating several receptors simultaneously. One such mechanism could be the modulatory effect of Zn^{2+} on P2X receptors, particular $P2X_3$ and $P2X_{2/3}$ receptors, which are implicated in nociception (Chen *et al.*, 1995; Cook *et al.*, 1997; Cockayne *et al.*, 2000; Souslova *et al.*, 2000). It has also been found that ATP can excite P2X receptors on central terminals of primary afferents (Gu & MacDermott, 1997) and on second-order sensory neurons of the dorsal horn in the spinal cord (Jahr & Jessell, 1983). However, the modulation of nociceptive transmission by Zn^{2+} observed in behavioural studies (Larson & Kitto, 1997) can not be explained solely by the reduction in responses at $P2X_3$ receptors, because Zn^{2+} only had a modest inhibitory effect at $P2X_3$ receptors. Although Zn^{2+} appears to potentiate ATP responses at $P2X_{2/3}$ receptors, this effect is likely to enhance nociception rather than reduce it. Therefore, $P2X_3$ and $P2X_{2/3}$ receptors are unlikely to be involved in the reported antinociceptive activity of Zn^{2+}.

8.5 THERE ARE SEVERAL DISTINCT NOCICEPTIVE ION CHANNELS

Ion channels are critical mediators in the transduction of noxious stimuli into depolarizations in primary afferent terminals and conduction of the resultant action potentials from peripheral sensory sites to central terminals. Over the past decades, studies of ion channels of nociception have focused on ATP-gated ion channels (P2X receptors), capsaicin-activated receptors, proton-gated channels and

tetrodotoxin (TTX)-resistant Na^+ channels (see reviews, Wood & Docherty, 1997; McCleskey & Gold, 1999;).

8.5.1 Capsaicin-gated ion channels

Capsaicin excites a subset of primary sensory neurons in DRGs and trigeminal ganglia (Liu & Simon, 1994; Oh *et al.*, 1996). As a general rule, these capsaicin-sensitive neurons are small-sized neurons and give rise to thin, unmyelinated C fibers (see review, Holzer, 1991). Activation of these sensory neurons by capsaicin produces sensations of burning pain or irritation. In addition, $P2X_3$ and VR1 receptors are colocalized in a subset of small-sized DRG neurons (see Section **1.4.6.3**), suggesting a possible interaction between two distinct nociceptive ion channels. In Chapter 4, coexpression of $P2X_3$ and VR1 in *Xenopus* oocytes has been employed to study the functional interaction between ATP-activated currents at $P2X_3$ and capsaicin-activated currents at VR1 receptors.

8.5.1.1 Functional inhibition between $P2X_3$ and VR1 receptors

As discussed in the section **1.4.6.1 and 1.4.6.2**, $P2X_3$ receptors are expressed in IB4-positive, non-peptidergic, small-sized DRG neurons. Of the IB4-positive profiles, $P2X_3$-immunoreactivity was frequently colocalized with VR1-like proteins (Guo *et al.*, 1999). Ueno and colleagues (1999) first demonstrated that approximately 70% of the capsaicin-sensitive DRG neurons responded to both ATP and α,β-meATP with rapidly-inactivating inward currents, possibly mediated by homomeric $P2X_3$ receptors, whilst about 48% of capsaicin-insensitive neurons gave rise to slowly-inactivating currents, mediated by heteromeric $P2X_{2/3}$ receptors. These authors also found that neurons with fast responses were predominately small in size, whilst those with slow responses were mainly

medium-sized neurons. These findings have been confirmed and extended by subsequent studies (Li *et al.*, 1999; Petruska *et al.*, 2000). Some of the capsaicin-sensitive neurons also responded to ATP with slow and mixed currents (Petruska *et al.*, 2000). In addition, a subset of small-sized DRG neurons responded to ATP with slowly-inactivating currents. The distribution pattern of $P2X_3$ and VR1 receptors in DRG neurons detected by immunohistochemical and electrophysiological studies suggest the possible interaction between these two distinct receptor populations.

With $P2X_3$ or VR1 receptors expressed in oocytes individually, I found that ATP failed to activate or modulate VR1 receptors and capsaicin did not gate or modulate $P2X_3$ receptors. However, with $P2X_3$ and VR1 expressed together, responses to ATP were attenuated by prior activation of VR1 receptors. The inhibition persisted after VR1 receptors desensitized. Therefore, the inhibition of ATP-responses required VR1 receptors at a ligand-bound active or desensitized state. In contrast, responses to capsaicin were not affected by the prior activation or desensitization of $P2X_3$ receptors. This one-way inhibition of ATP responses was dependent on the level of functional expression of VR1 receptors.

Responses to ATP in individual DRG neurons may be a rapidly-desensitizing current ($P2X_3$-like), or slowly-desensitizing current ($P2X_{2/3}$-like), or a mixture of these response types depending on the complement of receptors that are available in the cell (Burgard *et al.*, 1999; Grubb & Evans, 1999). Therefore, it is possible that the different interaction may occur depending on the P2X receptor subtype in individual sensory neurons. Nevertheless, one-way inhibition between $P2X_3$ and VR1 receptors may represent an important physiological mechanism by which the function of nociceptors can be regulated. It is a well documented phenomenon that desensitization occurs following excitation of sensory neurons by vanilloids (see reviews, Holzer, 1991; Szallasi & Blumberg, 1999). During this

refractory state, neurons do not respond to a subsequent capsaicin challenge and are resistant to various stimuli, ranging from noxious heat to mechanical pressure. Data presented in Chapter 4 suggest that desensitization induced by capsaicin challenge could also result in the resistance of sensory neurons to an endogenous chemical mediator ATP. These findings may have important implications *in vivo* where the excitation of VR1 receptors may suppress the activity of $P2X_3$ receptors following tissue damage.

8.5.1.2 Differential actions of protons on $P2X_3$ and VR1 receptors

Protons exert a complex action on cloned VR1 receptors (Chapter 4). For instance, a reduction in the pH from 7.5 to 6.5 failed to open VR1 receptors. Nonetheless, it caused a 2-fold increase in currents evoked by capsaicin (1 μM). These results are consistent with the previous reports that low pH potentiated responses to capsaicin in cultured primary sensory neurons (Bevan & Yeats, 1991; Petersen & LaMotte, 1993; Liu & Simon, 1994; Kress et al., 1996) and at heterologously expressed VR1 receptors (Caterina *et al.*, 1997; Tominaga *et al.*, 1998). I also found that a further reduction in pH to 5.5 evoked a current through VR1 receptors, which is in general agreement with the previous report (Caterina *et al.*, 1997). VR1 receptors have been suggested to be an integrator of painful chemical and physical stimuli because protons can also increase the response of VR1 receptors to noxious heat (Caterina *et al.*, 1997).

Regulation of nociceptive ion channels by protons may be of physiological and pathological importance because tissue acidosis occurs during hypoxia, ischaemia and inflammation. Acidic pH consistently enhanced the potency of VR1 receptors. In contrast, P2X receptors showed different sensitivity to extracellular pH (Chapter 3 and 4). I found that $P2X_3$ receptors was relatively insensitive to

protons compared with the $P2X_2$ receptor, the latter exhibiting a full sensitivity to acidic pH (King *et al.*, 1996c). With $P2X_3$ and VR1 receptors expressed together, a combined application of capsaicin and ATP gave rise to a biphasic response consisting of an initial rapidly-inactivating ($P2X_3$-like) response and a secondary slowly-inactivating (VR1-like) response. Lowering extracellular pH to 6.5 selectively enhanced the VR1-like responses, leaving the $P2X_3$-like responses unchanged. It is likely that the sensitivity of VR1 and $P2X_3$ receptors may be altered during local acidosis following tissue inflammation and hypoxia.

8.5.2 Proton-gated ion channels

Krishtal and Pidoplichko (1980; 1981) first discovered acid-sensing ion channels in sensory neurons and proposed that these channels may be associated with nociception. So far, five proton-gated channels have been cloned from mammalian tissues. An acid-sensing ionic channel (ASIC/ASIC1), mediates a rapidly-inactivating H^+-activated current, which requires a rapid drop of the extracellular pH (Waldmann *et al.*, 1997b). ASIC/ASIC1 are present in both sensory neurons and in neurons of the CNS. ACICβ/ASICb, a splice variant, has only been found in sensory ganglia, though the pH dependency and desensitizing kinetics of ACICβ/ASIC1b are similar to those described for ASIC/ASIC1 (Chen *et al.*, 1998). Dorsal root acid-sensing ionic channel (DRASIC/ASCI3) mediates a non-inactivating current, that responds to a slow decrease of the extracellular pH (Waldmann *et al.*, 1997a). A mammalian degenerin homologue MDEG1/ASIC2, colocalized with ASIC/ASIC1in the CNS, has been identified as an acid-sensing channel which requires higher proton concentrations for activation (pH 6.0-3.5) and has slower inactivating kinetics (Lingueglia *et al.*, 1997). Coexpression of ASIC/ASIC1 and MDEG1/ASIC2 subunits in *Xenopus* oocytes generates an

amiloride-sensitive H^+-gated Na^+ channel with novel kinetics, ionic selectivity, and pH sensitivity (Bassilana *et al.*, 1997). MDEG2/ASCI2b, a splice variant form of MDEG1/ASIC2, has also been cloned from mouse and rat brain (Lingueglia *et al.*, 1997). Heterologously expressed MDEG2/ASIC2b failed to be activated by protons, but might be associated with DRASIC/ASIC3 to form heteromeric assemblies with distinct kinetics.

These ASIC channels belong to the degenerin/ENaC channel superfamily, and are composed of two hydrophobic transmembrane domains and a large extracellular loop with the amino and carboxyl termini inside the cell (Waldmann *et al.*, 1997b). This structure is similar to the predicted membrane topology of the P2X receptor, even though there is no sequence homology between these two families. In contrast, the topology of the ASIC and $P2X_3$ receptors is completely different from that of the VR1 receptor, which has six transmembrane domains and the short hydrophobic region between transmembrane domain 5 and 6 may contribute to the ion permeation path (Caterina *et al.*, 1997). Although an abrupt drop of extracellular pH to 5.5 activated a slowly-desensitizing inward current at VR1 receptors expressed in oocytes (Chapter 4), the VR1 receptor does not formally belong to the family of acid-sensing ion channels.

Four out of five cloned acid-sensing channel mRNAs have been found in sensory neurons (Lingueglia *et al.*, 1997; Waldmann *et al.*, 1997a; 1997b; Chen *et al.*, 1998). ASIC-like proteins have also been detected in superficial dorsal horn, DRGs and peripheral nerve endings (Olson *et al.*, 1998). It is possible that ASICs and VR1 receptors may functionally interact with each other in that ASIC receptor immunoreactivity has been found in capsaicin-sensitive sensory neurons (Olson *et al.*, 1998). As DRASIC/ASIC3 is a DRG-specific isoform, future studies of possible crosstalk between the DRASIC/ASIC3 and $P2X_3$ or VR1 receptors will

provide important information in understanding how painful mediators including H^+ and ATP are transduced and interacted at the receptor level.

8.5.3 TTX-resistant Na^+ channels

Multiple voltage-gated Na^+ channels (VGSCs), some tetrodotoxin (TTX)-sensitive and others TTX-resistant, have been observed in DRG neurons (Elliott & Elliott, 1993; Rizzo *et al.*, 1994). TTX-resistant Na^+ channels seem to be particular important because an increased activity in these Na^+ channels is likely to cause nociception and contribute to injury-induced nociceptor hyperexcitability (Matzner & Devor, 1994). Two TTX-resistant Na^+ channels, the sensory-neuron specific Na^+ channel (SNS/PN3, Akopian *et al.*, 1996; Sangameswaran *et al.*, 1996) and previously unidentified Na^+ channel (NaN/SNS-2, Dib-Hajj *et al.*, 1998b), were recently identified in peripheral sensory neurons. It has been demonstrated that heterologously expressed SNS receptors produce a slowly inactivating TTX-resistant current (Akopian *et al.*, 1996; Sivilotti *et al.*, 1997). A persistent TTX-resistant Na^+ current was evoked in DRG neurons of SNS-null mutant and wild-type mice, indicating that NaN might mediate this persistent TTX-resistant current (Cummins *et al.*, 1999).

Several lines of evidence suggest that TTX-resistant Na^+ channels are involved in the initiation and maintenance of inflammatory and neuropathic pain. Firstly, SNS Na^+ channels have been found to be exclusively expressed in a subpopulation of small-sized sensory neurons, most of them are nociceptors (Akopian *et al.*, 1996; Dib-Hajj *et al.*, 1998b). Secondly, conventional local anaesthetic drugs are non-selective voltage-dependent Na^+ blockers, and have been found to be effective in some of animal pain models. For example, lidocaine at a concentration far below that needed for causing action potential block, reversed

mechanical allodynia in neuropathic pain models (Devor *et al.*, 1992; Sinnott *et al.*, 1999). Thirdly, electrophysiological evidence shows that the expression level of TTX-resistant Na^+ channels in sensory neurons is modulated by several different inflammatory mediators (Gold *et al.*, 1996; Tanaka *et al.*, 1998). Inflammatory mediators including prostaglandin E2 produced tenderness or hyperalgesia *in vivo* and significantly increase the amplitude of TTX-resistant Na^+ currents in isolated DRG neurons (Gold *et al.*, 1996). Transcripts of SNS Na^+ channels were up-regulated following peripheral inflammation induced by local injections of carrageenan (Tanaka *et al.*, 1998). Fourthly, expression of Na^+ channels in peripheral mammalian axons detected by immunohistochemical techniques has been found to be increased following nerve injury (Devor *et al.*, 1993), which was thought to be mediated by NGF, a trophic factor released at sites of nerve injury (Woolf *et al.*, 1994).

Subsequent studies have shown that NGF is an important mediator in the regulation of membrane excitability in small DRG neurons by pathways that include opposing effects on different sodium channel genes (Black *et al.*, 1997). Transcripts of SNS and NaN Na^+ channels and TTX-resistant sodium currents were significantly down-regulated, whilst transcripts of the TTX-sensitive Na^+ channel and a fast TTX-sensitive Na^+ current were up-regulated in small DRG neurons following chronic constriction injury (Dib-Hajj *et al.*, 1999). When the target organ-derived NGF is interrupted, as in the cause of axotomy, TTX-resistant Na^+ current density decreased. This axotomy-induced down-regulation of TTX-resistant Na^+ channels can be prevented by exogenous NGF (Dib-Hajj *et al.*, 1998a). Taken together, inflammation and nerve injury (NGF) make sensory neurons abnormally excitable, which might be the direct consequence of changes in the expression level of different types of VGSCs. Despite the plastic change in

response to nerve injury and tissue inflammation, TTX-resistant Na^+ channels might also be interacted with other nociceptive ion channels including the $P2X_3$ receptor. Studies to investigate the possible functional interaction between the $P2X_3$ and SNS Na^+ channels may be of physiological importance because both receptors are almost exclusively expressed in nociceptors.

8.6 NOVEL ANTAGONISTS OF P2X RECEPTORS

Potent and subtype selective P2X antagonists are needed for studies of native ATP-gated ion channels and their therapeutic potential. In the present study, the antagonist activity of a series of diinosine polyphosphate (Ip_nI, where n= 3, 4, 5) has been examined on ATP-activated currents at homomeric $P2X_{1-4}$ (Chapter 5) and heteromeric $P2X_{2/3}$ receptors (Chapter 6) expressed in *Xenopus* oocytes. These studies were initially prompted by the observation that diinosine pentaphosphate (Ip_5I) selectively antagonized P2X-mediated contractions in the guinea-pig vas deferens (pA_2, 6.5±0.1), leaving contractile responses evoked by noradrenaline unchanged (Hoyle *et al.*, 1997). These authors also found that Ip_5I (up to 100 μM) did not antagonize P2Y receptors in the guinea-pig taenia coli, nor P1 or P2 receptors in the guinea-pig left atrium. In the present study, Ip_5I was found to be an effective antagonist of agonist responses at recombinant $P2X_1$ and $P2X_3$ receptors. It exhibited a reasonable selectivity for $P2X_1$ (pA_2, 8.2) over $P2X_3$ receptors (pA_2, 6.3). The blocking activity of the Ip_nI series decreased as the phosphate chain was reduced in length. Ip_4I appeared to be equipotent at $P2X_1$ (IC_{50}, 0.56±0.08 μM) and $P2X_3$ receptors (IC_{50}, 1.0±0.3 μM), whilst Ip_3I was a weak antagonist with the IC_{50} over 30 μM at both receptors. In contrast, the Ip_nI series lacked antagonist activity at $P2X_2$ and $P2X_4$ receptors. Contrarily, Ip_5I and Ip_4I potentiated agonist responses at $P2X_4$ receptors. A similar potentiating effect

of other known P2 antagonists (e.g. suramin, PPADS and Reactive blue 2) has been found at $P2X_4$ receptors (Bo *et al.*, 1995). Therefore, Ip_5I may be a useful pharmacological tool in bioassays of naturally-occurring $P2X_1$ receptors in the vas deferens and vascular smooth muscle. The $P2X_1$ receptor appears to be the dominant P2X subtype in the vas deferens and blood vessels (Valera *et al.*, 1994; Collo *et al.*, 1996; Nori *et al.*, 1998). However, in addition to $P2X_1$ receptors, transcripts for other P2X receptor subtypes have been detected in vascular smooth muscle (Soto *et al.*, 1996a; Nori *et al.*, 1998). For example, $P2X_2$ and $P2X_4$ mRNAs were detected in rat coronary arteries. As Ip_5I is a potent antagonist at $P2X_1$ receptors but has no inhibitory effect at $P2X_2$ and $P2X_4$ receptors, it should be useful in distinguishing the complement of P2X receptor subtypes in blood vessels.

The antagonist activity of Ip_5I has also been investigated in oocytes co-injected with cRNAs for $P2X_2$ and $P2X_3$ receptor subunits. The effect of Ip_5I was compared with that of TNP-ATP, a known antagonist at both $P2X_3$ and $P2X_{2/3}$ receptors (Chapter 6). α,β-meATP evoked a biphasic response composed of transient and sustained components in co-injected oocytes. TNP-ATP inhibited both the transient and sustained parts of the response. In contrast, Ip_5I selectively abolished the transient component of the response, while leaving the sustained current unchanged. These results were consistent with the data obtained from the study of $P2X_3$ and $P2X_{2/3}$ receptors in isolation. Both Ip_5I and TNP-ATP potently inhibited the agonist response at homomeric $P2X_3$ receptors. However, Ip_5I at concentrations up to 30 μM had no inhibitory effect at heteromeric $P2X_{2/3}$ receptors, whilst TNP-ATP at a submicromolar concentration (0.1 μM) completely blocked the slowly-inactivating response to α,β-meATP. Thus, Ip_5I is an effective antagonist at homomeric $P2X_3$ receptors, but is ineffective at heteromeric $P2X_{2/3}$

receptors. In contrast, TNP-ATP is a potent antagonist at both $P2X_3$ and $P2X_{2/3}$ receptors, consistent with the previous report (Virginio *et al.*, 1998). Electrophysiological studies on isolated sensory neurons suggest that at least two populations of P2X receptors are expressed on sensory neurons, heteromeric $P2X_{2/3}$ and homomeric $P2X_3$ (or $P2X_2$) receptors (see Section **8.2**). These receptors are expressed either separately or together on individual neurons. Taken together, Ip_5I should be a useful tool in elucidating P2X subunit composition because of its reasonable selectivity of $P2X_3$ over $P2X_2$ and $P2X_{2/3}$ receptors.

8.7 P2X RECEPTORS: POTENTIAL TARGETS FOR NOVEL ANALGESIC DRUGS

A number of chronic pain conditions (e.g. migraine, cancer and neuropathic pain) are not well understood and difficult to treat by nonsteroidal antiinflammatory drugs (NSAIDs) and opioid analgesics. There is increasing evidence showing that ATP functions as a physiological mediator of nociception presumably via activating $P2X_3$ receptors on primary afferents (Bleehen & Keele, 1977; Bland-Ward & Humphrey, 1997; Hamilton *et al.*, 1999b). Consistently, pain-related behaviour caused by injection of formalin was significantly decreased in $P2X_3$ null-mutant mice (Cockayne *et al.*, 2000; Souslova *et al.*, 2000). The analgesic effect of P2 receptor antagonists (e.g. suramin and PPADS) has been observed in several animal pain models (Driessen *et al.*, 1994; Hamilton *et al.*, 1999b; Tsuda *et al.*, 1999a; b). Moreover, ATP might function as a chemical mediator of pain in several pathological conditions. Evidence has accumulated that ATP is released from endothelial cells during ischaemia and vasospasm (see review, Burnstock, 1999). The released ATP may act on different populations of P2 receptors in blood vessels: P2Y on endothelial cells and P2X on adventitia (Burnstock *et al.*, 1994;

Hansen *et al.*, 1999). It has been suggested that ATP may mediate ischaemic pain during angina and migraine (see review, Burnstock, 1996a). ATP release as a result of cell lysis may also contribute to pain in malignancy. Cytosolic concentrations of ATP are in the millimolar range in living cells. In addition, some tumour cells contain even higher levels of ATP, which may be released during tumour cell lysis and causes pain (see reviews, Burnstock, 1996a; Wood & Burnstock, 1996). For these reasons, it may be important to investigate the analgesic effect of Ip_5I in physiological and pathophysiological processes, because my data have clearly shown that Ip_5I is an effective antagonist at the recombinant $P2X_3$ receptor, a subtype that is believed to be involved in nociception.

Neuropathic pain usually occurs following peripheral nerve injury and is largely resistant to commonly used peripherally acting analgesic drugs. Burnstock (1996a) proposed that synaptically released ATP might mediate neuropathic pain following nerve injury, which might give rise to an abnormal sympathetic-sensory coupling (Ramer *et al.*, 1999). Evidence has shown that sympathetic nerve fibers can grow into DRGs, where they form basket-like structures around sensory neurons. Animals with the spinal nerve ligated, a model for neuropathic pain, exhibited an increased response to mechanical stimuli and noxious heat (Kim *et al.*, 1993). Surgical sympathectomy effectively suppressed the mechanical allodynia and heat hyperalgesia, strongly suggesting those behavioural signs of neuropathic pain are sympathetically maintained In contrast, medical sympathetomy following an injection of guanethidine or phentolamine (α-adrenoceptor blocker) was largely ineffective in relieving pain (Kim *et al.*, 1993; Park *et al.*, 2000). This discrepancy has led to the searching for factors other than noradrenaline and adrenoceptors, which might also contribute to the generation of neuropathic pain. When suramin was given in combination with phentolamine, the

mechanical hypersensitivity was markedly reduced in neuropathic rats (Park *et al.*, 2000). These results imply that sympathetically released ATP and noradrenaline are involved in the maintenance of neuropathic pain. An increase in $P2X_3$ subtype-like proteins in DRG neurons and their central terminals have been observed in a rat model of neuropathic pain (Novakovic *et al.*, 1999). Functional studies so far suggest that it is the $P2X_3$ and $P2X_{2/3}$ subunits that are important for the activation of primary afferents (Kirkup *et al.*, 1999; Rong *et al.*, 2000). Nevertheless, the involvement of these P2X receptors in pathophysiological conditions still needs to be clarified.

8.8 ADRENAL CHROMAFFIN CELLS: A MODEL FOR THE NATIVE $P2X_2$ RECEPTOR

Rat nodose ganglion neurons have been studied as a model for the heteromeric $P2X_{2/3}$ receptor because these neurons give rise to homogeneous slowly-inactivating responses to both ATP and α,β-meATP ($P2X_{2/3}$-like response) (Khakh *et al.*, 1995; Lewis *et al.*, 1995; Thomas *et al.*, 1998). Cultured rat neonatal DRG neurons have been used to study the native $P2X_3$ receptor as these neurons respond to P2X agonists with a rapidly-inactivating inward current ($P2X_3$-like response) (Robertson *et al.*, 1996; Rae *et al.*, 1998). In this thesis, the functional expression of P2X receptors in adrenal chromaffin cells has been examined in an attempt to search for a model for the native $P2X_2$ receptor.

Due to their embryonic origin, adrenal chromaffin cells retain some neuron-like properties. For example, ATP is co-stored with catecholamines in secretory granules at a high concentration and is co-released from adrenal chromaffin cells during excitatory stimulation (Winkler & Westhead, 1980; Rojas *et al.*, 1985). ATP induces catecholamine secretion in PC12 cells and bovine chromaffin cells,

which has been suggested to be accomplished by increasing the influx of extracellular Ca^{2+} through ATP-gated ion channels (Inoue *et al.*, 1989; Castro *et al.*, 1995; Reichsman *et al.*, 1995). A $P2X_2$-like receptor has been found in PC12 cells, a cell line derived from rat pheochromocytoma (Inoue *et al.*, 1989; Nakazawa *et al.*, 1990; 1991; Nakazawa & Inoue, 1992). Indeed, the $P2X_2$ receptor was first cloned from rat PC12 cells (Brake *et al.*, 1994). For these reasons, the PC12 cell has been served as a model for studying properties of the neuronal $P2X_2$ receptor (Nakazawa *et al.*, 1990; 1991; 1997a; 1997b). In Chapter 7, a $P2X_2$-like receptor has been found functionally expressed in chromaffin cells isolated from guinea-pig adrenal glands. Guinea-pig adrenal chromaffin cells responded to ATP with a slowly-inactivating inward current, which blocked by suramin and PPADS. The evoked response was potentiated by extracellular protons, but interestingly not by Zn^{2+} at micromolar concentrations. The latter is contrasted with the strong potentiation action of Zn^{2+} at the rat $P2X_2$ receptor (Wildman *et al.*, 1998). Nevertheless, the guinea-pig chromaffin cell may be a useful model for the study of the native $P2X_2$ receptor. Surprisingly, rat adrenal chromaffin cells failed to respond to ATP at micromolar concentrations. Therefore, it appears that expression of functional P2X receptors in chromaffin cells exhibits some species dependence. Similar species dependence in expression of P2X receptors has been observed in DRG neurons as discussed in Section **8.2.1.**

8.9 FUTURE EXPERIMENTS

Ion channels on nociceptors are not really independent. It is likely that not a single receptor, but rather a combination of them, plays a role in pain transduction, because none of the tested receptor antagonists alone can achieve complete analgesia (see reviews, Dray & Urban, 1996; Wood & Docherty, 1997; McCleskey

& Gold, 1999). TTX-resistant Na^+ channels (such as SNS and NaN subtypes) and the $P2X_3$ receptor are almost exclusively expressed in small-sized DRG neurons and are believed to be involved in nociceptive transduction and transmission (Chen *et al.*, 1995; Akopian *et al.*, 1996; Dib-Hajj *et al.*, 1998b). I would like to study the possible functional interaction between these two structurally related ionic channels using *Xenopus* oocytes expression system. With TTX-resistant Na^+ channels and $P2X_3$ receptors expressed together, the effect of activation and desensitization of $P2X_3$ receptors on Na^+ channels or *vice versa* could be studied using whole-cell twin-electrode techniques. In addition, the DRASIC/ASIC3, a DRG-specific isoform of acid-sensing channels and the $P2X_3$ receptor belong to two unrelated gene families, but both channels are excitatory receptors expressed by nociceptors as discussed in Section **8.5.2.** The possible 'crosstalk' between these two channels should also be investigated with DRASIC/ASIC3 and $P2X_3$ expressed in *Xenopus* oocytes. The study in potential functional interactions between $P2X_3$ and TTX-resistant Na^+ channels, or DRASIC/ASIC3 receptors may be useful in understanding how painful information is integrated at the receptor level.

An increase in expression of $P2X_3$ receptor proteins has been detected in a rat neuropathic pain model caused by chronic constriction injury to the peripheral nerve (Novakovic *et al.*, 1999). However, the mechanism by which $P2X_3$ receptor expression is regulated remains unclear. The expression of TTX-resistant Na^+ channels appears to be regulated by NGF and therefore may be overexpressed in the state of hyperalgesia following tissue inflammation or nerve injury (see section **8.5.3**). I hope to study the plasticity of $P2X_3$ receptors in acutely isolated rat DRG neurons following chronic nerve injury using a combined electrophysiological and immunohistochemical techniques. The possible alteration in expression of $P2X_3$

receptors will be compared with that of TTX-resistant Na^+ channels using the same neuropathic pain model.

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