CHARACTERIZATION OF ATP ANALOGUE CROSS-LINKING COMPOUNDS AND BIOCHEMICAL ANALYSIS OF P2X RECEPTORS

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by

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The aim of this thesis was to test whether cross-linking ATP analogues could be used to provide direct evidence of the amino acid residues which contribute to the ATP binding domain of P2X receptors.

The UV irradiation of 2-azido ATP caused the compound to cross-link to the P2X<sub>1</sub> receptor and caused a significant reduction in the response to ATP. The reduction of cross-linking following the pre-treatment of cells in excess ATP suggested competition between ATP and 2-azido ATP for occupancy of the P2X receptor binding site. This was proven with the radio-labelling of the P2X<sub>1</sub> receptor with 2-azido [γ<sup>32</sup>P] ATP. Similar techniques were used to identify other photo-reactive compounds with activity at the P2X<sub>1</sub> receptor. These photo-reactive compounds had reactive sites at different coordinates around the ATP molecule and can potentially cross-link to different regions within the ATP binding domain.

In an attempt to localize the ATP binding site, the P2X<sub>1</sub> receptor was tagged with flag and his epitopes and purified. Protein mass fingerprinting showed that the digestion of the P2X<sub>1</sub> protein did not provide enough coverage of the protein to guarantee successful analysis by mass spectrometry. Additionally, initial studies using digestion to identify peptide fragments which had bound to 2-azido [γ<sup>32</sup>P] ATP showed that experimental conditions caused the cleavage of the label from the peptide.

This thesis has demonstrated that photo-reactive ATP analogues can be used to label the P2X receptors. Further work should identify a suitable combination of enzymes to efficiently fragment the protein and allow spectral analysis to identify the amino acid residues which covalently bind to each photo-reactive compound. This would give the first direct evidence of the contributing amino acid residues in the ATP binding domain of the P2X receptors.
Acknowledgements

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Abbreviations

A......................................................Adenine
ABC..................................................ATP binding cassette
αβMe-ATP...........................................alpha, beta-methyleneATP
βγMe-ATP............................................beta, gamma-methyleneATP
AmpR..............................................Ampicillin resistant
ATP................................................Adenosine 5’-triphosphate
ATP-AA.............................................Adenosine 5’-triphosphate [γ] 4-Azidoanilide
ATP-BP..............................................Adenosine 5’-triphosphate [γ] Benzophenone
Bz-ATP..............................................2’, 3’-O-(4-benzoylbenzoyl)-ATP
C........................................................Cytosine
CNBr..............................................Cyanogen Bromide
E.Coli.............................................Escherichia coli
EDTA..............................................Ethylenediaminetetra-acetic acid
EGTA..............................................Ethylene glycol-bis(2-aminoethylether)-NNN', N'- tetraacetic acid
ETot................................................Extracellular Solution
FSBA..............................................5’-O-(4-fluorosulfonylbenzoyl)-adenosine
G......................................................Guanine
G-148..............................................Geneticin
HEK293............................................Human embryonic kidney
His tag............................................Hexahistidine tag
HPLC................................................High Performance Liquid Chromatography
MEL Cells........................................Murine Erythroleukemia Cells
NA..................................................Noradrenaline
NTA...............................................Nitrilotriacetic acid
NTA-Ni beads.................................Nitrilotriacetic acid-Nickel beads
P-CMV.............................................Cytomegalovirus promoter
PBS................................................Phosphate buffered saline
PCR................................................Polymerase Chain Reaction
PIC................................................Protease Inhibitor Cocktail
PMF............................................Peptide Mass Fingerprinting
SDS-PAGE... Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
T.................................Thymine
TNP-ATP..........................Trinitrophenyl-ATP
UV light..........................Ultra-violet light
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CHAPTER 1
INTRODUCTION

1.1 Introduction

The physiological effects of adenosine and adenosine nucleotides have been well documented in the last 80 years or so. The role of ATP was initially thought to be as a provider of energy for the many biological functions that take place within the body. However evidence of an extracellular role started to accumulate from the 1950s onwards. This culminated in the seminal paper by Burnstock (1972) in which he introduced the term “purinergic”. Since then an abundance of research has confirmed ATP’s role as not only the critical source of energy for the body, but as a fundamental signalling molecule.

The main focus of this thesis was to determine whether cross-linking ATP analogues have any activity at P2X receptors. The following introductory chapter aims to briefly look at the history of some of the earliest observed extracellular actions of ATP. I will discuss ATP’s structure, synthesis, storage and release. I will then focus in on the P2X receptors, providing evidence for their membrane topology and some of their distinct characteristics. Finally I will examine the different regions of the P2X receptors and their molecular backgrounds which suggest specific roles in the normal function of the receptor. I will then conclude with a more detailed examination of the aims of the project.

1.2 Adenosine 5’-Triphosphate (ATP)

Since the early 20th century, purines and purine nucleotides have been known to elicit a wide range of effects in the body. Drury and Szent-Gyorgyi first reported the actions of adenosine in 1929. It was found that adenosine injected into anaesthetized animals caused a fall in blood pressure, coupled with bradycardia and vasodilation (Drury and Szent-Gyorgyi, 1929). ATP was shown to produce contraction in the guinea pig uterus (Deuticke, 1932) and relaxation in the ileum (Gillespie, 1933). Another example of

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ATP’s extracellular effects was shown by Buchtal and Kahlson (1944) who concluded their study on ATP’s contractile effects on mammalian skeletal muscle by noting “the energy rich phosphate bond in the form of ATP is not only the immediate source of energy recharge but also the agent for the release of contraction.”

1.2.1 Early Observations of ATP’s Role as a Signalling Molecule

The 1950s heralded the first evidence of a possible role for ATP as a neurotransmitter. In particular a series of papers by Holton and Holton provided insight into the possibility of multiple roles for ATP. Investigation of sensory innervation was made by studies on the capillary bed in the rabbit ear. After surgically removing the superior cervical ganglion, electrical pulses were applied to the great auricular nerve. The antidromic nerve stimulation of the great auricular nerve caused the release of a chemical substance and the subsequent vasodilation of the rabbit ear artery.

Investigation into the identity of the substance responsible for the antidromic vasodilation ruled out any part played by histamine or acetylcholine (Holton and Perry, 1951). In 1953 Holton and Holton first suggested that ATP was the transmitter at sensory nerve endings. They found ATP in spinal root extracts and observed the same antidromic vasodilatory effect in rabbit ear vessels after injection of ATP. They also found that this response was similar for ADP but non-existent for AMP or adenosine. It was also noted that stimulation of the great auricular nerve produced an ATP-like substance. This idea of ATP release from sensory nerves was expanded in 1954. Acetone-dried powders prepared from horse dorsal and ventral roots, as well as the caudate nucleus were analysed using different methods. They found high concentrations of ATP, ADP and its breakdown products in the samples (Holton and Holton, 1954). They tentatively suggested that in certain sensory nerves, ATP was released after the arrival of action potentials at the nerve ending.

It was in 1959 that Holton demonstrated the release of ATP from nerve terminals by both mechanical and electrical stimulation (Holton, 1959) and concluded that ATP must be a
synaptic neurotransmitter. Though the evidence was plentiful, the idea of ATP being anything other than a “biological battery” was met with scepticism.

1.2.2 The Purinergic Hypothesis

Burnstock and his colleagues, whilst using the sucrose gap technique to study nerve transmission in the smooth muscle of guinea-pig taenia coli, observed a persistent component of the nerve response that was resistant to both adrenergic and cholinergic antagonism (guanethidine and atropine respectively). They observed large, transient hyperpolarizations which led to relaxation of the smooth muscle. Tetrodotoxin (TTX) is a potent neurotoxin which blocks action potentials in nerves by binding to the pore of voltage-gated sodium channels in nerve cell membranes. It prevents the action potentials in nerves without affecting the excitability of smooth muscle cells. In the presence of TTX, these antagonistic-insensitive responses were abolished, thus discounting the possibility that the responses were due to direct muscle stimulation. The conclusion that these inhibitory junction potentials (IJPs) resulted from stimulation of non-adrenergic, non-cholinergic neurones (NANC) sparked a search for the transmitter likely to be released from these newly discovered nerves. Many substances were explored, but ATP seemed to most satisfy the criteria (see below) generally accepted as necessary for establishing a substance as a neurotransmitter (Burnstock et al, 1963; 1964; 1966; 1970).

Though a lot of evidence had been collated over a long period of time which showed that ATP and its derivatives had many physiological effects, what was needed was a unifying theory that would pull all these pieces of information together. In 1972 Burnstock wrote a seminal review which provided evidence to support the idea of these NANC autonomic nerves which supplied organs such as the gut and bladder, and used the purine nucleotide ATP as their principal neurotransmitter. Since it was based around a purine he coined the term “purinergic” and tentatively suggested a model for storage, release and inactivation of ATP (Burnstock et al, 1970; Burnstock 1972).

Though now accepted, this “purinergic hypothesis” met considerable resistance since it was established that ATP drives unfavourable chemical reactions, fuels biological
machinery (as observed in the sodium-potassium pump) and can regulate various processes via protein phosphorylation. The next section will look at the structure of ATP, its synthesis and study the evidence that ATP is a neurotransmitter.

1.2.3 ATP Structure and Synthesis

ATP is a purine nucleotide comprised of a nitrogenous purine base (adenine), joined to a ribose sugar by a glycosidic bond. Also attached to the ribose sugar is a chain of three ionized phosphate groups (see figure 1.1). ATP is formed primarily through oxidative phosphorylation in the mitochondria, but it can also be formed during the breakdown of glucose to lactic acid (anaerobic glycolysis) and within the citric acid cycle (Hames & Hooper, 2000).

![Figure 1.1. Structure of ATP.](image)

When the third phosphate group of ATP is removed by hydrolysis, a substantial amount of free energy is released. Intracellularly, ATP powers many of the energy-consuming activities of the cell such as active transport, muscle contraction, maintenance of cell volume by osmosis and most anabolic reactions.

Newly synthesised ATP is transported out of the internal membrane of the mitochondrion by an adenine nucleotide transporter that exchanges an ATP molecule for its metabolite ADP. ATP is then released into the cytosol through mitochondrial porins and possibly packaged in vesicles (Bodin & Burnstock, 2001).

Describing ATP as a neurotransmitter is no simple task. There are a large number of endogenous chemicals found in the CNS alone, and only a few serve in the process of neuronal transmission. There a number of criteria that must be fulfilled before a
particular substance can be labelled as a neurotransmitter. The following section will look at each criterion.

1.2.4 Storage
Firstly, the substance must be shown to be present in the presynaptic terminal or stored, ready for release. Though ATP has a ubiquitous systemic presence in all cells; the high ATP content of catecholamine secretory granules was found by analysis of the amine granules isolated following density gradient centrifugation (Hillarp et al, 1955). This high concentration packaged into secretory granules was observed in the chromaffin cells of the bovine adrenal medulla and the co-packaging and co-release of noradrenaline and ATP was reported (Blaschko et al, 1956; Hillarp et al, 1958). ATP storage was also reported in the neuronal cells of the adrenergic nerve (Euler et al, 1963) and in cholinergic synaptic vesicles (Dowdall et al, 1974). Su demonstrated the accumulation and release of $[^3]$H ATP in isolated rabbit aorta, ear artery and portal vein after these excised sections were incubated in Krebs solution containing $[^3]$H adenine derivatives (Su, 1975). Recently Silvia Coco and her colleagues demonstrated the presence of stored ATP secretory granules in cultured astrocyte cells (Coco et al, 2003) by successfully labelling astrocytes with quinacrine fluorescence dye (which stains high levels of ATP bound to peptides in large granular vesicles). In addition they used a Luciferin-luciferase assay to probe the ATP content in subcellular fractions obtained using a sucrose gradient. They discovered the presence of a purine peak which was associated with the protein secretogranin II, a well established marker of dense-core vesicles.

1.2.5 Release
The second criterion is that on nerve stimulation the substance must be released into the synaptic cleft. There is now abundant evidence to suggest that ATP (usually released with other neurotransmitters) is released from neuronal terminals, both centrally and peripherally, thus ATP release can occur between neuron to neuron synapses and neuron to effector synapses.
Centrally, Edwards et al were the first group to show that ATP was synaptically released in the central nervous system by recording evoked P2X currents in the rat medial habenula (Edwards et al, 1992). Peripherally, a role for ATP as an endogenous transmitter was established through the analysis of ATP-mediated fast excitatory postsynaptic currents in cultured coeliac ganglion neurons, which normally supply sympathetic input to various visceral organs (Evans et al, 1992; Silinisky & Gerzanich, 1992).

1.2.5.1 Co-transmission

The original purinergic hypothesis suggested that ATP was released from nerves which were distinct from those that released acetylcholine or noradrenaline (Burnstock, 1972). However, it is now known that ATP is co-released with other neurotransmitters both centrally (NA, 5-HT, glutamate, dopamine and GABA) and peripherally (ACh and NA) (reviewed by Burnstock, 2004). An example of peripheral co-transmission in rabbit isolated vas deferens is shown below (figure 1.2).

One of the earliest deduced examples of co-transmission is the biphasic contraction observed in the vas deferens (Fedan et al, 1981; see figure 1.2). Neurogenic release of ATP (tetrodotoxin sensitive) caused an initial, fast; transient contraction followed by a longer, slower, tonic component caused by noradrenaline. Blockade of the initial response was brought about by using the purine receptor antagonist and photoaffinity label, arylazido aminopropionyl ATP (ANAPP3). The secondary tonic component was antagonized by the alpha-adrenoceptor antagonist prazosin. Reserpine, which causes depletion of noradrenaline also inhibited the secondary component. Similar studies also showed evidence of co-transmission (Sneddon & Westfall, 1984; Von Kugelgen, 1989; Banks et al, 2006).

The exocytosis of ATP, after vesicle fusion with the plasma membrane, is considered to be the major release mechanism and has been shown to be present in neurons, neuroendocrine cells and mast cells (Burnstock, 1972). However, the mechanism by which vesicles become loaded with ATP is still not understood.
Figure 1.2. Co-transmission in the vas deferens. A. Diagram taken from Sneddon et al (1996). Trains of pulses at 16Hz for 30 seconds produced a biphasic contraction in the rabbit isolated vas deferens (left trace). The biphasic contraction was composed of an initial fast transient contraction (ATP) followed by a slower tonic contraction (noradrenaline). The initial component was selectively reduced by the P2 antagonist ANAPP3. The secondary component was selectively removed by the $\alpha_1$-adrenoceptor antagonist prazosin (right trace). B. Diagram pictorially shows the co-release of ATP and noradrenaline from the same nerve terminal and the resulting synergistic response.
1.2.5.2 Other Routes of ATP Release

In addition to vesicular exocytosis, the release of the large intracellular ATP stores after cell damage or death can lead to a number of pathological states. ATP can be released from endothelial cells, red blood cells and platelets and can induce the release of prostanoids and inflammatory cytokines (Bodin & Burnstock, 1998). Damaged cells can release ATP to induce currents in the peripheral sensory nerves which can be interpreted centrally as pain (see section 1.5.3).

There is also evidence to suggest ATP release from distended epithelia (mechanical stress); in response to hypoxia, inflammation and agonist application (Vlaskovska et al, 2001; Bodin & Burnstock, 2001; Burnstock, 2006). Other routes of ATP release from cells have been postulated, these include – ATP permeable release channels, adenine nucleotide transporters, adenine nucleotide/nucleoside exchangers as well as ATP-filled vesicles (reviewed in Bodin & Burnstock, 2001; Schwiebert & Zsembery, 2003).

1.2.6 Identity of Action

Thirdly, on release of the neurotransmitter from the presynaptic membrane, the binding of the neurotransmitter to its receptor should elicit a specific response in the postsynaptic membrane. In addition, the exogenous application of the transmitter substance must mimic the endogenous response.

The observed actions of ATP imply the presence of purinoceptors. These receptors are of two types – the P2X which are the fast acting ligand-gated receptors and the P2Y which are slower acting G-protein linked metabotropic receptors (Burnstock & Kennedy, 1985; North, 2002). This is discussed in more detail in section 1.3.

1.2.7 Inactivation

Finally, a mechanism must exist for the rapid inactivation or removal of the active substance. After release, ATP is rapidly degraded by a family of ectonucleotidases which work to limit the actions of ATP. Ecto-ATPases hydrolyze ATP to ADP, ectoapyrases
metabolise both ATP and ADP and convert them to AMP, ecto-5'-nucleotidase converts AMP to adenosine (Zimmermann, 2001; see figure 1.3).

**Figure 1.3.** All metabolically active cells use ATP for energy, via breakdown to ADP, AMP and adenosine. ATP and AMP that escape the cell are broken down to adenosine by ecto-nucleotidases. These enzymes are present in the cytosol and at the surface membrane. Therefore adenosine can be formed both intracellularly and extracellularly by the breakdown of AMP. ATP is replenished in cells by the uptake of adenosine and phosphorylation - this process requires oxygen (Shyrock and Belardinelli, 1999). Diagram taken from Polosa, 2002.

The ubiquitous nature of ATP's signalling role may hark back to a theorised primitive function, when not only was ATP used as a source of energy, but ATP and its derivatives may have been primordial transmitter substances (see review by Burnstock, 1996). Whether this theory can be proven or not, the idea of ATP as a signalling molecule has now been accepted after first being put forward over thirty years ago. These potent extracellular actions are mediated by specific purinergic receptors termed P2. The focus of the next section will be a brief history on the sub-divisions within the purinergic hierarchy with emphasis on P2X receptors.

1.3. P2X Receptors

1.3.1 *A brief historical discussion of P2 receptors*

The existence of purinergic receptors was implicit in the development of the purinergic hypothesis; however the differences in agonist and antagonist potencies in different
tissues, coupled with the differences in transduction mechanisms led to a division into two distinct classes of purinoceptor, the P1 receptor which is activated by adenosine and the P2 receptor activated by ATP and ADP (Burnstock, 1978). This division was still not adequate to quell the pharmacological differences observed in native tissue. Consequently Burnstock and Kennedy made a further sub-division of the P2 receptors to P2X and P2Y in 1985 (Burnstock and Kennedy, 1985; see figure 1.4). This was based on the differing potencies of various ATP analogues. They suggested preferential activation by the ATP analogues alpha, beta methyleneATP (αβMe-ATP) and beta, gamma-methyleneATP (βγMe-ATP) indicated the P2X receptor and differentiated them from the preferential activation of P2Y receptors by 2-methylthioATP. The eventual cloning of the P2 receptors allowed the division to be based on structural information. The ligand-gated ion channels were grouped into P2X receptors and the G-protein coupled receptors were P2Y receptors. The newly cloned P2X receptor sub-types were numbered sequentially as proposed by Abbracchio and Burnstock (1994). To this date seven distinct P2X receptors (P2X₁ through to P2X₇) have been identified.

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**Figure 1.4. Diagram of purinoceptor classification.** Initial differences in pharmacology led to a division between receptors activated by adenosine (P1) and receptors predominantly activated by ATP and ADP (P2). The P2 receptors were further sub-divided based on their distinctive pharmacological differences. Molecular cloning has allowed the division to be based on structure. The P2X receptor is an ATP-gated ion channel, whilst the P2Y receptors are G-protein coupled.
1.3.2 Molecular Cloning of the P2X Receptors

The cloning of the P2X receptor subunits has allowed researchers the ability to examine receptors in a number of different expression systems, which in turn has allowed the pharmacology and characteristics of each individual isoform to be deduced.

The first cDNAs encoding P2X receptor subunits were isolated in 1994 (Brake et al, 1994; Valera et al, 1994). The expression cloning from RNA extracted from rat vas deferens and urinary bladder was conducted by Valera and colleagues, whilst Brake and his colleagues performed expression cloning from RNA extracted from rat pheochromocytoma PC12 cells. Fast excitatory responses to ATP had been extensively characterized in each tissue type and both groups used their respective RNA samples to generate a complementary DNA expression library. From this they were able to screen injected oocytes for the production of characteristic ATP-evoked inward currents. Using this technique, both groups isolated a single clone encoding the P2X subtype from each tissue type. In both cases the current responses observed from *Xenopus* oocytes, injected with cRNA transcripts derived from each clone, mimicked those observed in their native tissue. Valera *et al* (1994) observed that the receptor isolated from vas deferens was sensitive to αβMe-ATP and produced quickly desensitizing currents after agonist application, whereas Brake *et al* (1994) recorded αβMe-ATP insensitive currents which underwent little to no desensitization upon agonist application. The discovery of these two subunits of the P2X receptor, later named P2X₁ (vas deferens) and P2X₂ (PC12 cells), allowed the first glimpses into the structure of the P2X receptor. Unlike other ligand-gated ion channels such as members of the cys-loop or glutamatergic families (which have four and three transmembrane domains respectively), the P2X receptor is a two transmembrane domain channel, with intracellular amino and carboxy termini. In addition, a large proportion of the channel exists extracellularly. This extracellular domain which contains 10 conserved cysteine residues, as well as consensus sites for glycosylation, also contains the ATP binding site.

Following the discovery of the first two P2X receptor subunits, other subunits were isolated in a short period of time. The P2X₃ receptor was isolated from dorsal root
ganglion (Chen et al, 1995), the P2X4 receptor was cloned from rat brain (Bo et al, 1995), the P2X5 receptor was cloned from rat coeliac ganglion and the P2X6 receptor was cloned from a superior cervical ganglion cDNA library (Collo et al, 1996). The P2X7 receptor was also isolated from rat brain (Suprenant et al, 1996). The P2X subunit proteins are 384 (P2X4) to 595 (P2X7) amino acids long and all seven receptors share 35-48% sequence homology. The first transmembrane domain begins about 30 amino acid residues from the amino terminus and the second near residue 320. The sequence between these domains (about 270 amino acids) contains 67 amino acids that are completely conserved throughout all seven receptors. The most abundant conserved amino acid residues are glycine (14 residues), cysteine (10 residues) and lysine (6 residues). There are two to six possible sites of N-linked glycosylation and the carboxy terminal domain ranges from 25-240 amino acids in length (North, 1996; North, 2002). A simple diagram showing the P2X receptor membrane topology is shown in figure 1.7.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Tissue first cloned from</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2X1</td>
<td>Vas deferens</td>
<td>Valera et al (1994)</td>
</tr>
<tr>
<td>P2X2</td>
<td>Pheochromocytoma cells (PC12)</td>
<td>Brake et al (1994)</td>
</tr>
<tr>
<td>P2X4</td>
<td>Brain (hippocampus)</td>
<td>Bo et al (1995)</td>
</tr>
<tr>
<td>P2X7</td>
<td>Superior cervical ganglion/brain (Medial habenula)</td>
<td>Suprenant et al (1996)</td>
</tr>
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</table>

**Figure 1.5.** Tissues from which P2X receptor subunits were first cloned

The summary table in figure 1.6 shows each receptor’s principle agonists and antagonists as well as some of the reported areas of distribution within the body.
<table>
<thead>
<tr>
<th>Receptor subtypes</th>
<th>Agonists</th>
<th>Antagonists</th>
<th>Principal tissue</th>
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<td>P2X&lt;sub&gt;1&lt;/sub&gt;</td>
<td>αβMe-ATP&lt;br&gt;ATP</td>
<td>PPADS&lt;br&gt;Suramin&lt;br&gt;TNP-ATP</td>
<td>Smooth muscle&lt;br&gt;Sensory ganglia&lt;br&gt;Cerebellum (Postsynaptic)&lt;br&gt;Heart; Platelets</td>
<td>Vulchanova et al, 1996; Collo et al, 1996; Vial et al, 1997;</td>
</tr>
<tr>
<td>P2X&lt;sub&gt;2&lt;/sub&gt;</td>
<td>ATP &gt; ATP-γ-S</td>
<td>PPADS&lt;br&gt;Suramin</td>
<td>Autonomic ganglia&lt;br&gt;Brain; Pituitary&lt;br&gt;Sensory ganglia&lt;br&gt;Smooth muscle&lt;br&gt;Retina</td>
<td>Vulchanova et al, 1996; Collo et al, 1996; Rubio &amp; Soto, 2001</td>
</tr>
<tr>
<td>P2X&lt;sub&gt;3&lt;/sub&gt;</td>
<td>2-MeSATP &gt; ATP</td>
<td>TNP-ATP&lt;br&gt;PPADS&lt;br&gt;αβMe-ATP (desensitizes) Suramin</td>
<td>Sensory ganglia&lt;br&gt;Solitary tract neurons&lt;br&gt;Sympathetic neurons</td>
<td>Collo et al, 1996; Dunn et al, 2001; Bradbury et al, 1998</td>
</tr>
<tr>
<td>P2X&lt;sub&gt;4&lt;/sub&gt;</td>
<td>ATP &gt; αβMe-ATP</td>
<td>*</td>
<td>Brain; Pancreas&lt;br&gt;Testes; Colon&lt;br&gt;Smooth muscle</td>
<td>Rubio &amp; Soto, 2001; Collo et al, 1996;</td>
</tr>
<tr>
<td>P2X&lt;sub&gt;5&lt;/sub&gt;</td>
<td>ATP &gt; αβMe-ATP</td>
<td>PPADS&lt;br&gt;Suramin</td>
<td>Heart; Gut&lt;br&gt;Bladder&lt;br&gt;Autonomic ganglia&lt;br&gt;Skin (Proliferating cells)&lt;br&gt;Thymus; Spinal cord</td>
<td>Bo et al, 1995; Collo et al, 1996</td>
</tr>
<tr>
<td>P2X&lt;sub&gt;6&lt;/sub&gt;</td>
<td>αβMe-ATP</td>
<td>---------</td>
<td>Skeletal muscle&lt;br&gt;Brain&lt;br&gt;Spinal motor neurons&lt;br&gt;Autonomic ganglia</td>
<td>Rubio &amp; Soto, 2001; Collo et al, 1996;</td>
</tr>
<tr>
<td>P2X&lt;sub&gt;7&lt;/sub&gt;</td>
<td>Bz-ATP &gt; ATP</td>
<td>KN62; KN04 Coomassie brilliant blue</td>
<td>Apoptotic cells&lt;br&gt;Macrophages&lt;br&gt;Lymphocytes&lt;br&gt;Microglia&lt;br&gt;Skin</td>
<td>Suprenant et al, 1996; Rassendren et al, 1997b;</td>
</tr>
</tbody>
</table>

PPADS: Pyridoxal-5-phosphate-6-azophenyl-2′,4′-disulfonic acid

(*) Dependent on species

**Figure 1.6.** The widespread expression of P2X receptors helps to indicate the enormous role extracellular ATP has in normal systemic function. Data taken from Burnstock, 2006.
1.3.3 Membrane Topology of the P2X Receptor

The P2X receptor membrane topology features two transmembrane-spanning segments, an extracellular loop (which makes up two thirds of the receptor’s mass) and amino and carboxy termini which exist intracellularly. Initial evidence for this structure came from hydrophobicity plots and analysis of the primary amino acid sequence. These plots suggested that there are two hydrophobic regions sufficiently long enough to span the plasma membrane (Valera et al, 1994; Brake et al, 1994). In addition, the absence of a leader signal peptide sequence suggests that the amino terminus is intracellular (Valera et al, 1994; Brake et al, 1994).

Vulchanova et al (1997) showed that positive detection of the carboxy termini by immunohistochemistry was only possible when the cell membranes were permeabilized. This suggested that the carboxy terminal existed intracellularly (Vulchanova et al, 1997). Torres et al (1998) theorized that if both the amino and carboxy termini existed on the same side of the lipid membrane, that two subunits placed in tandem as a single polypeptide should be able to fold correctly to create a functional receptor. They generated a tandem cDNA which resulted in a protein containing a P2X2 subunit linked
to a P2X3 subunit. Similar to the responses produced by the heteromeric P2X2/3 receptor, the resultant functional channel exhibited a non-desensitizing current with sensitivity to αβMe-ATP. The success of this construct indicated that the second (P2X3) subunit's N-terminus was on the same side of the membrane as the C-terminus of the first subunit (P2X2). Additional investigation showed that P2X2 receptors, tagged with the flag epitope at either the amino or carboxy termini, only gave detectable immunofluorescence when the transfected cells were permeabilized. This suggests that both structures exist on the intracellular side (Torres et al, 1998).

N-glycosylation is the process by which sugars are added to the protein at asparagine (N) residues in the consensus sequence of N-X-S/T. Glycosylation occurs during protein biosynthesis inside the endoplasmic reticulum and only occurs on domains that are destined to be extracellular. Valera et al’s study in 1994 showed that the higher molecular weight of the P2X receptor might reflect glycosylation. Torres and colleagues used N-glycosylation scanning mutagenesis to provide further evidence of the membrane topology. Analysis of the P2X2 primary sequence indicated three potential N-linked glycosylation sites at positions 182, 239, and 299. They produced mutant receptors which removed each of the sites individually and one construct that removed all of the sites. Western blot analysis (using a flag antibody) of cells expressing either variant of the P2X2 receptor showed a shift in the band patterns. Removal of one of the glycosylation sites did not affect the functionality of the resultant receptor, but it did cause a shift in the band size from 70kDa to 60kDa. When all three sites were removed the band shifted to 55kDa, which was of a similar size to the endoglycosidase H-treated P2X2 receptors. These results agreed with Valera et al’s original assertion of P2X receptor glycosylation and demonstrated that these residues are extracellular on functional proteins (Torres et al, 1998).

In addition, Torres et al also introduced two glycosylation consensus sites at the amino-terminus (preceding TM1, positions 9 and 22), the central loop (positions 206 and 300) and the carboxy-terminal domain (following TM2, positions 381 and 434). Results showed that the central loop mutant was successfully glycosylated, again proving that...
this domain exists extracellularly. However no glycosylation was observed in either the amino or carboxy termini mutants (the gel shift mirrored that of the mutant which had all glycosylation sites removed). These results suggested that these sites were not available for glycosylation and therefore reside intracellularly (Torres et al, 1998). An extensive study in P2X2 receptors by Newbolt et al (1998) showed that functional channels were formed by receptors which lacked a single glycosylation site, but not by channels mutated in two or three glycosylation positions. Further experiments using N-glycosylation scanning mutagenesis localized to different areas of the receptor allowed Newbolt and her colleagues to define the outer membrane boundary of both transmembrane domains (Newbolt et al, 1998).

These experiments, as well as others, such as the transfer of the extracellular loop from the P2X1 receptor to the P2X2 receptor conferring αβMe-ATP sensitivity; (Werner et al, 1996) and the introduction of point mutations to the extracellular loop affecting agonist and antagonist potency (Buell et al, 1996; Ennion et al, 2000) helped to confirm the general topology seen in figure 1.7. This also verifies the novel structure of the P2X receptor when contrasted with the topology of the nicotinic (four membrane-spanning segments) and glutamate (three membrane-spanning segments) superfamilies.

1.3.4 A Multimeric Channel

The cloning, successful expression and the production of functional receptors, which mimicked the characteristics observed in their native tissue, suggested that P2X receptors can exist as homomeric structures. However there were some observed differences between responses from specific P2X subtypes purported to reside in certain tissue and responses observed from the native tissue itself. Lewis and colleagues found that although they cloned the P2X3 receptor from rat dorsal root ganglia, electrophysiological investigation showed P2X3 receptor responses which were different from those recorded in native sensory neurons. It was only when both the P2X2 and P2X3 receptor was co-expressed in HEK293 cells that the responses mimicked those observed in the native tissue. The results suggested that both subunits co-assembled to form a slow desensitizing, αβMe-ATP sensitive channel. The characterisation of this channel which
encompasses features associated with the individual subtypes implies that functional receptors must be multimeric. Additionally, the co-infection of C-terminal tagged P2X$_2$ and P2X$_3$ (cloned into the baculovirus) cDNAs into insect cells were co-immunoprecipitated with antibodies specific for either tag. Similarly, to Lewis et al's study, Radford et al (1997) found that co-assembly of the P2X$_2$ and P2X$_3$ subunits led to a functional receptor with a phenotype unlike either of its constituent subunits. Again this provided evidence that functional channels are generated by the assembly of a number of subunits. Currently evidence indicates that a functional P2X receptor is composed of three subunits to form a trimeric structure.

1.3.5 The Functional Receptor is a Trimeric Complex

Evidence suggests that functional P2X receptors are formed through the assembly of three subunits or multiples of three. Concentration-response analysis has shown that the P2X receptor has a Hill coefficient greater than one; thereby suggesting that more than one ATP molecule might be required for activation (Brake et al, 1994; Ding and Sachs, 1999). Whilst studying current responses from rat and bullfrog dorsal root ganglia neurons, Bean (1990) found that at low concentrations of ATP (0.3-1μM), the concentration-response relationship was much steeper than that predicted for a 1:1 binding correlation; where one molecule of ATP would activate the receptor. He found that the binding relationship was much better fitted by a model in which three ATP molecules are required to bind to three independent, non-interacting binding sites for the channel to open (Bean, 1990). Nicke et al (1998) investigated the quaternary structure of the P2X receptor by using two distinct methods – chemical cross-linking and blue native Polyacrylamide Gel Electrophoresis (blue native PAGE). His tagged P2X$_1$ (and P2X$_3$) receptors were heterologously expressed in *Xenopus* oocytes and the oocytes were labelled with $^{35}$S methionine. Purified P2X$_1$ protein samples were chemically cross-linked to bi-functional PPADS analogues and resolved by SDS PAGE. Autoradiography revealed that cross-linking of either receptor generated dimers and trimers; the relative amount of either being determined by the distance between the two reactive groups on each respective cross-linking compound. The second method made use of the separation method, blue native PAGE. Results showed that non-reduced His-P2X$_1$ samples
appeared as a single protein band at a mass of about 200kDa – this corresponds with a P2X₁ trimer. These results were the same when only the membrane surface receptors were used as the sample. Homotrimeric architecture was also found to be the constituent form of P2X₃ receptors expressed in *Xenopus* oocytes (Nicke et al., 1998). Aschrafi et al used similar methods to show that P2X₂, P2X₄ and P2X₅ receptors are also trimers of identical subunits. In addition, they also showed that heteromeric P2X₁/₂ receptors also form trimeric structures.

The characterization of single channel currents from the P2X₂ receptor (expressed in *Xenopus* Oocytes) also suggests a model of receptor activation based on the binding of three ATP molecules (Ding and Sachs, 1999). Again this suggests the presence of three ATP binding sites contributed by three subunits.

Definitive evidence was recently reported by Barrera et al (2004). They used atomic force microscopy to visualize purified P2X₂ receptors and provide more evidence consistent with a trimeric structure. (Barrera et al, 2004). Crude cell extracts of normal rat kidney cells, transiently transfected with P2X₂ receptors, separated on SDS-polyacrylamide gels showed receptor migration as a single band of 70kDa. However, when the crude cell extracts were incubated with the cross-linking compound disuccinimidy suberate (DSS), new bands at 140kDa (dimer) and 210kDa (trimer) were observed. P2X₂ receptors with N-terminal his₆ tags on each subunit, were incubated with anti-his antibodies and subsequently formed complexes with none, one, two or three antibodies. Receptors with two bound antibodies were found to have a bond angle between subunits of ~ 120° (123°), again consistent with a trimeric arrangement (figure 1.8).
Figure 1.8. Trimeric Model of the P2X$_2$ Receptor. Incubation of the N-terminal his$_6$ tagged P2X$_2$ subunits with anti-his$_6$ antibodies, led to the binding of antibodies to the receptor. Complexes which formed with two antibodies were found to have a bond angle of about 120°. This is consistent with an assembly of three subunits forming a functional channel.

1.3.6 Heteromeric Receptors

The assembly of identical subunits into a multimeric structure results in the formation of a homomeric receptor with defined characteristics based on the particular subtype. The formation of a functional receptor from non-identical subunits can result in a receptor with an amalgamation of characteristics and a novel phenotype (Brown et al, 2002; Torres et al, 1998b; Lewis et al, 1995; Le et al, 1998). Work conducted by Torres et al (1999) has shown which subunits have the ability to co-assemble not only with themselves, but also with other subunits. The seven P2X receptor subunits were differentially epitope-tagged (FLAG or HA) and expressed in various combinations in HEK 293 cells. Using an antibody specific to one of the transfected receptors they carried out an immunoprecipitation and then analyzed the sample with western blot to determine whether the appropriate receptor was co-immunoprecipitated with the receptor with the
opposing tag. Co-immunoprecipitation was indicative of successful co-assembly of differing subunits. This systematic examination of all the seven members of P2X allowed the table below (figure 1.9) to be constructed (Torres et al, 1999).

<table>
<thead>
<tr>
<th></th>
<th>P2X₁</th>
<th>P2X₂</th>
<th>P2X₃</th>
<th>P2X₄</th>
<th>P2X₅</th>
<th>P2X₆</th>
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<td>P2X₁</td>
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<td>P2X₂</td>
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<td>P2X₃</td>
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**Figure 1.9. The co-assembly of P2X receptor subunits.** (+) Denotes positive interactions between specified subunits. (-) Denotes unsuccessful interactions.

Results showed that all subunits were able to co-assemble with others to form heteromeric structures, with the exception of P2X₇. Apart from the P2X₆ subunit, all subunits have the ability to form functional homomeric channels. They have not been shown to form in transiently transfected HEK293 or COS cells nor in *Xenopus* oocytes (North, 2002). Aschrafi *et al* (2004) showed that P2X₆ subunits could not form homomeric structures, but instead, congregated in the endoplasmic reticulum and results from atomic force microscopy also show a failure of the P2X₆ subunits to form a stable homomeric receptor (Barrera *et al*, 2005). However Jones *et al* (2004) showed the functional expression of a P2X₆ receptor with a slowly desensitizing, αβMe-ATP sensitivity. Cell lines stably expressing the receptor were generated and (one successful clone from 200 attempts) they subsequently found that the receptor was only functional when the subunits were fully glycosylated.

The cloning and pharmacological characterization of the various P2X subunit combinations has allowed the identification of the P2X receptors responsible for the
observed responses in native tissue. However responses from native tissue which do not conform to those described characteristics have been reported (Lewis et al, 1995; Stoop et al, 1997; Le et al, 1998; King et al, 2000). The confirmation of these novel phenotypes resulting from the association of different P2X subunits may allow the identification of P2X receptor expression in all tissue.

### 1.3.7 Possible Subunit Stoichiometry in the P2X\textsubscript{2/3} Receptor

Jiang et al (2003) looked at subunit arrangement in P2X receptors. They found inter-subunit disulfide bond formation between engineered cysteine residues. They were able to demonstrate the close proximity between the outer ends of the first transmembrane domain of one subunit and the second transmembrane domain of another. This made it possible to understand the relative arrangement of the transmembranes within a subunit and the quaternary organization of subunits within a receptor. Results allowed them to propose a "head-to-tail" subunit arrangement in the quaternary structure of P2X receptors and show that a trimeric P2X\textsubscript{2/3} receptor would have the composition P2X\textsubscript{2} (P2X\textsubscript{3}) \textsubscript{2}. They also suggest that the three subunits are arranged around a central pore with the movement of one P2X subunit relative to another being a key part of gating in both homo- and heteromeric channels (see figure 1.10).

![Figure 1.10. Proposed “head-to-tail” arrangement of the transmembranes around a central pore.](image)
1.3.8 P2X₁ Receptor

The molecular background of the P2X receptors is reflected in their systemic distribution and function. The main focus of this thesis is the P2X₁ receptor and its various roles will be discussed further.

The P2X₁ receptor was first cloned from rat vas deferens and urinary bladder in 1994 (Valera et al., 1994) and the sequence of the 399-amino acid human protein is 89% identical to that of the rat and mouse homologues. The receptor has most of its distribution within the muscle lining of a variety of hollow organs, where they regulate smooth muscle contractility (Vial et al., 2000; Mulryan et al., 2000). These organs include the urinary bladder, arteries and vas deferens (Valera et al., 1994; Collo et al., 1996; Vulchanova et al., 1996; Lewis & Evans, 1995; Mulryan et al., 2000; Vial et al., 2000). They are also found to be well expressed in platelets (Vial et al., 1997) where they serve an important role in platelet physiology and haemostasis (Vial et al., 2002). Knock-out studies can be used to further clarify the roles that the P2X₁ receptor has in normal function.

1.3.9 Knock-out Studies

Mulryan et al. (2000) found that the creation of mice with targeted P2X₁ receptor gene deletion caused a curious phenotype - male infertility. It was found that male fertility was reduced by approximately 90%, while female and heterozygous mice were unaffected. This reduction was not due to sperm dysfunction but a reduction in the amount of sperm in the ejaculate. It appears that a substantial component of the contractile response of the vas deferens to sympathetic nerve stimulation, which propels sperm into the ejaculate, is mediated through the P2X₁ receptor (Burnstock & Holman, 1961; Fedan et al., 1981). In P2X₁ receptor-deficient mice, contraction of the vas deferens to sympathetic nerve stimulation was reduced by up to 60%, and responses to P2X receptor agonists were abolished. Therefore it can be concluded that P2X₁ receptors are essential for normal male reproductive function.
Further work with knock out models has also shown roles in blood clotting, with the P2X\textsubscript{1} receptor playing a priming role in the subsequent activation of the P2Y\textsubscript{1} receptor (Vial \textit{et al}, 2002). Vial \textit{et al} studied the role of P2X\textsubscript{1} receptors in megakaryocytes and platelets by comparing the properties of megakaryocytes from normal and P2X\textsubscript{1} receptor knock out mice. Megakaryocytes are bone marrow cells which are responsible for the production of blood platelets. Their normal distribution and numbers in knock-out mice suggested that P2X\textsubscript{1} receptors are not necessary for normal development; however, electrophysiological and intracellular calcium recordings show a significant reduction (~50\%) in the current associated with P2Y activation. The reduction in the P2Y current implies a synergistic relationship occurs between the receptors.

Additionally, knock-out mice have been used to confirm the presence and mediation of contractile response in arterial and urinary bladder smooth muscle (Vial \textit{et al}, 2002; Vial \textit{et al}, 2000\textsuperscript{b}).

\textit{1.3.10 Therapeutic possibilities}

Knock-out studies have shown that P2X receptor activation can be the underlying factor in many processes. Since these receptors can be subjected to pharmacological intervention, they can be viable drug targets in various maladies.

\textit{1.3.10.1 Blood Haemostasis}

The complex interaction between numerous factors within the haemostatic system helps preserve intravascular integrity by achieving a balance between haemorrhage and thrombosis. A thrombus or blood clot is the final product of blood coagulation brought about by the aggregation of platelets. The formed thrombus can be physiologic in cases of injury, but pathologic during thrombosis. During thrombosis, the formation of a blood clot inside a blood vessel can lead to partial or full occlusion thereby obstructing normal blood flow and leading to various pathological states such as deep vein thrombosis, stroke and myocardial infarction. When a blood vessel is wounded there are several steps which help the cessation of blood flow:-
Initially, platelets bind to collagen in the exposed walls of the blood vessel (subendothelial matrix) thereby forming a haemostatic plug.

Coagulation then occurs. This involves a complex cascade of events which ultimately concludes with the transformation of fibrinogen into polymerized fibrin and the creation of a blood clot.

The clot attracts and stimulates the growth of fibroblasts and smooth muscle cells within the vessel wall, and begins the repair process which ultimately results in clot lysis.

ATP and ADP play a crucial role in this process. Large amounts of ATP and ADP are stored in platelet dense granules and are released when platelets adhere to the exposed walls of the blood vessel and become activated. The P2 receptors, P2X₁, P2Y₁ and P2Y₁₂ have been shown to have crucial roles in transducing the increase in ATP and ADP concentration into platelet shape change, aggregation and thrombus growth (Gachet, 2006; Hechler et al, 2003).

The P2X₁ receptor is well expressed in blood platelets. Though its fundamental role has been difficult to ascertain (possibly due to rapid desensitization), and was once denied (Takano et al, 1999), it is now known that it is responsible for rapid calcium influx which leads to transient shape change in response to ATP. The P2X₁ receptor also contributes to platelet activation induced by low concentrations of collagen, and though unable to trigger platelet aggregation by itself, it has been shown to participate in collagen- and shear-induced aggregation (Hechler et al, 2003). Using P2X₁ knock-out mice, Hechler and her colleagues observed decreased platelet aggregation and a decrease in thrombus growth at high shear rates in knock-out P2X₁ mice.

These P2 receptors are therefore potential targets for the development of anti-thrombotic compounds. Currently, the most widely used anti-platelet agents are the COX-inhibitor aspirin, blockers of fibrinogen binding and clopidogrel – the selective P2Y₁₂ inhibitor. Atherosclerosis and accompanying thrombosis is one of the leading causes of mortality.
and morbidity in western countries. Consequently, a specific P2X₁ antagonist could play an important role in various vascular maladies.

1.3.10.2 Hypertension

Artery smooth muscle has been shown to be sensitive to the ATP analogues α, β methylene and L- β, γ methylene ATP. These responses have also been found to quickly desensitize during agonist application. These characteristics are indicative of expression of P2X₁ receptor subunits. Since these P2X-mediated responses are found to cease in P2X₁-receptor deficient mice, this suggests that the P2X₁ receptor is the dominant isoform in some types of arterial smooth muscle (Vial and Evans, 2002; Inscho et al, 2003).

The presence of the P2X₁ receptor in the smooth muscle walls of small diameter arteries and sub-mucosal resistance arterioles accounts for a significant component of vasoconstriction. Since sympathetic nerves release ATP and noradrenaline, thus mediating vasoconstriction through activation of P2X₁ and α₁-adrenoceptors, this provides an extra therapeutic target for the regulation of vascular tone that is resistant to α₁-adrenoceptor and calcium channel antagonists. This novel drug target may have applications in the treatment of hypertension and heart disease.

1.3.11 Further P2X Receptor Drug Targets

One of the primary observations of ATP’s extracellular role was its increased concentration after cell trauma. Bleehen and Keele (1977) found that the application of ATP to a blister base in man induced pain. It has now been found that ATP release from damaged cells activates P2X receptors on primary afferent nerves, which in turn induce action potentials that are interpreted centrally as pain.

Studies, including those with P2X₃ knock-out mice have shown that the receptors are most likely P2X₃ or P2X₂/₃. Consequently, the use of targeted P2X₃ or P2X₂/₃ antagonists should provide a novel method of pain relief from chronic inflammatory pain and some features of neuropathic pain (North, 2003). McGaraughty et al (2003) have observed...
good anti-nociceptive results in rat models of chronic inflammatory and neuropathic pain. Using a novel antagonist (A-317491) highly selective for blocking P2X$_3$ homomeric and P2X$_{2/3}$ heteromeric channels they found intrathecal administration to be more effective than intraplantar (sole of foot/paw) administration.

The role of P2X$_4$ in neuropathic pain has also been extensively reviewed (Inoue et al, 2005). Neuropathic pain, which can develop after nerve damage, bone compression, diabetes or infection, can lead to a hypersensitive pain sensation. Neuropathic pain can be so severe that innocuous events like touch can be intensely painful and anti-nociceptive drugs such as morphine do not exhibit adequate therapeutic effects. The P2X$_4$ receptor, the subtype most commonly found in the brain, has been revealed to have a potentially substantial role in the appearance of neuropathic pain. The subtype is expressed in spinal microglia, which, as shown in animal models (Coyne, 1998), proliferates within the spinal dorsal horn after nerve damage. The change in morphology and increase in number does not seem to fully abate even after the injury has healed. It is proposed that the activation of P2X$_4$ receptors causes an increase in intracellular calcium which in turn leads to the activation of mitogen activated protein kinase. This causes the release of cytokines and chemokines which may interact with the excitatory and inhibitory synapses of neighbouring dorsal horn neurons leading to hypersensitivity and neuropathic pain. The development of new therapeutic agents based on this knowledge would fundamentally change pain management (Inoue et al, 2005).

Additionally, knock-out mice have been generated to show the role of the P2X$_7$ receptor in the neuropathic and inflammatory pain state. The P2X$_7$ receptor is predominantly expressed on immune cells such as macrophages and astrocytes (Suprenant et al, 1996). Its activation can trigger a series of cellular responses such as membrane permeabilization, cytokine release, cell proliferation and/or apoptosis (North 2002) Chessell et al (2005) have demonstrated that the inflammatory and neuropathic hypersensitivity to both mechanical and thermal stimuli was completely absent in P2X$_7$ knockout mice whilst normal nociceptive processing was preserved. They have also
reported the up-regulation of the P2X<sub>7</sub> receptor in human dorsal root ganglia and injured nerves obtained from chronic neuropathic pain patients (Chessell et al, 2005).

P2X<sub>3</sub> null mice have been found to have a very interesting phenotype in addition to the observed reduction in pain response. It has been found that the bladders of these knockout mice can fill up to almost twice their normal capacity before voiding (Cockayne et al, 2000). Subsequent studies have shown that the mice exhibit bladder hypo-reflexia due to the reduced pelvic afferent response to bladder distension. Hence this leads to a reduction of voiding frequency and an increase in bladder capacity (Cockayne et al, 2000; Vlaskovska et al, 2001). It was previously unknown whether this characteristic was due to the loss of P2X<sub>3</sub> homomers or heteromeric receptors containing the P2X<sub>3</sub> subunit in bladder afferents. Zhong et al (2003) have however shown, through the use of immunohistochemistry and patch clamping, that the predominant receptor is the P2X<sub>2/3</sub> heteromeric receptor. Specific P2X<sub>3</sub> and P2X<sub>2/3</sub> antagonists would therefore be very useful in treating bladder instability disorders such as incontinence.

In the next section I will examine the different regions of the P2X receptor and discuss some of the roles that have been determined through various biochemical techniques and extensive mutational analysis.

1.4 Roles of the Different Regions of the PX Receptor

The molecular basis of the properties of P2X receptors are determined by the amino acid composition. Their contribution to the function of the receptor can be exploited through the use of site-directed mutagenesis. The mutagenesis of key conserved amino acid residues can produce changes in agonist potency, time course of the response, amplitude of the response, the rate of desensitization and can affect the ability of the receptor to function. Many of the regions critical to the activity of the channel have been probed and subsequent changes in normal receptor function may imply specific roles to these regions. Evidence for a few of these proposed roles are discussed in this section.
1.4.1 Trans-membrane Spanning Segments

1.4.1.1 Lining the Pore
In a study using the substituted cysteine accessibility method (SCAM) Rassendren et al (1997) tried to identify the parts of the structure that form the ionic pore of the P2X2 receptor. This method involved replacing individual amino acids with cysteine residues. Any exposed side-chains of these newly inserted residues were then probed with water-soluble sulfhydryl-reactive agents. The method assumes that if the side-chain of the inserted cysteine residue is exposed to the aqueous environment of the water-filled pore, it will bind to the sulfhydryl-reactive agents when they come into contact. The binding of the sulfhydryl-reactive agent could cause alterations in the current response and offer clues as to the position of the residue in the ionic pore. In their investigation, Rassendren and his colleagues used the methanethiosulfonate (MTS) derivatives MTS ethylammonium (MTSEA), which is a small molecule with a positive charge, MTS ethyltrimethylammonium (MTSET) which is a large positively charged molecule and MTS ethylsulfonate (MTSES), which is a negatively charged molecule of intermediate size.

Mutations in amino acids preceding and throughout the second hydrophobic domain (V316-T354) were made (individually) and then expressed in HEK293 cells. All the mutant receptors, apart from S340C and D349C, produced responses comparable to the wildtype P2X2 receptor when ATP was applied. Results showed that residues I328C, N333C and T336C were accessible to all three reagents resulting in inhibition of ATP evoked currents as a consequence of binding to the free cysteine residue and inhibition of normal ion flow through the channel. Since this response was similar for all three, it was concluded that these three residues lie in the wide part of the pore - the outer vestibule. In addition, MTSEA and MTSET were found to cause more inhibition than MTSES. The positive charge associated with both MTSEA and MTSET was concluded to be the critical factor. Thus, this may be a region of charge selection. The small size of MTSEA allows it to be more accessible to the deeper regions of the pore. Mutants L338C and D349C were also found to have an inhibited current response when modified.
by MTSEA. L338C showed full inhibition whether or not ATP opened the channel, but D349C was inhibited only when ATP was concurrently applied. The full inhibition observed with D349C is dependent on the channel being open, thus implying that this residue must reside on the intracellular side of the channel gate. Since channel opening was required for inhibition by MTSEA at D349C but it was not needed for inhibition at L338C, this would place the gate between these residues (see figure 1.11). Similar results were reported by Egan et al (1998). They used the small, inorganic ion, Ag⁺ in addition to MTSEA. Though they reported slightly different observations, they placed the channel gate in the same region. They concluded G342 resides at or near the channel gate (Egan et al, 1998). Residues G342 and D349 are conserved among all seven P2X receptors.

Similar studies have shown that the first transmembrane region (L29-V51, P2X₂) also contributes to the pore and ionic permeation (Jiang et al, 2001; Haines et al, 2001). It would be sensible to think that the negative charges on residues lining the pore may very well contribute to ionic permeation. However Migita et al (2001) found that mutation of D315 and D349 did not affect cation permeation. It was subsequently found that the size of side chains exposed to the pore played a significant role. Mutation of polar threonines at 336 and 339 as well as serine 340 affected cation permeation greatly. The largest effect was found when these residues were replaced with the bulky aromatic side chain of tyrosine. It seems that addition of this chain prevents any cation entry (Schwiebert & Zsembery, 2003). Thus, the two transmembrane domains co-operate in both the formation of the pore and in the conduction of monovalent and divalent cations through the pore. It could mean during the association of three subunits to form the functional channel, all six transmembrane domains could form around the pore with each contributing specific residues to form the channel gate. However, the pore may also be formed by only a sub-set of the available transmembrane domains. Additionally, some individual domains may go through conformational change and an alteration of their role after ligand binding. This characteristic is significantly different to other ligand-gated ion channels whose pore is formed by the second transmembrane region only. Recently Li et al (2004) have revealed, using alanine point mutations across both transmembrane domains (P2X₂), that both domains participate in the conformational changes that occur
following agonist binding. The movement of each of the domains relative to each other is most likely the process by which the open pore is exposed and an influx of ions occurs. Taken together this evidence suggests that both transmembrane segments contribute to the pore.

Figure 1.11. At residue 325, no change in current is observed when ATP and MTSEA are co-applied. At residues 328, 333, 336 & 338 the effects are dependent on time of exposure to MTSEA. A block of up to 75% is observed. However at residue 349 the effects are dependent on the number of times the channel is activated by ATP. Since the channel gate can only open when ATP is bound this places D349 on the intracellular side of the gate. Work by Rassendren et al (1997) suggests that the channel gate is between residues 338 and 349. Egan et al (1998) placed the gate at or near residue G342. Studies have also shown that transmembrane 1 also contributes to the pore (Jiang et al, 2001; Haines et al, 2001).

1.4.1.2 Desensitization
Desensitization can be described as the decline (or decay) of current (or response) caused by the continued presence of an agonist. The transmembrane domains, along with other structures, play a fundamental role in determining the rate of desensitization in a given receptor. Pharmacological study has shown that P2X₁ and P2X₃ act similarly in that they
desensitize quickly, while P2X2, P2X4, P2X6 and P2X7 exhibit slow desensitization or not at all (P2X7). Werner et al (1996) studied this phenomenon by using chimeric receptors. They were able to exchange different domains from the P2X1, P2X2 and P2X3 receptors and reported any subsequent changes in the phenotype. They found that chimeric swapping of either one of two 34-residue segments, these segments included the first or the second hydrophobic domain, from the P2X2 receptor to the P2X1 receptor removed the desensitization. In contrast, desensitization was only introduced into the P2X2 receptor when both these segments of the P2X1 receptor were added. Thus it was concluded that desensitization was difficult to produce, requiring two interacting segments to be substituted. While desensitization was easy to lose, requiring only one segment to be substituted (Werner et al, 1996). These results may suggest that desensitization requires interaction between the two transmembrane domains of the receptor. Equally it may simply suggest that disruption of the transmembrane domains leads to alterations of the desensitization phenotype. Other studies (as discussed below) have shown that other regions within the receptor regulate the time course.

1.4.1.3 Subunit Assembly
Using a co-immunoprecipitation assay, Torres et al (1999b), investigated which domains were critical in functional subunit assembly of the P2X receptor. Epitope-tagged deletion mutants and chimeric constructs were tested for their ability to co-associate with full-length P2X subunits. The inability of certain chimeras to co-associate was also recorded. The systematic examination of the amino and carboxyl terminus and the transmembrane domains led to the discovery that the second transmembrane (or motifs present within) is important for determining the interaction between subunits and the assembly of the P2X receptor. Nicke et al (1998) also reported that the removal of the second transmembrane region in truncated P2X3 receptors (expressed in Xenopus oocytes) prevented the assembly of the channel (Nicke et al, 1998). This discovery also means that ligand-gated ion channels do not follow a generic process whilst undergoing assembly since the nicotinic acetylcholine receptor (4TM) is dependent on the amino terminus for subunit assembly (Torres et al, 1999 b).
1.4.2 *Intracellular Amino Terminal*

The length of the amino terminal domain is less variable than the carboxy terminal and contains a conserved protein kinase C (PKC) site. Phosphorylation, the addition of a phosphate group to a protein or a small molecule, can help to control many enzymes and receptors by causing functional changes within the target protein. These changes can switch them "on" or "off" (dephosphorylation can also bring about these changes) thereby regulating them. In Boué-Grabot *et al*'s (2000) study, they introduced mutations to disrupt the PKC site. They found the disruption caused changes in the time course of the response. They reported that amino acids 18-20 in the N-terminus of the P2X$_2$ receptor were critical for regulation of receptor desensitization. Mutation of residue T18 to T18A, T18N or K20T caused rapid desensitization in the P2X$_2$ receptor. In the P2X$_1$ receptor, mutations at the PKC site have generated receptors with a ten fold increase in the rate of desensitization. A substantial decrease in current amplitude (reduced by >99%) was also observed (Ennion and Evans, 2002). A reduction in current was also observed by Liu and his colleagues (2003). They transfected and recorded current response from HEK293 cells transfected with WT P2X$_1$ or P2X$_1$ with mutations at the PKC site or P2X$_1$ with mutations at sites adjacent to the PKC site. They found that the mutants T18A and T18N produced undetectable current amplitudes in response to 100µM ATP application, P19V produced comparable results to the WT and R20T had an intermediate response between the two. Mutations of adjacent residues showed that responses in D17E were unaltered whilst V22L showed a potentiation of the current response (Liu *et al*, 2003).

These results suggest that phosphorylation of the P2X$_1$ receptor could play an important role in regulating channel function (Ennion and Evans, 2002; Liu *et al*, 2003); similar results have also been shown for the P2X$_3$ receptor (Paukert *et al*, 2001).

1.4.3 *Intracellular Carboxy Terminal*

Sequences of the C-terminal regions of P2X subunits are quite variable in length and are minimally related to each other. A number of splice variants exist for the P2X$_1$, P2X$_2$,
P2X4 and P2X6 genes. These splice variants have been shown to have different properties when compared to their full length counterparts (Brandle et al, 1997; Simon et al, 1997; Koshimizu et al, 1998). In P2X2 (b), which contains a shorter C-terminus region (lacks a stretch of 69 amino acid residues), Koshimizu et al (1998) found that the receptor expressed a faster rate of desensitization than the wild-type P2X2 receptor; this was in agreement with previous reports (Brandle et al, 1997; Simon et al, 1997). Subsequent investigation suggested that the absence of a six amino acid region (Arg^{371} - Pro^{376}) was responsible for this phenotype (Koshimizu et al, 1998^b). Further investigation showed that substitution of this stretch of amino acids into wild-type P2X3 and P2X4 receptors caused desensitization phenotypes which mimicked that of the wild-type P2X2 receptor (P2X3 partial change and P2X4 full change) (Koshimizu et al, 1999).

In addition to its role in desensitization, Chaumont et al, (2004) identified a conserved motif (YXXXXK) within the C-terminal which has a role in the surface expression of the receptor. Using a chemiluminescent assay they found that disruption of the motif resulted in a marked reduction in the surface expression of the P2X2 receptor (similarly for the P2X3, P2X4, P2X5 and P2X6 receptors). Conversely, the introduction of this motif into an unrelated membrane protein (CD4) caused an increase in its surface expression. They suggested that these alterations in cell surface expression were not due to trafficking errors, but were due to rapid internalization of the receptor due to a lack of association with cytoskeletal proteins.

1.4.4 Extracellular Loop

1.4.4.1 ATP binding site

The ATP binding site of P2X receptors shows no sequence homology with any other ATP-binding proteins. The absence of a common amino acid sequence such as the Walker motif A (GXXXXGKT/S) (Walker et al, 1982) means that mutagenesis of conserved amino acids within the extracellular loop must be relied upon to determine the residues involved in the ligand binding process. In the extracellular loop of the P2X receptors, >90 of the ~270 amino acids are identical in at least five of the seven P2X
receptor subtypes. The rationale proposes that the replacement of an important residue to
the action of ATP would cause a change in the properties of the receptor. Conversely, a
lack of effect of the mutation would suggest that the conserved amino acid was not
essential for normal receptor function. The ATP binding site and the important amino
acid residues therein constitutes the focus of this thesis. In the following section I shall
look at some of the important conserved amino acid groups that have previously been
suggested to play a role in the ATP binding site of the P2X1 receptor.

**Charged Amino Acids**

Studies have shown that the ATP binding pocket is indeed on the extracellular domain
and possibly close to the channel vestibule (Ennion and Evans, 2000). Separate studies
by Ennion and Evans (2000) and Jiang *et al* (2000) suggested the importance of some of
the conserved positively charged residues in the extracellular loop. It is thought that
these residues would attract the negatively charged phosphate groups of ATP allowing it
to bind in the agonist binding site.

There are eleven conserved positively charged amino acids in the extracellular loop of all
P2X subtypes. Ennion and Evans (2000) systematically mutated each residue and
recorded ATP-evoked responses from the receptors expressed in *Xenopus* oocytes.
Mutations were made to keep the positive charge (K to R or R to K) or to neutralize it
(K/R to A). The majority of changes had little or no affect on ATP potency, but when the
positive charge was lost at K68 (1800 fold reduction), K70 (5 fold reduction), K309
(1400 reduction) and R292 (90 fold reduction), significant reductions in ATP potency
were observed. K68, K70, R292, and K309 are close to the predicted transmembrane
domains of the receptor. The reductions in ATP potency at K68 and K70 (external to
transmembrane domain 1) and at R292 and K309 (external to transmembrane domain 2),
suggest that these residues are fundamental to ATP recognition. Jiang *et al* (2000) found
similar results in the P2X2 receptor.

It’s possible that the anionic triphosphate group of ATP is attracted to and bound by these
lysines and the flanking serine and isoleucine help stabilize the interaction. Upon
binding, ATP may mask these closely coupled lysines with positively charged side chains, removing steric hindrance and allowing cations to move from the external channel vestibule and into the pore (Schwiebert & Zsembery, 2003).

The potential association of the negatively charged phosphate group of the ATP molecule with the positively charged amino acids near the channel vestibule encouraged further study into the orientation of the ATP molecule during binding. The role of the conserved negatively charged amino acids were examined. It was previously proposed that these residues may play a role in the binding of the positively charged magnesium – which is complexed with ATP.

Eleven conserved amino acids residues (aspartic and glutamic acid) were mutated in the P2X₁ receptor and none of the mutants altered ATP potency significantly. This lack of effect suggested that none of the individual amino acids were essential for ATP action at the P2X₁ receptor (Ennion and Evans, 2001).

**Aromatic and Polar Amino Acids**

Roberts and Evans (2005) studied the possibility that conserved aromatic amino acids could associate with the adenine and ribose moieties of ATP. Aromatic acids have previously been shown to coordinate the binding of both regions of ATP in other ATP-binding proteins (Skorvaga et al, 2002). There are 20 conserved aromatic amino acids in the extracellular loop of at least 6 of the 7 P2X receptors and alanine point mutagenesis was used to systematically replace these conserved amino acids in the extracellular loop of the P2X₁ receptor. The vast majority of the mutants produced ATP concentration-dependent responses which were either indistinguishable from the wild-type response or had a <6 fold decrease in ATP potency. Mutants F195A and W259A failed to form detectable channels at the cell surface. Since receptor expression was rescued with the substitution of tyrosine at these positions, this suggests the importance of these residues in channel processing prior to trafficking.
The mutants F185A and F291A produced 10- and 160-fold decreases in ATP potency without significant alteration in the time course of the response. The significant reduction in ATP potency at these two mutants suggested that they may be involved in ATP binding. However ATP potency is the combination of ligand binding and the process of gating. To distinguish between the two, the partial agonists 2',3'-O-(4-benzoyl)-ATP (Bz-ATP) and P1,P5-di(adenosine 5')-pentaphosphate (Ap5A) were used. ATP is very efficacious therefore a substantial change or mutation is needed before the potency of the compound is affected. Since Bz-ATP and Ap5A cannot produce maximal currents in the P2X1 receptor, the peak current that they evoke is more sensitive to changes. This allows a greater range of amino acid residues which contribute to the ATP binding site to be identified. The agonist activity of Bz-ATP was reduced to < 5% for the mutants F185A, R292A, F291A, K68A, and K309A and it acted as an antagonist when co-applied with ATP (inferring competition for occupancy of the binding site). The response to Ap5A was almost abolished in a majority of the mutants but increased in K70A. Co-application with ATP caused a reduction in ATP response in F291A, K68A and K309A, indicating antagonistic action; whilst causing a potentiation of the ATP response at the R292A mutant.

As well as providing corroborating evidence for the involvement of the positively charged amino acids K68, K309 and R292, Roberts and Evans also showed that the aromatic phenylalanine at positions 185 and 291 play a significant role in the potency of ATP and may coordinate the binding of the adenine ring (Roberts and Evans, 2005).

Uncharged polar amino acids can also play important roles in the agonist action by hydrogen bonding with the agonist or stabilizing conformational changes. Alanine point mutations at asparagine position 290 and threonine at position 186 had 60- and 6-fold decreases in ATP potency respectively. The partial agonists Bz-ATP and Ap5A failed to evoke any responses. There was a significant reduction in the ATP response when Bz-ATP was co-applied with ATP at either the N290A or T186A mutant receptor. This suggested that the partial agonist still interacted with the agonist binding site. Taken together, two motifs which affect ATP potency have been found. The FT (F185 and
T186) is totally conserved throughout the seven P2X receptor subtypes and the NFR (N290, F291 and R292) which is also conserved in all seven subtypes (Roberts and Evans, 2006). These residues may work together to coordinate binding of the adenine ring and the ribose moiety respectively (see figure 1.12).

**Structural Amino Acids**

Ten cysteine residues have been shown to be conserved in the P2X receptor family (Ennion and Evans, 2001b). Cysteine is an amino acid that contains sulphur in its attached side chain. This gives cysteine a highly reactive characteristic that can result in the formation of disulphide bonds with other cysteine residues, thereby conferring structural constraints on the receptor. Using site-directed mutagenesis and electrophysiological and biochemical methods, Ennion and Evans (2001b) were able to show that cysteine residues form disulfide bonds in the extracellular loop of the human P2X1 receptor and that some of these bonds are essential for normal trafficking of the receptor to the cell surface. Cysteine pairings at C117-C165, C126-C149, C132-C159, C217-C227, and C261-C270 were found in the P2X1 receptor and disruption of the C261-C270 disulfide bond or disruption of C117-C165 together with another bond were found to severely disrupt trafficking to the cell membrane. Similar results were reported in the P2X2 receptor (Clyne, 2002).

Digby et al, (2005) studied the importance of the glycine residues in the extracellular loop. Glycine is the simplest amino acid with only a hydrogen atom for its side chain. This enables it to confer a high degree of flexibility in a given protein. This flexibility may play a role in ATP binding or provide the flexibility necessary for conformational change initiated by agonist binding. Eleven glycine residues are completely conserved and an additional five are conserved in at least five of the seven P2X subtypes. Again, individual mutations were made in the P2X1 receptor and the glycine residues were replaced with alanine. ATP evoked concentration-dependent responses were not significantly different from the wild-type responses in the majority of the mutants tested. However ATP (up to 10mM) was ineffective at mutants G96A, G250A and G301A. Subsequent study of receptor expression showed these three mutants failed to form
detectable channels at the cell surface. Functionality was returned for two of these mutants (G96A and G301A) when the alanine residue was replaced with proline or cysteine. However substitution of G250 with a number of other amino acids (representative members of the classes of amino acids) did not, with the exception of G250S, produce a functional receptor. This lack of function was not attributable to receptor expression as the majority of substitutions were detected at the cell surface. This study suggests that the unique structural properties of glycine are necessary at position 250 and that the amount of flexibility afforded by the presence of the residue is necessary for normal functioning of the P2X₁ receptor. The conformational changes resulting from ligand binding may center in this region of the extracellular loop.

Analysis of the amino acid sequence also shows that the extracellular loop of P2X₁ receptors contains four prolines that are conserved across the P2X receptor family (P93, P166, P228 and P272) and other prolines that are less conserved – P196 (six of seven family members) and P174 and P225 (five of seven family members). Prolines are structural amino acids that can introduce “kinks” or tight turns in the protein. The majority of proline to alanine point mutations, conducted by Roberts and Evans (2005), had no effect on the potency of ATP, and thus are not essential for normal channel function. However, P272 proved to be highly resistant to this change - the altered receptor became non-functional. A similar phenotype was observed when the proline was replaced with lysine or aspartic acid. The increase in ATP potency after replacement with glycine or phenylalanine led to the conclusion that individual prolines are not essential in the region. However the receptor conformation around P272 seems to contribute to activation of the P2X₁ receptor. This conformational change after agonist binding, which eventually leads to channel opening and influx of ions was probably facilitated when proline was replaced with glycine or phenylalanine. Unlike the lysine, arginine residues and the aromatic residues that directly contribute to agonist binding, the proline at 272 may prove to have a purely structural role.
1.4.4.2 Trafficking to the cell surface

All the P2X receptor subunits have consensus sequences for N-linked glycosylation (N-X-S/T). Glycosylation has already been shown to be essential for trafficking of proteins from the ER to the cell surface (Fiedler & Simon, 1995). This is also the case with the P2X receptor as is observed upon the removal (by tunicamycin) or prevention (by mutagenesis) of glycosylation (Torres et al, 1998').

Rettinger et al (2000) used site-directed mutagenesis to eliminate glycosylation sites in the rat P2X1 receptor. The experiment allowed them to conclude that not only does glycosylation have roles in trafficking, but also the loss of specific glycosylation sites relates to changes in P2X1 function. There are five glycosylation sites on the rat P2X1 receptor, N153, N184, N210, N284 and N300. Investigation showed that the asparagine at 284 has no role at all due to the close proximity of a proline residue. They also found that any two from the remaining residues were necessary for strong functional protein expression. Ligand recognition also seems to be affected when the N-210 residue is removed. A reduction in ATP potency was observed which could mean that the N-glycan imposes structural limitations to the protein. Its normal role could be to help stabilize the ATP binding site and aid it in correct folding. Similar to Rettinger et al’s study, Roberts and Evans (2006) found that glycosylation is necessary for the efficient trafficking and expression of functional channels at the cell membrane. They found the human P2X1 receptor to be glycosylated at N153, N184, N242 and N300. This shows a slight difference to the rat isoform, where, N153, N184 and N300 is shared, but the site corresponding to N242 in the human is absent in the rat and an additional N210 is present instead.

In the P2X2 receptor, there are three glycosylation sites (asparagines 182, 239, and 298). Receptors in which any two of the three sites are glycosylated appear at the cell surface and are fully functional. However, receptors in which only one site is glycosylated give barely detectable currents in response to ATP, and channels with no sites glycosylated give no current. These mutant receptors are retained within the cell, and were detected by

Since P2X family members can be implicated in many physiological processes, the identification and development of competitive P2X receptor antagonists would be of great benefit. Unfortunately the P2X ATP-binding site shows little sequence homology with other protein classes. By and large, the areas pertaining to the site of ATP binding have been elucidated by systematic mutagenesis and consequent analysis. In the P2X1 receptor the positive amino acids at lysine 68, lysine 70, arginine 292 and lysine 309 have been shown to effect peak currents as well as the time constant. The aromatic amino acid phenylalanine at positions 185 and 291 has also been found to have great importance in ATP binding (Roberts and Evans, 2004). This extensive mutagenesis approach has led to the development of a model of the receptor (Vial et al, 2004; figure 1.12). Friest et al (1998), using secondary structure predictions and sequence alignments, also proposed a model for the ATP binding site of the P2X4 receptor. This was based on the similarities between the second half of the P2X4 receptor (170-330) and the catalytic domains of class II aminoacyl-tRNA synthetases. In their model they predicted that asparagine 280 coordinates binding of the magnesium ion complexed with ATP and the adenine ring binds to phenylalanine 230 and lysine 190. Their model suggests the binding of the phosphate groups by histidine 286 and arginine 278. Residues predicted from that model to be involved in ATP binding have been shown to reduce ATP potency when they were mutated (Yan et al, 2005). Yan et al (2005) found significant reductions in ATP potency from mutations of 3 of these residues — lysine 190, phenylalanine 230 and asparagine 280. Although both models show similarities; the mutants equivalent to lysine 190 results in a decrease in ATP potency at P2X1 and P2X2 receptors, there are also some differences. Mutations of the residue equivalent to phenylalanine 230 did not have an affect on ATP potency in the P2X1 receptor. This however may be indicative of the pharmacological differences that exist between P2X receptor subtypes.

In conclusion it seems that assigning specific roles to each region of the P2X receptor is not a simple task. Desensitization for example seems to be a multi-factorial process,
Figure 1.12. Diagram of the P2X$_1$ receptor based on evidence acquired from some of the mutation studies carried out. Systematic mutagenesis of conserved amino acid residues has allowed the development of a molecular model of the receptor. The diagram shows the suggested contribution of the positively charged and aromatic amino acids in the coordination of ATP binding. The structural importance of the cysteine residues in the formation of disulphide bridges is also shown. In addition, the glycine residue at 250 and the proline residue at 272 is also shown. The protein kinase C (PKC) region is highlighted in the amino terminal, as is the trafficking motif which is conserved in all P2X subtypes in the carboxy terminal.
where a number of regions work in concert to produce the desired effect. This is also the most likely scenario in the translation of ATP binding to the opening of the channel gate.

1.5 Project Aim

This thesis aims to test and identify ATP analogues which can cross-link at P2X receptors thereby providing direct binding evidence for the first time. I then aim to develop a method of purifying the receptor so that cross-linked protein can be enzymatically digested and analysed by mass spectrometry to identify amino acid residues in and around the ATP binding site. The identification of new amino acid residues will help the refinement of a P2X receptor model and in addition may provide corroborating evidence acquired from the extensive mutational analysis studies.
CHAPTER 2
IDENTIFICATION OF ATP ANALOGUES WITH CROSS-LINKING ABILITY AT THE P2X₁ RECEPTOR

2.1 Introduction

A greater understanding of the molecular model of P2X receptors would aid in the production of various pharmaceutical therapies by rational drug design. The extracellular domain of P2X receptors contains a distinct ATP binding site that has yet to be fully elucidated. One of the complications is that there is little sequence homology with other ATP-binding protein classes i.e. none of the "classical" nucleotide-binding motifs have been detected in P2X receptors. For example the motif GXXXXGKT/S (where X = any residue), more commonly known as the Walker motif A (Walker et al, 1982) is not present. This motif is found in many ATP-requiring enzymes, ABC (ATP binding cassette) transporters and the muscle protein myosin. Indeed, a search conducted with the Brookhaven protein data bank has shown that over 600 polypeptide chains have been found to contain this sequence (Ramakrishnan et al, 2002). Though not all have any nucleotide interactions, the motif can still be used in a predictive capacity to locate specific areas thought to bind to ATP and other associated nucleotides.

For P2X receptors, site-directed mutagenesis of conserved amino acid residues in the extracellular loop and the structural homology to other nucleotide binding proteins have been used to propose different models of the ATP binding site (Vial et al, 2004b; Freist et al, 1998). Though mutagenesis is a powerful tool in directing attention to specific residues that appear to influence ATP potency (Ennion et al, 2001; Roberts & Evans, 2005), discrimination between residues involved in agonist binding and residues involved in the process of gating is extremely difficult. Direct binding evidence can corroborate data from previous studies as well as indicate other residues which may play important roles in the ATP binding site. Reactive ATP analogues have been used previously to identify residues within the binding sites of the Na⁺, K⁺ ATPase (Tran et, 1994), Kₐ₅P channels (Tanabe et al, 1998), natriuretic peptide receptor (Joubert et al, 2005) and the ADP/ATP carrier (AAC) from yeast (Mayinger et al, 1989) as well as other nucleotide binding proteins.
The initial aim of this chapter was to develop protocols which could aid in the identification of ATP analogues with cross-linking ability at the P2X₁ receptor. This first chapter examined the whether the ATP analogues FSBA and 2-azido ATP would be useful for this purpose.

2.1.1 Cross-linking

Cross-linking can be defined as the establishment of chemical bonds (usually covalent) between two or more compounds. Cross-linking compounds often contain reactive groups that can be activated chemically, by heat, catalysis or irradiation thus resulting in the formation of a chemical bond. Cross-linking ATP analogues should be particularly effective in this project since their resemblance to ATP should allow them to bind and activate (or antagonise) the P2X₁ receptor. Thus, it is likely that the analogue should label the receptor at the binding site and subsequently allow the identification of residues that are in and around the binding site.

2.1.2 Chemical cross-linking

The ATP analogue 5'-[(p-fluorosulfonyl)benzoyladenosine (FSBA) has a reactive sulfonyl fluoride group that is an electrophilic agent in covalent reactions with several types of amino acids; these include tyrosine, lysine, histidine, serine and cysteine (Colman, 1983). Nucleotide binding sites as diverse as the ADP receptor on platelets (Figures et al, 1987), the binding domain on the ArsA protein of E. Coli (Ramaswamy and Kaur, 1998) and the nucleotide-binding site on the enzyme pyruvate kinase (Wyatt and Colman, 1977) have been successfully labelled with FSBA. Positive labelling with radioactive FSBA has also been shown in the Na⁺/K⁺ ATPase (extracted from dog kidney). This integral membrane enzyme is responsible for the active transport of Na⁺ and K⁺ through cell membranes. In a study by Ohta et al (1986), it was shown that the activity of the enzyme was significantly reduced after prolonged exposure (90mins) to FSBA. The incorporation of FSBA was specific to the ATP binding site since the presence of ATP, but not GTP, CTP or ADP, caused a reduction in labelling and protection from inactivation (Ohta et al, 1986). The wide spread activity of FSBA at different nucleotide binding sites made the compound a possible candidate to use as a cross-linker at the ATP binding site of P2X₁.
2.1.3 Photoactive Compounds

Photolabelling compounds such as 2-azido ATP contain a photoactive azido (-N₃) group that is chemically inert until activation by UV light. Upon irradiation, a highly reactive nitrene is formed, this inserts into either the peptide backbone or the amino acid side chains of the protein to which it is bound. This insertion forms a covalent linkage between the compound and the protein allowing it to be permanently tagged for identification. Photoactive compounds have been extensively used in the past to identify nucleotide binding sites (Kon and Suhadolnik, 1996), identify amino acid residues that can be mutated to form proteins with altered activity (Weber et al, 1993) and help in detecting alterations in nucleotide binding of proteins in various disease states (David et al 1998). Tran et al (1994) used 2-azido ATP to identify amino acid residues within the ATP binding site of Na⁺, K⁺ ATPase. The use of 2-azido ATP as a substrate by Na⁺, K⁺ ATPase suggested that 2-azido ATP bound to the enzyme from within its active site. When the enzyme was UV irradiated in the presence of 2-azido ATP there was an inhibition of enzymatic activity by ~ 50%. Protection by ATP against enzyme inhibition due to photoactivation of 2-azido ATP suggested competition between the azido nucleotide and ATP for available binding sites on the Na⁺, K⁺ ATPase.

The 2-azido ATP molecule contains an azido (-N₃) substitution at the C-2 position of the adenine ring. Previous study has shown that ATP analogues with alterations at the C-2 position of the adenine base often show no significant change in the EC₅₀ in subtypes of the P2X receptor (Cusack and Hourani, 1990; Evans et al, 1995). This
tolerance of analogue alteration and the continued activity of such compounds make 2-azido ATP an attractive ATP analogue which can be investigated for any ability to cross-link to the P2X₁ receptor.

**Figure 2.2. Diagram of 2-azido ATP.** The azido group (N₃) can be positioned at different co-ordinates across the ATP molecule. Upon irradiation a highly reactive nitrene is formed, the lone pairs of electrons can help form a covalent linkage between the photoprobe and the receptor.

In this chapter I have conducted experiments to test the cross-linking ability of FSBA at the P2X₁ receptor. I have also established protocols for the UV irradiation of cells and have tested 2-azido ATP as a cross-linking compound at the P2X₁ receptor.
2.2 Materials and Methods

2.2.1 Cell Culture
Native Human embryonic kidney (HEK293) and HEK293 cells stably expressing the human P2X1 receptor (Vial et al., 2004) were cultured in 80cm² flasks in growth medium composed of Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, U.K.) containing Earle’s salts, glutamax 1, 10% foetal calf serum and 1% non-essential amino acids. The human P2X1 plasmid construct was contained within the pcDNA3.1 vector. This vector also contained G418 resistance. Cells were maintained at 37°C in humidified 5% CO₂/95% air and P2X1 stable cells were under permanent selection in 600µg/ml G418 (Invitrogen, U.K.). Cells were plated onto 13mm glass coverslips and electrophysiological recordings were made 24-48hrs later. These coverslips had previously been stored in ethanol and were flame cleaned to remove any debris. The preparation of plates and cell culture all took place in a laminar flow hood under sterile conditions.

2.2.2 Electrophysiological recordings
Electrophysiological recordings were made using the whole-cell patch clamp configuration on single cells at room temperature (21°C-26°C). Microelectrodes were filled with internal solution composed (in mM) of K gluconate, 140; EGTA, 10; HEPES, 10; NaCl, 5; (pH 7.3, adjusted with KOH) and had resistances in the range of 2 – 6 MΩ. The bath was continuously perfused with E_total extracellular solution containing (in mM) NaCl, 150; HEPES, 10; glucose, 11; KCl, 2.5; MgCl₂, 1; CaCl₂, 2.5; (pH 7.3, adjusted with NaOH). Cells were voltage clamped at −70 mV (after correction for the tip potential) and the data was low pass-filtered at 1kHz and digitized at a sampling interval of 200µs. Using an Axopatch 200B amplifier, data was collected with pClamp 8.1/9.0 software.

Agonists were applied for one second via a fast-flow U-tube (Evans and Kennedy, 1994) and P2X₁ receptor responses were quantified by measuring the peak current amplitude relative to the base-line holding current immediately preceding agonist application. The effects of antagonists were determined by allowing the cells in the bath to be perfused for 5mins (unless stated otherwise) prior to agonist application.
2.2.3 Drugs
ATP and FSBA were obtained from Sigma (U.K.). 2-azido ATP was obtained from Affinity Labelling Technologies Inc (Kentucky, U.S.). Prior to application, all drugs were dissolved in \( E_{\text{total}} \) to form stock solutions.

2.2.4 Cross-linking with 2-azido ATP
Wild-type P2X₁ cells, mounted on 13mm cover slips, were placed in an 8.8cm² petri dish (Nunclon, U.K.) and were pre-treated with 2ml of 30μM 2-azido ATP. An irradiation box was constructed to house the UV lamp (Uvitech, U.K., LF206.MS, 50 x 150mm). The interior of the box was maintained at room temperature and constant humidity (figure 2.3). The petri dish was placed inside the box and the wavelength was set at 312nm. This was done in a similar fashion previously used by Ward and Cavieres (1998). The UV lamp was positioned 7.5cm above the cells and the cells were continually irradiated for 3min. After irradiation, cells were washed for 5min in extracellular solution and were ready for electrophysiological recording.

![Figure 2.3. The experimental setup used to apply UV irradiation to the cells. Experiments were carried out with a stable HEK293 cell line expressing the P2X₁ receptor. The cells were placed inside the chamber at room temp and constant humidity. The UV lamp was set at 312nm.](image)

2.2.5 Radio-labelled Cross-linking
Native HEK293 cells and wild-type P2X₁ cells were cultured in 24-well plates in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) with Earle’s salts, glutamax 1, 10% foetal calf serum and 1% non-essential amino acids. Wild-type P2X₁ cells were maintained under permanent selection in 600μg/ml G418 (Invitrogen). When cells reached 80% confluence the growth media was removed and replaced with 1μM or 10μM 2-azido \( [\gamma^{32}P] \) ATP (0.32MBq or 3.2MBq) (Affinity Labelling Technologies...
Inc) at 0.5ml/well. The 24-well plate containing these cells was placed in the UV box and continually irradiated by 312nm of UV light for 3min. The 24-well plate was placed on ice and unbound $^{32}$P labelled 2-azido ATP was removed by washing the cells twice with 1ml PBS. The cells were then lysed into a homogenising buffer (100μl) containing 150mM NaCl, 1mM EDTA, 1mM EGTA, 40mM Tris HCl (pH7.4), 8mM Tris base, 1% triton X and a mixture of protease inhibitors (1:100 dilution) (Sigma). The cell homogenate was centrifuged for 5min at 13,000rpm (4°C) on a benchtop centrifuge, the cell debris was discarded and the supernatant was stored on ice. A stock solution of P2X<sub>1</sub> antibody (0.3mg/ml) (Alomone, Israel) was prepared and 3μl of the solution was added to 85μl of the supernatant. The mixture was allowed to incubate on ice for 1hr. Protein A-sepharose beads (30mg/ml) (Amersham, U.K.) were pre-washed in PBS and the resultant antibody-antigen complex was precipitated with the addition of 75μl protein A-sepharose beads and rolled at 4°C overnight. 1ml homogenising buffer was added to the protein A-sepharose beads (complexed with the antibody and antigen) and the mixture was spun on a benchtop centrifuge at 13,000rpm for 5min. The supernatant was removed with a pump aspirator and discarded. The process was repeated three times in total to enable the beads to be washed of any unbound radioactive material. 20μl of sample buffer (with 5% β-mercaptoethanol) was added to the protein A-sepharose P2X<sub>1</sub> pellet. The pellet was heated to 95°C for 5min to allow the protein to elute from the beads. 20μl of the sample was removed and loaded onto a 10% SDS-PAGE gel and run at 120V. For autoradiography, the gels were dried and exposed to x-ray film for 48-72hrs at -80°C.

2.2.6 Data Analysis
The results are expressed as mean ± standard error of mean and analyzed using the appropriate Student’s T-test with P values ≤ 0.05, when compared to the control considered as significant (*). P values ≤ 0.01 were considered very significant (**) and P values ≤ 0.001 were considered highly significant (***)). Data analysis was carried out with the computer package, “Excel” and the graphics package “Origin 6” was used to construct the graphs.
2.3 Results

2.3.1 Wild-type P2X<sub>1</sub> responses to ATP application

ATP application evoked concentration-dependent inward currents which desensitized rapidly in the continued presence of the agonist. These characteristic responses have been previously reported (Valera et al, 1995; Evans et al, 1995). The application of 0.1μM ATP to P2X<sub>1</sub> cells produced inwardly transient currents which had a mean current amplitude of -0.24 ± 0.07nA (n = 19). An increase in concentration to 1μM ATP evoked mean peak amplitude currents of -2.84 ± 0.28nA (n = 10). 10μM ATP application caused mean peak amplitudes of -5 ± 0.41nA (n = 11) (figure 2.4). Repeated application of ATP causes a reduction in the peak current amplitude of the response. These characteristics have been described previously (Evans et al, 1995; Rettinger & Schmalzing, 2004). Consequently only one ATP response was recorded per cell. This rule was applied throughout the investigation. The EC<sub>50</sub> concentration of ATP with regards to the P2X<sub>1</sub> receptor has been recorded previously as 1μM (Evans et al, 1995; North, 2002). This was used as a control concentration throughout this chapter to reflect changes in the ATP binding site.

The concentration dependence was also evident in the change in time course of the response. An increase in the concentration caused a speeding of the time course. The application of 0.1μM ATP produced a rise time (10 – 90%) of 431 ± 46ms and a decay time (peak to 50%) of 644 ± 88ms (n = 19). When 1μM ATP was applied there was a mean rise time of 146 ± 26ms and a decay time of 617 ± 48ms (n = 10). The application of 10μM ATP produced a rise time of 76 ± 4ms and a decay time of 103 ± 12ms (n = 11). These basic properties of the wild-type P2X<sub>1</sub> current (emphasis on the peak current amplitude) were used as a control to monitor any changes that were caused by cross-linking of a compound to the P2X<sub>1</sub> receptor.

2.3.2 Testing FSBA Activity at the P2X<sub>1</sub> Receptor

The ATP analogue 5'-O-(4-fluorosulfonylbenzoyl)-adenosine (FSBA) has a reactive sulfonyl fluoride group on the tail end of the molecule. Its ATP-like structure, which includes both a nitrogenous base and ribose sugar, is shown in figure 2.1. 1μM ATP application evoked desensitizing inward currents in wild-type P2X<sub>1</sub> receptors. The
Figure 2.4. Current responses evoked by ATP application at the P2X₁ receptor  

A. Whole cell patch clamp traces show the desensitizing, inward current evoked by ATP application at the P2X₁ receptor (application of ATP is indicated by open bar). B. The pooled data showed the response to ATP is concentration-dependent. The mean peak current recorded from P2X₁ cells applied with 0.1µM ATP was -0.24 ± 0.07nA (n = 19). Application of 1µM ATP evoked mean current amplitudes of -2.84 ± 0.28nA (n = 10). The mean current amplitude increased to -5 ± 0.41nA (n = 11) when 10µM ATP was applied.
mean responses recorded were \(-2.76 \pm 0.42\text{nA} \ (n = 6)\). To test if FSBA was an agonist, 100\(\mu\)M FSBA was applied to wild-type P2X1 receptors. FSBA application had no effect on the holding current of these cells. 100\(\mu\)M FSBA produced mean peak currents of \(-0.038 \pm 0.01\text{nA} \ (n = 6)\). This result was indistinguishable from the background noise and indicated that FSBA had no action as an agonist at the P2X1 receptor (figure 2.5).

Previous study has described that FSBA’s cross-linking effects are due to a spontaneous reaction encouraged by the incubation of the compound with the receptor/enzyme of interest (Ohta et al., 1986; Figures et al., 1987). When wild-type P2X1 receptors were incubated in 100\(\mu\)M FSBA for 1 hour prior to 1\(\mu\)M ATP application, there was no significant change from the control response. The mean response recorded was \(-2 \pm 0.3\text{nA} \ (n=8)\) (figure 2.5). This suggests that FSBA has no cross-linking ability at the P2X1 receptor.

FSBA is a chemical cross-linker that has been extensively used in previous studies (see section 2.1.2). Its inability to inhibit the ATP response means that it has no activity at the P2X1 receptor and thus implies that the P2X1 receptor must contain a distinct agonist binding site. The results from these set of experiments show that FSBA has no further use as a cross-linking compound in this investigation.

2.3.3 2-azole ATP as an Agonist
The photo-labelling compound 2-azole ATP contains an N3 substitution at the 2’ position of the adenine ring. The rest of the structure resembles ATP. 1\(\mu\)M ATP application to P2X1 receptors produced transient inward currents of \(-3.47 \pm 0.38\text{nA} \ (n = 9)\). Similarly, 2-azole ATP also evoked desensitizing inward currents at P2X1 receptors. When 1\(\mu\)M 2-azole ATP was applied to cells expressing the P2X1 receptor, the peak current amplitude was \(-1.91 \pm 0.56\text{nA} \ (n = 8)\). These currents were concentration-dependent. When the concentration of 2-azole ATP was increased to 10\(\mu\)M, the subsequent mean amplitude response was \(-5.42 \pm 0.59\text{nA} \ (n = 18)\). An increase to 30\(\mu\)M produced responses of \(-6.12 \pm 0.64\text{nA} \ (n = 14)\) (figure 2.6). The concentration dependent response was also expressed as an increase in the speed of the time course. The rise time of the response following 1\(\mu\)M 2-azole ATP application was 86 \pm 20\text{ms} and the decay time was 499 \pm 43\text{ms} \ (n = 8). The rise time
Figure 2.5. An investigation of the activity of FSBA at the P2X₇ receptor  A. Whole cell patch clamp traces show the inactivity of FSBA at the P2X₇ receptor. 1μM ATP evoked a desensitizing, inward current (application of 1μM ATP is indicated by open bar). Conversely, 100μM FSBA application does not produce any change in the holding current (application of FSBA is indicated by the black bar). When cells were incubated in 100μM FSBA for 1hr, then washed for 5min in extracellular solution, 1μM ATP application still evoked desensitizing inward currents. B. The pooled data from the investigation. The mean peak current recorded from control cells applied with 1μM ATP was -2.76 ± 0.42nA (n = 6). When 100μM FSBA was applied to P2X₇ receptors there was no current. Recorded responses were -0.04 ± 0.01nA (n = 6). 1hr incubation of cells in 100μM FSBA for 1hr had no effect on the response evoked after 1μM ATP application. The mean current recorded was -2 ± 0.3nA (n = 8). All recorded responses were measured from single cells.
Figure 2.6. An investigation of the activity of 2-azido ATP at the P2X₁ receptor

A. Whole cell patch clamp traces showed the concentration-dependent activity of 2-azido ATP at the P2X₁ receptor. 1μM ATP evoked a desensitizing, inward current (application of 1μM ATP is indicated by open bar). The application of 2-azido ATP (application of 2-azido ATP is indicated by the black bar) produced a similar desensitizing inward current that increased when the applied concentration of 2-azido ATP was increased. B. The pooled data from the investigation. The mean peak current recorded from P2X₁ receptors applied with 1μM ATP was -3.47 ± 0.38nA (n = 9). When 1μM 2-azido ATP was applied to P2X₁ receptors there was a significant smaller response. Recorded responses were -1.91 ± 0.56nA (n = 8). The mean amplitude of P2X₁ receptors after 10μM 2-azido ATP application was -5.42 ± 0.59nA (n = 18). When 30μM 2-azido ATP was applied the mean amplitude was -6.12 ± 0.64nA (n = 14). (* = P ≤ 0.05 and show significant difference from the 1μM ATP control).
was 18 ± 3ms and the decay time was 268 ± 65ms (n = 18) when 10µM was applied. 30µM 2-azido ATP caused a rise time of 9 ± 2ms and a decay time of 392 ± 100ms (n = 14).

These results showed that 2-azido ATP worked effectively as an agonist at the P2X₁ receptor and had potential as a cross-linking compound at the receptor. Its effectiveness as an agonist indicated that it resembled the ATP molecule enough to activate the P2X₁ receptor. This suggests that the C-2 position is not critical in the recognition of potential agonists at the P2X₁ receptor.

2.3.4 Optimizing the UV Protocol
To establish protocols for the UV irradiation of cells, preliminary experiments were conducted to find the optimum conditions that allowed the cells to be UV irradiated without being damaged. The starting parameters were used previously by Ward & Cavieres, 1998. In their study they constructed a UV box and positioned the UV lamp 50cm from their sample. They then varied the UV irradiation time. In this study the UV lamp was fixed 7.5cm above the cells and the optimal UV irradiation time was investigated. The optimal irradiation time was deemed to be the maximum amount of time that the cells could be UV irradiated without an adverse effect on the current response to 1µM ATP application. To study the effect of UV, the 1µM ATP responses from UV irradiated P2X₁ cells were compared to the current responses evoked by 1µM ATP application of unexposed P2X₁ cells.

Cells (plated on 13mm coverslips and placed in a petri dish) were bathed in 2ml extracellular solution (Etq) and placed in the UV box (figure 2.3). Initially, cells were continually irradiated for 5min before being transferred to the recording chamber and patch clamped. Recorded responses to 1µM ATP application revealed a 30% reduction in the mean peak amplitude. The mean peak amplitude response from unexposed P2X₁ cells was -2.54 ± 0.33nA (n = 15). However 1µM ATP evoked significantly reduced currents of -1.79 ±0.2nA (n = 34) in P2X₁ cells UV irradiated for 5min (figure 2.7). This suggested that UV irradiation had a damaging effect on P2X₁ cells exposed to UV irradiation for 5min. Consequently the UV irradiation time was reduced to 3min and subsequent results showed no change in the current response to 1µM ATP application. 1µM ATP evoked mean currents of -2.43 ± 0.52nA (n =
Figure 2.7. The effect of UV irradiation time on cell response. A. Whole cell patch clamp traces show the effect of UV irradiation time on the cell response (application of 1μM ATP is indicated by open bar). HEK 293 cells expressed wild-type P2X1 receptors. These cells were irradiated in the UV box for 3min or 5min and then transferred to the recording chamber and patch clamped. B. The pooled data from the investigation showed that 3min UV irradiation of cells caused no effect on the mean peak amplitude. The control responses were measured at -2.54 ± 0.33nA (n = 15). 1μM ATP application after 3min irradiation produced peak currents of -2.43 ±0.52nA (n = 13). There was a significant reduction in the peak response when cells were UV irradiated for 5min. The recorded responses were -1.79 ± 0.2nA (n = 34). ( * = P≤ 0.05 and shows significant difference from the 1μM ATP control).
The result suggested that P2X₁ cells can be UV irradiated for 3min without incurring any cellular or receptor damage. This 3min UV irradiation time was used for all subsequent cross-linking experiments.

2.3.5 UV Irradiation of 2-azido ATP

The rapidly desensitizing inward currents caused by 2-azido ATP application indicate it is an effective agonist at the P2X₁ receptor. The potencies of 2-methylthio ATP and 2-chloro ATP are essentially the same at the P2X₁ receptor (Evans et al, 1995). The similarity of 2-azido ATP's potency suggests that the P2X₁ receptor readily accepts ATP analogues with alterations at the 2' position. Once UV irradiated, the 2-azido ATP should bind to the receptor leaving it in its desensitized state (figure 2.8).

Figure 2.8. Cartoon describing the theory behind photoactive cross-linking. In normal ATP binding (A) at the P2X₁ receptor, ATP molecule/s bind at the agonist binding site and cause an opening of the channel. The receptor desensitizes and wash-off of the agonist causes the ATP to dissociate from the receptor. This allows the receptor to recover. After receptor recovery (5-10min), subsequent agonist applications again cause inward currents. A photoactive compound (B) resembles the parent compound and contains a reactive group that, whilst inert under normal conditions, can be activated under UV light. UV activation causes the compound to bind to the receptor and maintain the receptor in its desensitized state. The receptor response to subsequent agonist applications is inhibited.

ATP application to P2X₁ cells caused rapid desensitization in the continued presence of the agonist. Subsequent current responses from these cells were markedly
inhibited unless there was a period of time in which the cells could be washed with extracellular solution. This allowed the receptors to recover.

As a control, cells were treated in 30\(\mu\)M ATP and were UV irradiated for 3min before they were removed from the UV box. They were then placed on the electrophysiology rig and perfused with extracellular solution for 5min to allow recovery. The application of 1\(\mu\)M ATP elicited responses of -3.03±0.57nA (\(n=6\)); this was not significantly different from cells which were not UV irradiated. 1\(\mu\)M ATP application to untreated P2X\(_1\) cells produced currents of -3.47 ±0.9nA (\(n = 9\)). The results showed that UV irradiation in the presence of 30\(\mu\)M ATP caused no change in the subsequent response to 1\(\mu\)M ATP application. It also shows that any reductions in the ATP response from cells UV irradiated in the presence of 2-azido ATP suggest successful cross-linking of the compound to the receptors (figure 2.9).

To test the cross-linking ability of 2-azido ATP, cells were treated in 30\(\mu\)M 2-azido ATP and UV irradiated for 3min. The cells were then placed on the electrophysiology rig and perfused with extracellular solution for 5min. Whole cell patch clamp recordings were made from single cells over a 45min time period. Results showed that the response produced by 1\(\mu\)M ATP application was reduced by 86\%. Recorded currents averaged at -0.49 ±0.09nA (\(n = 30\)) a decrease from -3.47 ±0.9nA (\(n = 9\)) which was recorded from cells which had not been UV irradiated (figure 2.9).

This reduction in the 1\(\mu\)M ATP response may suggest cross-linking of the compound to the receptor binding site and preservation of the P2X\(_1\) receptors in their desensitized state. The cross-linking of the compound to a majority of receptors would prevent further binding by ATP and produce reduced peak amplitudes in response to 1\(\mu\)M ATP application.

### 2.3.6 Cell Recovery

It has already been shown that 2-azido ATP evoked concentration-dependent inward currents that desensitized quickly during the continued presence of the drug (figure 2.6). The reduced 1\(\mu\)M ATP response from cells UV irradiated in the presence of 2-
Figure 2.9. Does UV irradiation in the presence of 2-azido ATP block P2X₃ receptor responses? A. Cells were treated in 30μM 2-azido ATP (or ATP; UV irradiation in the presence of 2-azido ATP is indicated by the yellow vertical bar) and UV irradiated at room temp for 3min. Cells were then placed in the recording chamber and washed in extracellular solution for 5min prior to patch clamp recording. The response to 1μM ATP application (application indicated by open bar) provided evidence to the inhibitory effects of cross-linking. B. Whole cell patch clamp traces showed that the P2X₃ receptor was not effected by UV irradiation in the presence of 30μM ATP. The ATP response showed no significant difference from the control. However, when cells were UV irradiated in the presence of 30μM 2-azido ATP there was a significant reduction in subsequent cellular response. C. The pooled data from the investigation. The mean peak current recorded from control cells applied with 1μM ATP was -3.47 ± 0.38nA (n = 9). When cells were treated in 30μM 2-azido ATP and then UV irradiated there was an 86% decrease in subsequent cell responses; -0.49 ±0.09nA (n = 30). This highly significant reduction is not observed when cells are treated in 30μM ATP and then UV irradiated (-3.03±0.57nA (n=6)).
azido ATP could be interpreted as the continued effect of desensitization. It has been previously reported that the P2X₁ receptor requires a period of recovery after desensitization before it can produce responses (Rettinger & Schmalzing, 2004) similar to the primary response. The reduced response recorded after UV irradiation (figure 2.9) could simply be the result of desensitization of the receptor. Over time the response to 1μM ATP application could return.

To investigate this, the ATP (1μM) responses from cells UV irradiated in the presence of 30μM 2-azido ATP were measured over time. These measurements were compared to recordings taken from cells treated in 30μM ATP and cells treated in 30μM 2-azido ATP without UV irradiation. After treatment, cells were placed on the electrophysiology rig and perfused with extracellular solution for 5min to allow wash-off of the respective compound. Patch clamp recordings were continuously taken (from different single cells) every 2min, over a 45min time period.

As a control, the wild-type P2X₁ receptor response to 1μM ATP application was recorded at -3.6 ±0.48nA (n = 13). Over 45min, recordings were compared from cells treated in 30μM ATP, 30μM 2-azido ATP (no exposure to UV) and UV irradiated 30μM 2-azido ATP. When treated in 30μM ATP, results showed that between 1-5mins the average current was -1.9 ±0.15nA (n = 7) this increased to -2.96 ±0.21nA (n = 9) after 10min. Over the course of the 45min time period, recordings generally remained within -2.6 and -3nA, meaning 85% of the current produced by the control was recovered. A similar result was also observed when cells were treated in 30μM 2-azido ATP. Again currents were shown to recover within 10min, with the first 5min averaging -0.71 ±0.04nA (n = 5). After 10min full recovery was achieved. The mean recordings measured were -4.07nA ±0.09nA (n = 7) (figure 2.10).

However, when 30μM 2-azido ATP was UV irradiated, the reduced current was maintained over the whole 45min time period. After 10min, the average current recorded was only 36% of the 1μM ATP control (-1.3 ±0.45nA (n = 5)). After 45min the ATP (1μM) response was only 5% of the control (-0.18 ±0.01nA (n = 5); figure 2.10).
Figure 2.10. Is the block maintained over time or can it be attributed to desensitization? A. Wild-type P2X₁ cells were plated onto 13mm coverslips and placed in a 8.8cm² petri dish. The media was replaced with either 2ml of 30µM ATP or 30µM 2-azido ATP and were left for 3min to equilibrate (green bar). Cells were then placed in the recording chamber and were continuously perfused with extracellular solution for 45min. Patch clamp recordings were continuously taken from different single cells every 2min during this 45min time period. Alternatively, wild-type P2X₁ cells were plated onto 13mm coverslips and placed in a 8.8cm² petri dish. The media was replaced with 2ml of 30µM 2-azido ATP and the cells were left to UV irradiate for 3min. The cells were recorded in the same manner as established previously. In both protocols single cells were whole cell patch clamped and their current amplitude response to 1µM ATP application was recorded. For each time period, the current responses from 3 different cells were recorded. B. Whole cell patch clamp traces show a sample of recordings taken between 1-5mins of wash-off. C. Bar graph shows summary data which compares cell response between different pre-treatment regimes. Bar graph shows that within 10mins, recovery of current was possible after pre-treatment in 30µM ATP or 30µM 2-azido ATP. However when cells, treated in 30µM 2-azido ATP, were UV irradiated, the reduction was maintained over time.
The results showed that when P2X₁ cells were treated in either 30μM ATP or 2-azido ATP (without UV exposure) for 3min their peak amplitudes, in response to 1μM ATP application, recovered within 10min. It is the UV irradiation of 30μM 2-azido ATP which is the significant factor that reduced the P2X₁ response to 1μM ATP application. These results again suggest that 2-azido ATP effectively cross-links to the P2X₁ receptor and maintains the receptor in its desensitized state.

2.3.7 Receptor Protection

If the receptors can be blocked by the physical attachment of 2-azido ATP at the agonist-binding site, can they be protected from such a process? To further test 2-azido ATP's validity as a cross-linking compound at the P2X₁ receptor excess ATP or suramin were used. These drugs could protect the receptors by competing for unoccupied receptors. If the majority of the receptors are not occupied by 2-azido ATP when the cells are UV irradiated, the majority of the receptors will not be permanently blocked and the response to ATP application will not be inhibited, thus the receptor would be protected (figure 2.11).

![Figure 2.11. Cartoon describing the theory behind receptor protection with excess ATP. A. If the receptors are co-treated with excess ATP or suramin in conjunction with 2-azido ATP, the majority of receptors should be occupied by ATP or suramin. B. Hence UV irradiation should only cause the cross-linking of a minority of receptors, leaving the vast majority free to be activated. To some extent, the responses garnered from subsequent ATP application should be recovered.](image)

Wild-type P2X₁ cells were plated onto 13mm coverslips and placed in an 8.8cm² petri dish. The growth media was removed and was replaced with 1.5ml of either
300μM ATP or 100μM suramin. 1.5ml of 30μM 2-azido ATP was immediately added to the petri dish and the mixture was allowed to equilibrate for 5min. The dish was placed inside the UV box and was UV irradiated for 3min at 312nm. As before, 1μM ATP applications were made and whole cell patch clamp recordings were made. As previously, UV irradiation in the presence of 30μM 2-azido ATP resulted in a near 90% reduction in the peak amplitude following 1μM ATP application (-0.49 ±0.1nA (n = 30)). P2X₁ receptors which were treated in 300μM ATP, prior to UV irradiation in the presence of 30μM 2-azido ATP, produced mean peak amplitudes which were only reduced by 57%. The mean peak amplitude in response to 1μM ATP application was -1.58 ±0.48nA (n = 12). P2X₁ receptors which were treated in 100μM suramin, prior to UV irradiation in the presence of 30μM 2-azido ATP, had mean peak amplitudes that were only reduced by 13%. The mean response after 1μM ATP application was -3.2 ±0.42nA (figure 2.12).

One possibility for the protection of the current response is that the addition of ATP or suramin caused an increased absorbance of UV light. This is called molar extinction. Molar extinction refers to the potential absorbance of the UV light by the molecules of ATP or suramin. If the light was absorbed by these molecules, then less light would have been able to penetrate down to the P2X₁ receptors themselves. To test this theory, 300μM adenosine was used to protect the P2X₁ receptors. Adenosine has no pharmacological action at the P2X receptors (Burnstock, 1978) and so should not provide protection from the effects of UV irradiated 2-azido ATP. However if molar extinction played a major factor in the previous results, adenosine should absorb the UV light and no significant reduction in the 1μM ATP response would be observed.

Results showed that 300μM adenosine did not protect the P2X₁ receptors from significant reduction of the current produced by 1μM ATP application. Currents were reduced 86% which is similar to the results recorded from cells treated in 30μM 2-azido ATP and UV irradiated. Recorded currents were -0.53 ±0.17nA (n = 20) (figure 2.12).
Figure 2.12. 2-azido cross-linking reduced by pre-treatment with excess suramin or ATP

A. Wild-type P2X<sub>1</sub> cells were plated onto 13mm coverslips and placed in a 8.8cm<sup>2</sup> petri dish. The media was replaced with 1.5ml of 100µM suramin or 300µM ATP which were dissolved in extracellular solution (pre-treatment with 300µM ATP is indicated by green bar). 1.5ml of 30µM 2-azido ATP was added (indicated by red bar) and the cells were left to equilibrate for 5min. After 5min the cells were UV irradiated for 3min using the established protocol (UV irradiation indicated by yellow vertical bar). Cells were then placed in the recording chamber and were continuously perfused with extracellular solution for 5min before patch clamp recordings were made. The peak amplitude responses to 1µM ATP application were recorded (ATP application indicated by open bar). B. Current traces showed that cells, UV irradiated in the presence of 2-azido ATP, produce reduced responses to 1µM ATP application. However these currents were protected when the cells were pre-treated with 300µM ATP or 100µM suramin (in addition to 30µM 2-azido ATP) prior to UV irradiation. C. Summary bar graph shows that pre-treating cells with either 300µM ATP or 100µM suramin can prevent significant current inhibition. This receptor protection was specific to P2X<sub>1</sub> since pre-treatment with 300µM adenosine did not provide the receptor with protection after UV exposure in the presence of 30µM 2-azido ATP. (* * = P ≤ 0.01 and ** * = P≤ 0.001 and show significant difference from UV irradiation with 2-azido ATP).
These results indicate that both ATP and suramin are capable of protecting P2X₁ receptors from the inhibitory effects of cross-linking and that the protection was not due to the effects of molar extinction. This provides more evidence in the identification of 2-azido ATP as a cross-linking compound at the P2X₁ receptor.

### 2.3.8 Radiolabelling at the P2X₁ receptor

Electrophysiological recordings have provided a great deal of evidence to indicate that 2-azido ATP successfully binds to P2X₁ receptors and cross-links after UV irradiation. However the binding of 2-azido ATP can be visualized with the use of a radiolabelled version of 2-azido ATP. In addition, the protective effects of excess ATP can also be visualized. A corroboration of the previous results would finally confirm that 2-azido has been identified as a cross-linking compound at the P2X₁ receptor.

To detect the binding of 2-azido ATP to the receptor, 2-azido [$γ^{32}\text{P}$] ATP was used. Due to financial constraints and the reduced availability of the compound, HEK293 cells stably expressing the P2X₁ receptor were treated with either 1μM (0.32MBq) or 10μM (3.2MBq) 2-azido [$γ^{32}\text{P}$] ATP instead of 30μM which had been previously established. To determine the level of non-specific binding, non-transfected HEK293 cells were treated similarly. Both sets of cells were UV irradiated and the cells were homogenised. To remove background levels and increase specificity, the cross-linked P2X₁ receptor was isolated by immunoprecipitation with the P2X₁ antibody (Alomone) and the samples were run on a 10% SDS gel. Exposure of the dried gel to autoradiography film produced intense bands which corresponded to the sizes of the glycosylated P2X₁ monomer (~55kDa) and dimer (~110kDa). The low background also suggests that the immunoprecipitation step was very effective. The lack of binding at HEK293 cells shows the specificity of 2-azido ATP and the P2X₁ receptor. Intense bands of the same size were observed after labelling in 1μM or 10μM 2-azido ATP (figure 2.13) and the intensity was markedly reduced when P2X₁ cells were pre-treated with 10μM ATP (in addition to 2-azido ATP). This indicated that ATP protected the receptors from cross-linking to 2-azido ATP by occupying the majority of sites prior to UV irradiation (figure 2.11). The reduced level of radiolabelling is visualised as a reduction in the level of intensity in the bands. In conclusion this
Figure 2.13. Radiolabelled cross-linking with P2X\(_1\) protein. Samples of non-transfected HEK293 cells and HEK293 cells stably expressing the P2X\(_1\) receptor were treated in 1\(\mu\)M (0.32MBq) or 10\(\mu\)M (3.2MBq) 2-azido-\(\gamma^{32}\)P ATP respectively. In addition, cells stably expressing the P2X\(_1\) receptor were co-treated with 10\(\mu\)M ATP and then 1\(\mu\)M 2-azido ATP. All samples were UV irradiated for 3 mins. After lysis, the protein was immunoprecipitated with the P2X\(_1\) antibody (Alomone) and ran on 10% SDS PAGE. After drying, the gel was exposed to autoradiography film for 3 days at -80°C. Intense bands appeared at a similar weight range for the glycosylated monomer (-55kDa) and dimer (-110kDa) of P2X\(_1\) protein and suggest successful labeling by 2-azido ATP. 1\(\mu\)M 2-azido treatment shows intense binding to the P2X\(_1\) receptor but, as indicated in the electrophysiological study, this binding is significantly reduced if the receptor is protected with excess ATP (10\(\mu\)M).
evidence corroborates the findings made earlier and also directly suggests that ATP and 2-azido ATP compete for the same binding site on the P2X₁ receptor. It also confirms the identification of 2-azido ATP as a cross-linking compound at the P2X₁ receptor.
2.4 Discussion

The P2X<sub>1</sub> receptor was first cloned from rat vas deferens and urinary bladder in 1994 (Valera et al, 1994) and the sequence of the 399-amino acid human protein is 89% identical to that of the rat and mouse homologues. The receptor has been found to regulate smooth muscle contractility in a number of organs such as the urinary bladder, arteries and vas deferens (Valera et al, 1994; Collo et al, 1996; Vulchanova et al, 1996; Lewis & Evans, 1995; Mulryan et al, 2000; Vial et al, 2000). They are also found to be well expressed in platelets (Vial et al, 1997) where they serve an important role in platelet physiology and haemostasis (Vial et al, 2002). Recombinant P2X<sub>1</sub> receptors heterologously expressed in either oocytes or mammalian cells show that the agonist actions of ATP can be mimicked by the ATP analogue, alpha beta methylene ATP (αβme-ATP) (Evans et al, 1995). With an EC<sub>50</sub> close to 1 μM, both ATP and αβmeATP can elicit approximately equal currents (North & Suprenant, 2000; North, 2002). The currents elicited by ATP (and other agonists) quickly desensitize in the continued presence of ATP and the receptors require periods >5min between repeat applications for reproducible currents to be obtained (Valera et al, 1994; Rettinger & Schmalzing, 2000).

The results described in this chapter have identified 2-azido ATP as a successful cross-linking compound at the P2X<sub>1</sub> receptor. Application of 2-azido ATP evoked concentration-dependent inward currents in the P2X<sub>1</sub> receptor that rapidly desensitized. When P2X<sub>1</sub> cells were pre-treated with 2-azido ATP and UV irradiated for 3min, the amplitude of P2X<sub>1</sub> receptor current, following 1μM ATP application, was reduced by ~ 90%. This reduction in the ATP response was only observed when P2X<sub>1</sub> cells were UV irradiated in the presence of 2-azido ATP. When P2X<sub>1</sub> receptors were treated in 2-azido ATP and UV irradiated, the reduction in response to ATP was maintained throughout the recorded time period and so was not attributable to simple desensitization of the receptors. The significant reduction in the amplitude of the ATP response was prevented by pre-treatment with either suramin or ATP. Finally, direct evidence of 2-azido ATP cross-linking was provided with the use of 2-azido [γ<sup>32</sup>P] ATP.
The P2X₁ receptor does not contain any commonly occurring consensus ATP binding motifs such as the Walker A motif. This suggests an ATP binding site that is uncharacteristic and distinct. Reactive ATP analogues have been successful in probing various enzymes and receptors to allow analysis of some the amino acid residues within their nucleotide binding sites. The identification of a cross-linking ATP analogue which can permanently label the P2X₁ receptor may help subsequent studies reveal the identity of some of the amino acid residues which contribute to the ATP binding site. In addition, mutational analysis studies have implicated certain conserved amino acid residues in the extracellular loop which contribute to the ATP binding site. ATP analogues may provide evidence to corroborate those findings.

FSBA is an analogue of ATP that cross-links to a number of amino acid residues in the ATP-binding sites of a wide range of enzymes. FSBA has intact adenosine and ribose moieties but does not have a triphosphate tail (figure 2.14). In place of the first phosphate group there is a carbonyl group, the second phosphate is replaced by a benzene ring and instead of the terminal phosphate of ATP there is a sulfonyle fluoride moiety. This reactive sulfonyle fluoride moiety confers the ability of FSBA to bind to a broad range of nucleotide binding sites. Though this is a marked alteration from the structure of ATP, FSBA has been shown to competitively bind to many kinases and proteins that have nucleotide-binding domains.

**Figure 2.14.** A comparison between the structures of (A.) ATP and (B.) FSBA.

This chapter has shown that FSBA was unable to cause any change in the recordings taken from the P2X₁ receptor. Though having been incubated with 100μM FSBA for 1 hour, FSBA had no effect on the amplitude of the response to 1μM ATP application. The unaltered current responses showed that FSBA either has no binding ability at the
ATP binding site of the P2X<sub>1</sub> receptor or it binds to the P2X<sub>1</sub> receptor in another area that does not affect the function of the receptor. FSBA binds specifically to tyrosine, lysine, histidine, serine and cysteine (Colman, 1983). It is also possible that FSBA binds within the ATP binding pocket but does not come into close enough contact with either of these specific residues and therefore does not permanently attach itself to the receptor. Any of these different scenarios could explain the inactivity of FSBA at the P2X<sub>1</sub> receptor. If the inactivity was a reference to the change in the structure (excised phosphate tail) this would suggest the importance of an intact phosphate tail in the activation of the P2X<sub>1</sub> receptor. The importance of the phosphate tail is already implied in the inability of activation or antagonism of the P2X<sub>1</sub> receptor by ADP (Mahaut-Smith et al, 2000). Additionally, GTP has also been shown to be inactive at the P2X<sub>1</sub> receptor (Valera et al, 1994; Chen et al, 1995). This suggests that both the adenine group and the phosphate tail are important co-ordinates necessary for recognition of the compound.

Photoaffinity labels have been increasingly popular since the late 1970s. As noted in a paper by Williams et al (1986), “When successfully designed and applied, these molecular probes seek out selective domains on macromolecular targets for which they possess a substrate-like affinity, and upon irradiation with actinic light, form covalent cross-linkages at these specific binding sites.” This high affinity, irreversible binding to the active site allows an investigator to examine the primary sequence of the active site. Photoaffinity cross-linkers have advantages over chemical counterparts, such as FSBA. The respective structures are and can be extremely close to the parent compound. The attached reactive group (carbenes and nitrenes) can indiscriminately react with a wide variety of chemical groups; therefore there is less restriction with the number of amino acids that can be affected. Most importantly, photoaffinity agents remain chemically unreactive until deliberate activation, thereby allowing the investigation of its initial affinity to the protein in question.

The photoactive cross-linking compound 2-azido ATP contains a substitution at the C-2 position of the adenine ring. Substitutions at the 2-position have already been shown to be tolerated and compounds such as 2-chloro ATP and 2-methylthio ATP have been shown to be equipotent agonists at recombinant P2X<sub>1</sub> receptors (EC<sub>50</sub> ~ 1μM). These results agreed with the original study of the activity of ATP analogues
with C-2 substitutions on tissue from the guinea pig urinary bladder (Cusack & Hourani, 1990). Investigation of 2-azido ATP's agonist ability showed that 1μM application produced currents which were about half of the 1μM ATP control. Results showed that it produced quickly desensitizing inward currents similar to those caused by ATP. A concentration-dependent increase was observed when the concentration of 2-azido ATP was increased to 10μM and 30μM.

UV irradiation causes a chemical change in photoactive compounds leading to the formation of covalent bonds in and around the binding site and causing inhibition to the normal agonist response. The initial optimization of the experimental setup shown in figure 2.3 fully ensured that any reduction in the 1μM ATP response was due to cross-linking and not due to cell/receptor damage brought about by UV exposure.

The results shown in figure 2.9 clearly show that the significant reduction in the peak current amplitude response to ATP application was caused by UV irradiation of cells treated in 2-azido ATP. When cells were treated in ATP or extracellular solution and UV irradiated (for 3mins), no such reduction took place. This suggests that the majority of P2X<sub>1</sub> receptors were cross-linked to 2-azido ATP and were held in their desensitized state.

A similar reduction in function was observed in whole tissue. In 1982 Jeffery Fedan and his colleagues used an azido-bound, photo-active compound to study the biphasic tissue response of the guinea-pig vas deferens (Fedan et al, 1982). They used the P2X receptor antagonist arylazido aminopropionyl ATP (ANAPP<sub>3</sub>) to investigate the mechanisms by which ATP causes the tissue to contract. Though conducted in whole tissue, the results were similar to those reported in this chapter. They UV irradiated vas deferens tissue treated in ANAPP<sub>3</sub> and they observed a significant decrease in the initial phase of the response usually brought by ATP application. Thus the native biphasic response was reduced to a monophasic response. The P2 receptors, now known to be P2X<sub>1</sub>, responsible for the initial phase of contraction were permanently cross-linked. This allowed the secondary phase to continue (Fedan et al, 1982).
Although the significantly reduced ATP response served as a good piece of evidence in the favour of the cross-linking ability of 2-azido ATP, further control experiments were necessary to prove without a doubt that the results observed were the result of permanent photo-insertion of the compound within the ATP binding pocket.

The activation of wild-type P2X₁ receptors causes desensitization. After wash-off of the agonist there is a period of recovery that is required before reproducible responses can be recorded. This long refractory closed state has been shown previously (Rettinger and Schmalzing, 2004). Investigation showed that pre-treatment of P2X₁ cells in 2-azido ATP or ATP did reduce the subsequent ATP response. However the response returned within 10min of continuous wash with extracellular solution. The UV irradiation of P2X₁ cells pre-treated with 2-azido ATP caused a reduction in response that did not return after 45min of continuous washing. This again suggests that the receptors were cross-linked and so were unable to be occupied and activated by ATP.

Further evidence for the action of 2-azido ATP at the ATP binding site was provided with the study on receptor protection. When P2X₁ cells were pre-treated in excess suramin or ATP (in addition to 2-azido ATP) and UV irradiated, the decrease in the ATP response was significantly less than that observed from UV irradiation in the presence of 2-azido ATP. This suggested that the P2X₁ receptors were prevented from cross-linking to the photoactive compound. This protection was not influenced by the effects of molar extinction. Molar extinction can be defined as the fraction of light lost to scattering and absorption per unit distance. Thus the protection of the response could have been due to a reduction in UV light reaching 2-azido ATP. The failure of adenosine to prevent the reduction in response emphasized the non-existent effect of molar extinction and confirmed that the majority of the receptors were indeed protected.

The results from the protection study suggest that the excess concentrations of ATP and suramin protected the receptors by competing for occupancy of the ATP binding site (figure 2.11). Since there were more molecules of ATP or suramin there was a greater probability that receptors would be occupied by either compound instead of 2-azido ATP. Upon UV irradiation, only a minority of receptors would have had 2-
azido ATP covalently attached to the receptor. This would have left the majority of receptors free to bind with ATP after receptor recovery from desensitization. The results also showed that the protection provided by excess suramin was more effective than that provided by excess ATP. This may suggest that suramin binds to an alternative site and causes non-competitive inhibition by altering the ATP binding site.

Positive radio-labelling of the P2X<sub>1</sub> receptor with 2-azido [γ<sup>32</sup>P] ATP provided the final piece of evidence. The intense bands which corresponded to P2X<sub>1</sub> protein directly showed positive association of 2-azido [γ<sup>32</sup>P] ATP and the receptor. Radio-labelling also substantiated the results recorded from the protection study. The decrease in the intensity of the bands after pre-treatment with excess ATP (in addition to 2-azido ATP) again suggest that ATP and 2-azido ATP competed for occupancy of P2X<sub>1</sub> receptor agonist binding site.

In conclusion, these results suggest that 2-azido ATP is a suitable compound which can be used to probe the ATP binding site of the P2X<sub>1</sub> receptor. In addition to 2-azido ATP there are other ATP analogues which contain reactive sites at different positions around the ATP molecule. The protocols developed in this chapter can be used to identify other potential cross-linking compounds with activity at the P2X receptors.
2.5 Summary and Conclusion

The evidence describing the effectiveness of 2-azido ATP as a cross-linking compound at the P2X<sub>1</sub> receptor is as follows:

- 2-azido ATP, a photoactive compound (with similarities to the parent compound ATP), can act as an agonist at the P2X<sub>1</sub> receptor.
- UV irradiation of cells (expressing the P2X<sub>1</sub> receptor) treated in 2-azido ATP caused a significant reduction in the peak amplitude current associated with 1μM ATP application. This implied cross-linking of the compound to the receptor.
- When cells were exposed to a corresponding concentration of ATP or 2-azido ATP, cells recover within 10mins. However, the reduction caused by UV irradiation in the presence of photoactive compound caused a stable reduction that remained over time. Therefore the reduction in ATP response cannot be attributed to desensitization.
- 2-azido ATP seemed to compete directly with ATP for occupancy of the binding site. Receptors can be protected if cells are exposed to an excess of ATP or suramin prior to UV irradiation in 2-azido ATP. This allowed the cell response to ATP to be recovered.
- Further evidence was provided via the use of a radio-labelled version of 2-azido ATP. Specificity of binding to the P2X<sub>1</sub> receptor was observed, as was the decrease in photo-insertion brought about by pre-treatment of cells in micro-molar levels of ATP. This is in agreement with the electrophysiological study and provides direct evidence of the permanent association of 2-azido ATP with the P2X<sub>1</sub> receptor.

This chapter concluded with the identification of 2-azido ATP as a successful cross-linking compound at the P2X<sub>1</sub> receptor. There are other photoactive compounds which may be effective at the P2X<sub>1</sub> receptor. The difference in the positioning of their reactive groups may allow for greater definition of the ATP binding site. Consequently, the development of the optimized cross-linking protocol and the further control methods will help identify other potential ATP analogues with similar ability.
CHAPTER 3
FURTHER IDENTIFICATION OF PHOTOACTIVE CROSS-LINKING
COMPOUNDS AND THEIR ACTIVITY AT OTHER P2X RECEPTOR
SUBTYPES

3.1 Introduction

The previous chapter concluded with the successful development of a UV cross-linking assay and the identification of 2-azido ATP as a photoactive ATP analogue which can bind and cross-link to the P2X$_1$ receptor. There are a range of compounds which have reactive sites at different co-ordinates around the ATP molecule. In addition to alterations at the adenine ring, substitutions at the ribose and phosphate tail may help to reveal other amino acid residues which contribute to the binding site within P2X receptors.

In this chapter I shall again use compounds containing the azido group. These compounds are 8-azido ATP and ATP azidoanilide (ATP-AA). They both contain the reactive azido group (–N$_3$) and are activated by UV light in the same manner as 2-azido ATP (figure 3.1).

![8-azido ATP and ATP azidoanilide](image)

Figure 3.1. A. 8-azido ATP. B. ATP azidoanilide. The azido group (N$_3$) can be positioned at different co-ordinates across the ATP molecule. Upon irradiation a highly reactive nitrene is formed, the lone pairs of electrons can help form a covalent linkage between the photo-probe and the receptor.

ATP benzophenone (ATP-BP) contains the reactive benzophenone group. Unlike azido, upon UV irradiation the oxygen double bond is broken, leaving lone pairs of electrons. This highly reactive species allows the hydrogen, from the protein to which
the nucleotide is bound, to be extracted resulting in a covalent linkage between the
two molecules. Bz-ATP (2', 3'-O-(4-benzoylbenzoyl)-ATP) also works in a similar
manner (figure 3.2). It too has been shown to have the ability to incorporate itself into
nucleotide binding sites after UV irradiation (Williams et al, 1986; Erb et al, 1993).

![Chemical structures](image)

**Figure 3.2.** A. Bz-ATP. B. ATP benzophenone. Another photoactive moiety that
can be attached to ATP is the benzophenone group. Upon exposure to UV light, the
benzophenone group's double-bonded oxygen (highlighted in the diagram) forms a
highly reactive species that can abstract hydrogen from the protein to which the
nucleotide is bound, ultimately causing the two molecules to be covalently linked.

The pharmacological characteristics of heterologously expressed P2X receptors have
been comprehensively reviewed in Gever et al (2006). The characteristics of P2X
subunits can be classified into four main groups: (i) agonist selectivity; (ii) time
course of desensitization; (iii) antagonist selectivity and (iv) ionic modulation. P2X₁
and P2X₃ receptors exhibit α,βMe-ATP sensitivity and both desensitize quickly in the
continued presence of the agonist (Valera et al, 1994; Chen et al, 1995). They are
also both sensitive to inhibition with nanomolar concentrations of TNP-ATP (Virginio
et al, 1998), and suramin and PPADS are potent inhibitors that have been shown to
block receptor current (Evans et al, 1995). In contrast, P2X₂ and P2X₄ receptors
desensitize slowly and are insensitive to α,βMe-ATP (Brake et al, 1994; Bo et al,
1995; Evans et al, 1995). They are also significantly less sensitive to TNP-ATP,
suramin and PPADS (P₂X₄ sensitivity dependent on species type). One of the many
reasons that may account for the similarities and differences between P2X receptor
subtypes is the amino acid residues that contribute to the ATP binding site of each
respective subtype. The success or failure of different photoactive cross-linking
compounds may reflect some of these similarities or differences and may in turn
reveal some of these amino acid residues.
In this chapter I aim to use the protocols previously developed to identify other photoactive compounds which successfully cross-link to the P2X₁ receptor. In addition, I will investigate their cross-linking abilities at P2X receptor subtypes P2X₂, P2X₃ and P2X₄.
3.2 Materials and Methods

3.2.1 Cell Culture
Human embryonic kidney (HEK293) cells stably expressing the human P2X$_1$ or human P2X$_4$ receptors were cultured as described in chapter 2 (section 2.2.1).

Wild type HEK293 cells, at 80-90% confluence, were plated onto 40mm cell culture dishes. For each dish of cells, transient transfection was conducted using 1μg DNA (rat P2X$_2$ and rat P2X$_3$) and 10μl lipofectamine 2000 (invitrogen) in 500μl of serum-free Opti-MEMI. To confirm the presence of transfected protein, GFP (green fluorescent protein) was co-transfected. After 6-10 hrs incubation, cells were plated onto 13mm coverslips and left to grow in DMEM cell culture medium. Cells were subjected to experiments 24-48hrs after transfection. Single cells that expressed green fluorescence implied expression of the transfected gene (P2X$_2$ or P2X$_3$) and were subjected to electrophysiological recording. The preparation of plates, cell culture and transfection all took place in a laminar flow hood under sterile conditions.

3.2.2 Electrophysiological recordings
Electrophysiological recordings were performed as previously described in chapter 2 (section 2.2.2).

3.2.3 Drugs
ATP, FSBA, Bz-ATP, 8-Bromo ATP, 2-methylthio ATP and 2-chloro ATP were obtained from Sigma. 2-azido ATP, ATP γ 4-azidoanilide, ATP γ benzophenone and 8-azido ATP were obtained from Affinity Labelling Technologies Inc. Prior to application, all drugs were dissolved in Et$_2$O to form stock solutions.

3.2.4 Cross-linking Protocol
The cross-linking protocol was performed as previously described in chapter 2 (section 2.2.4).

3.2.5 Radio-labelled binding study
Native HEK293 cells and wild-type P2X$_1$ cells were cultured in 24-well plates in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) with Earle’s salts, glutamax 1, 10% foetal calf serum and 1% non-essential amino acids. Wild-type P2X$_1$ cells
were maintained under permanent selection in 600µg/ml G418 (Invitrogen). When cells reached 80% confluence the growth media was removed and replaced with 1µM ATP azidoanilide-[γ³²P] (0.92MBq, Affinity Labelling Technologies Inc) at 0.5ml/well. The 24-well plate containing these cells was placed in the UV box and continually irradiated by 312nm of UV light for 3min. The 24-well plate was placed on ice and unbound ³²P labelled ATP azidoanilide was removed. The cells were then washed twice in 1ml PBS and were lysed into a homogenising buffer (100µl) containing 150mM NaCl, 1mM EDTA, 1mM EGTA, 40mM Tris HCl (pH7.4), 8mM Tris base, 1% triton X and a mixture of protease inhibitors (1:100 dilution) (Sigma). The cell homogenate was centrifuged for 5min at 13,000rpm (4°C) on a benchtop centrifuge and the cell debris was discarded. A stock solution of P2X₁ antibody (0.3mg/ml) (Alomone) was prepared and 3µl of the solution was added to 85µl of the supernatant. The mixture was allowed to incubate on ice for 1hr. The resultant antibody-antigen complex was precipitated with the addition of 75µl protein A-sepharose beads (30mg/ml) (Amersham) and rolled at 4°C overnight. 1ml homogenising buffer was added to the protein A-sepharose beads (complexed with the antibody and antigen) and the mixture was spun on a benchtop centrifuge at 13,000rpm for 5min. The supernatant was removed with a pump aspirator and discarded. The process was repeated three times in total to enable the beads to be washed of any unbound radioactive material. 20µl of sample buffer (with 5% β-mercaptoethanol) was added to the protein A-sepharose P2X₁ pellet. The pellet was heated to 95°C for 5min to allow the protein to elute from the beads. 20µl of the sample was removed and loaded onto a 10% SDS-PAGE gel and run at 120V. For autoradiography, the gels were dried and exposed to x-ray film for 48-72hrs at -80°C.

3.2.6 Data Analysis

The results are expressed as mean ± standard error of mean and analyzed using the appropriate Student’s T-test with P values ≤ 0.05, when compared to the control considered as significant (*). P values ≤ 0.01 were considered very significant (**) and P values ≤ 0.001 were considered highly significant (***) Data analysis was carried out with the computer package, “Excel” and the graphics package “Origin 6” was used to construct the graphs.
3.3 Results

3.3.1 Cross-linking Compounds as Agonists

The ATP analogue Bz-ATP has already been well established as a partial agonist at the P2X1 receptor (Bianchi et al, 1998; Roberts & Evans, 2004). As a control, the mean peak amplitude response to 1μM ATP application was -3.68 ±0.38 nA (n = 5). Bz-ATP application to wild-type P2X1 cells evoked inwardly transient currents that desensitized rapidly in the continued presence of the agonist. 1μM Bz-ATP application produced mean currents of -1.85 ±0.43 nA (n = 14). These currents were about half of the 1μM ATP control (figure 3.3).

In contrast 1μM 8-azido ATP had no effect on the holding current of P2X1 cells. Mean recorded currents were -0.05 ±0.03 nA (n = 6). Similarly, 1μM application of ATP azidoanilide or ATP-benzophenone didn’t produce any changes in the holding current of P2X1 cells. Mean current responses were measured at -0.04 ±0.01 nA (n = 4) and -0.036 ±0.02 nA (n = 5) respectively (figure 3.3). These results suggested that these ATP analogues had a lower potency at the P2X1 receptor. An increase in the concentration to 100μM was tried. 100μM 8-azido ATP application produced transient inward currents. The mean peak amplitude was -0.862 ±0.36 nA (n = 4) (figure 3.3). Similar rises in the response were observed with 100μM ATP azidoanilide or 100μM ATP-benzophenone application. They both produced transient inward currents with mean peak amplitudes of -2.09 ±0.45 nA (n = 10) and -2.51 ±0.48 nA (n = 18) respectively (figure 3.3). The reduced response from the application of 100μM 8-azido ATP suggested that the compound was a weak agonist at the P2X1 receptor.

3.3.2 Substitutions at the 2 and 8 Position of the Adenine Ring

Both 2-azido ATP and 8-azido ATP have substitutions located in the adenine moiety, however 1μM application of each compound to P2X1 cells produced significantly different results (figure 3.3). To briefly investigate whether the reduction in the activity of 8-azido ATP was indicative of substitutions at this position, further ATP analogues were tested at P2X1 cells.
Figure 3.3. The agonist activity of the varying photoactive compounds at the P2X$_1$ receptor.

A. Whole cell patch clamp traces show application of photoactive compounds to the P2X$_1$ receptor. 1μM ATP evoked a desensitizing, inward current (application of 1μM ATP is indicated by open bar). The application of each photoactive compound evoked different responses in the P2X$_1$ receptor (1μM application indicated by the black bar). B. Summary bar graph shows the average P2X$_1$ response after 1μM application of each photoactive compound. C. Whole cell patch clamp traces comparing the 1μM ATP control with 100μM applications of 8-azido, ATP-AA and ATP-BP. D. Summary bar graph shows the average currents produced by 100μM application of 8-azido, ATP-AA and ATP-BP.
The ATP analogues 2-methylthio ATP and 2-chloro ATP were tested for their agonist activity at 1µM. The application of 1µM 2-methylthio ATP evoked inward currents that desensitized rapidly. The mean peak amplitude was -0.89 ±0.24nA (n = 10), this was ~ 35% of the mean peak amplitude recorded after the application of 1µM ATP (-3.09 ±0.24nA (n = 5)). When 1µM 2-chloro ATP was applied a larger inward current was produced. The mean peak current measured was -3.34 ±0.85nA (n = 7) (figure 3.4).

Similarly to 8-azido ATP, 8-bromo ATP has a substitution at the 8th position of the adenine ring. Application of 1µM 8-Br ATP to wild-type P2X1 cells produced no significant change in the holding current. The mean current amplitude was -0.08 ± 0.03nA (n = 13), this was ~ 4% of the peak amplitude recorded from the 1µM ATP control (figure 3.5). Indeed, there had to be an increase to 100µM before comparable currents were observed. At 100µM, 8-Br ATP evoked transiently inward currents that rapidly desensitized. The mean peak amplitude was -2.47 ±0.46nA (n = 11) (figure 3.5). These results, which may have been influenced by a contamination with ATP, suggest the P2X1 receptor can accept changes to the ATP molecule at the 2 position whilst changes at the 8 position are not as readily tolerated. This may indicate the importance of this position during binding of the compound with the ATP binding site of the P2X1 receptor.

3.3.3 UV Irradiation of ATP analogues
UV irradiation of photoactive compounds converts the compound from its stable state to a reactive intermediate. If the compound successfully binds within the agonist site a covalent bond can be formed in the area. This should allow it to be labelled for further analysis. The concentration at which each ATP analogue produced activity comparable to 1µM ATP was used as the concentration to test whether the analogue could cross-link to the P2X1 receptor. Hence Bz-ATP was tested at 10µM and ATP-azidoanilide, ATP-benzophenone, 8-azido ATP and TNP-ATP were tested at 100µM.

The control response from 1µM ATP application to untreated P2X1 cells was -4.5 ±0.66nA (n = 14). The UV irradiation of P2X1 cells pre-treated in 10µM Bz-ATP reduced the subsequent peak amplitude response to 1µM ATP. The mean peak
Figure 3.4. Do substitutions at the 2 position of the adenosine ring cause changes in a compound's activity? 

A. Whole cell patch clamp traces and B. bar chart show the change in the activity of compounds with substitutions at the 2-position of the ATP molecule. At 1μM all the compounds investigated (2-azido, 2-methylthio and 2-chloro ATP) caused a response at the P2X<sub>1</sub> receptor. Only methylthio ATP gave a significantly smaller current than the ATP control. (* * * = P≤ 0.001 and shows significant difference from the 1μM ATP control).
Figure 3.5. Do substitutions at the 8 position of the adenosine ring cause changes in a compound’s activity? A. Whole cell patch clamp traces and B. bar chart show the change in activity of compounds with substitutions at the 8-position of the ATP molecule. At 1μM both 8-azido ATP and 8-Bromo ATP did not evoke a substantial response in P2X<sub>1</sub> cells after application. The concentration had to be increased to 100μM before comparable currents were observed. (** * = P≤ 0.001 and shows significant difference from the 1μM ATP control).
amplitude was \(-0.5 \pm 0.12\) nA (\(n = 52\)) (figure 3.6). This was an 89% reduction in the P2X₁ current response.

When P2X₁ cells were UV irradiated in the presence of 100μM ATP azidoanilide, reductions of 67% were observed; currents were reduced from \(-3.66 \pm 0.65\) nA (\(n = 10\)) to \(-1.2 \pm 0.43\) nA (\(n = 30\)) (figure 3.7). Similarly, 100μM ATP benzophenone caused a 69% reduction in response when cells were UV irradiated in its presence. The average peak current was reduced from \(-3.78 \pm 0.56\) nA (\(n = 11\)) to \(-1.19 \pm 0.17\) nA (\(n = 35\)) (figure 3.8).

The irradiation of P2X₁ cells treated in 100μM 8-azido ATP did not cause any change in the subsequent response of the P2X₁ cells to 1μM ATP application. Untreated P2X₁ cells responded to 1μM ATP application with a mean peak amplitude of \(-3.38 \pm 1.3\) nA (\(n = 4\)). P2X₁ cells pre-treated in 100μM 8-azido ATP and UV irradiated subsequently produced peak amplitude currents of \(-3.08 \pm 0.53\) nA (\(n = 6\)) in response to 1μM ATP application (figure 3.9).

TNP-ATP is a known potent inhibitor at the P2X₁ receptor (Lewis et al, 1998) and a fluorescent compound (Hiratsuka, 2003). When 100μM TNP-ATP was left to equilibrate with wild-type P2X₁ cells for 5min, it reduced the 1μM ATP response from \(-4.4 \pm 0.87\) nA (\(n = 11\)) to \(-0.12 \pm 0.02\) nA (\(n = 11\)) (figure 3.10). To further confirm that the inhibitory effect of cross-linking was specifically caused by UV irradiation of photoactive compounds, TNP-ATP was also tested for its cross-linking ability at the P2X₁ receptor. The mean peak current produced from P2X₁ cells pre-treated in 100μM TNP-ATP and UV irradiated, were indistinguishable from those recorded from untreated P2X₁ cells. Untreated P2X₁ cells produced mean peak currents of \(-3.22 \pm 0.56\) nA (\(n = 14\)). When P2X₁ cells were UV irradiated in the presence of 100μM TNP-ATP, the peak amplitude current in response to 1μM ATP application was \(-3.64 \pm 0.44\) nA (\(n = 26\)) (figure 3.10). Similarly to the results recorded from UV irradiated 30μM ATP, the inability of TNP-ATP to cross-link to the P2X₁ receptor confirmed that it was specifically the UV irradiation of particular photoactive compounds which caused the inhibition of the ATP response in P2X₁ receptors.
Figure 3.6. Does UV irradiation in the presence of Bz-ATP block P2X<sub>1</sub> receptor responses? A. P2X<sub>1</sub> cells were treated in 10μM Bz-ATP (UV irradiation in the presence of Bz-ATP is indicated by the yellow vertical bar) and UV irradiated at room temp for 3min. Cells were then placed in the recording chamber and washed in extracellular solution for 5min prior to patch clamp recording. The response to 1μM ATP application (application indicated by open bar) provided evidence to the inhibitory effects of cross-linking. B. Whole cell patch clamp recordings shows that UV irradiation in the presence of 10μM Bz-ATP leads to a reduction of subsequent 1μM ATP responses. C. Summary bar graph shows > 80% reduction in recorded 1μM ATP responses after cells were UV irradiated in the presence of 10μM Bz-ATP. (* * * = P≤0.001 and shows significant difference from the 1μM ATP control).
Figure 3.7. Does UV irradiation in the presence of ATP-AA effect P2X$_1$ receptor responses? A. Cells were treated in 100μM ATP-AA (UV irradiation in the presence of ATP-AA is indicated by the yellow vertical bar) and UV irradiated at room temp for 3min. Cells were then placed in the recording chamber and washed in extracellular solution for 5min prior to patch clamp recording. The response to 1μM ATP application (application indicated by open bar) provided evidence to the inhibitory effects of cross-linking. B. Whole cell patch clamp traces show the ability of ATP-AA, when UV irradiated, to reduce the ATP response from HEK293 cells stably expressing the P2X$_1$ receptor. C. Summary bar graph shows that currents are reduced by 67% after UV irradiation in the presence of 100μM ATP-AA. (* = $P \leq 0.01$ and show significant difference from the 1μM ATP control).
Figure 3.8. Does UV irradiation in the presence of ATP-BP affect P2X<sub>1</sub> receptor responses? A. Cells were treated in 100µM ATP-BP (UV irradiation in the presence of ATP-BP is indicated by the yellow vertical bar) and UV irradiated at room temp for 3min. Cells were then placed in the recording chamber and washed in extracellular solution for 5min prior to patch clamp recording. The response to 1µM ATP application (application indicated by open bar) provided evidence to the inhibitory effects of cross-linking. B. Patch clamp traces show that cells UV irradiated in the presence of 100µM ATP-BP cause a significant reduction in the normal response evoked by 1µM application. C. Summary bar graph shows that ATP-BP reduces subsequent ATP currents by 69% following UV irradiation in its presence. (* * * = P ≤ 0.001 and show significant difference from the 1µM ATP control).
Figure 3.9. Does UV irradiation in the presence of 8-azido ATP effect P2X\textsubscript{1} receptor responses? A. Cells were treated in 100μM 8-azido ATP (UV irradiation in the presence of 8-azido ATP is indicated by the yellow vertical bar) and UV irradiated at room temp for 3min. Cells were then placed in the recording chamber and washed in extracellular solution for 5min prior to patch clamp recording. The response to 1μM ATP application (application indicated by open bar) provided evidence to the inhibitory effects of cross-linking. B. Unlike 2-azido ATP, patch clamp traces show that 8-azido ATP does not reduce the ATP response after UV irradiation in its presence. C. Summary bar graph shows that currents are maintained at -3.08 ± 0.53nA (n = 6) despite cells being UV irradiated in the presence of 100μM 8-azido ATP. This suggests that the agonist action observed was probably due to ATP contamination.
Figure 3.10. Does UV irradiation in the presence of TNP-ATP effect P2X<sub>1</sub> receptor responses? A. P2X<sub>1</sub> cells were treated in 100μM TNP-ATP (UV irradiation in the presence of TNP-ATP is indicated by the yellow vertical bar) and UV irradiated at room temp for 3min. Cells were then placed in the recording chamber and washed in extracellular solution for 5min prior to patch clamp recording. The response to 1μM ATP application (application indicated by open bar) provided evidence to the inhibitory effects of cross-linking. B. Patch clamp traces show TNP-ATP that the ATP response is not reduced when cells are pre-treated with 100μM TNP-ATP and UV irradiated. C. Summary bar graph shows that there is absolutely no reduction in the 1μM ATP response following UV irradiation in the presence of TNP-ATP. Currents remained at -3.64 ± 0.44nA (n = 26) after UV irradiation. This experiment confirmed that UV irradiation of specific photoactive compounds resulted in cross-linking at the P2X<sub>1</sub> receptor and a reduction in the subsequent ATP response.
The reduction in the ATP response following UV irradiation of P2X₁ cells in the presence of Bz-ATP or ATP azidoanilide or ATP-benzophenone suggested successful cross-linking of the analogue with the P2X₁ receptor. The failure of 8-azido ATP and TNP-ATP to cause any change in the subsequent ATP response suggested that neither analogue cross-linked to the ATP binding site of the P2X₁ receptor.

### 3.3.4 Cell Recovery

The previous chapter described control experiments which can aid in the identification of a compound’s cross-linking ability at the P2X₁ receptor. The potential influence of desensitization can be recognized by investigating receptor recovery. The peak current amplitude recorded from P2X₁ cells (after 1μM ATP application) which were UV irradiated in the presence of photoactive compound was measured over time. These measurements were compared to recordings taken from P2X₁ cells treated in an equivalent concentration of ATP and P2X₁ cells treated in an equivalent concentration of photoactive compound without UV irradiation. After treatment, cells were placed in the recording chamber and were perfused with extracellular solution to allow wash-off. This wash-off lasted 45min, during which patch clamp recordings were continuously taken from different single cells.

As a control, the mean peak current amplitude recorded after 1μM ATP application to P2X₁ cells was measured at -4.5 ±0.48nA (n = 14). When P2X₁ cells were treated in 10μM ATP for 3min there was a recovery in response to 1μM ATP within 6 - 10min of wash. The response increased from -1.26 ±0.73nA (n = 43) in the first 5min to -3.77 ±0.19nA (n = 6) after 10min of continuous wash. Similarly, P2X₁ cells which were pre-treated in 10μM Bz-ATP for 3min showed a recovery of response within 10min. Recordings taken within the first 5min showed that 1μM ATP evoked a mean response of -1.94 ±0.43nA (n = 5). This increased to -2.68 ±0.54nA (n = 6) after 10min wash. This level of recovery was generally maintained throughout the 45min wash period (figure 3.11). This recovery in response was not observed when P2X₁ cells were pre-treated in 10μM Bz-ATP and UV irradiated for 3min. The significant reduction in current was maintained throughout the 45min wash period. After 10min the mean peak response to 1μM ATP application was -0.08 ±0.22nA (n = 5) (figure 3.11). As previously described in section 2.3.5, this suggests that the UV irradiation
Figure 3.11. Is the block maintained over time or can it be attributed to desensitization?
A. Wild-type P2X7 cells were plated onto 13mm coverslips and placed in a 8.8cm² petri dish. The media was replaced with either 2ml of 10µM ATP or 10µM Bz-ATP and were left for 3min to equilibrate (green bar). Cells were then placed in the recording chamber and were continuously perfused with extracellular solution for 45min. Patch clamp recordings were continuously taken from different single cells every 2min during this 45min time period. Alternatively, wild-type P2X7 cells were plated onto 13mm coverslips and placed in a 8.8cm² petri dish. The media was replaced with 2ml of 10µM Bz-ATP and the cells were left to UV irradiate for 3min. The cells were recorded in the same manner as established previously. In both protocols single cells were whole cell patch clamped and their current amplitude response to 1µM ATP application was recorded. B. Patch clamp traces and bar graph compare the wash-off over a 45min time period between 10µM ATP, 10µM Bz-ATP and UV irradiated 10µM Bz-ATP. Sample traces taken between 11-15mins. C. Summarized data shows that pre-treatment in 10µM ATP can lead to current recovery within 10mins, the same can be noted for 10µM Bz-ATP. However when 10µM Bz-ATP is UV irradiated, the reduction in current is maintained throughout the 45min period.
of Bz-ATP caused permanent cross-linking of the compound with the P2X₁ receptor and this maintains the receptor in its desensitized state.

Though ATP azidoanilide showed cross-linking ability at the P2X₁ receptor, it did not show the same high level of inhibition over the 45min time period as Bz-ATP did. As a control the mean peak current amplitude for wild type P2X₁ cells following 1μM ATP application was -3.7 ±0.65nA (n = 10). The recovery of the peak current amplitude following 3min treatment in 100μM ATP was, as previously observed with 10μM ATP, within 6 - 10min. The mean peak amplitude recorded after 10min wash was -2.88 ±0.6nA (n = 4) (figure 3.12). When P2X₁ cells were pre-treated in 100μM ATP azidoanilide for 3min peak amplitude currents comparable to the control returned within 11 - 15min wash. The average current recorded was -2.71 ±0.1nA (n = 5) (figure 3.12). When P2X₁ cells were UV irradiated in the presence of 100μM ATP azidoanilide, there was a significant reduction in the peak amplitude response to 1μM ATP. However, this reduction was not as high as that observed with 2-azido ATP or Bz-ATP. After 10min wash the mean peak amplitude was recorded at -1.37 ±0.41nA (n = 6). Although an apparent aberrant result was recorded between 11-15min (-2.61 ±0.31nA (n = 5)), this level of inhibition was maintained over the entire wash period. Similarly, UV irradiation of ATP-benzophenone showed a 56% inhibition of the current after 11 - 15min of wash. The mean peak response to 1μM ATP application was -1.55 ±0.07nA (n = 7) Again this level of inhibition was maintained over time (figure 3.13).

In summary these results suggest that Bz-ATP, ATP azidoanilide and ATP-benzophenone all maintain the inhibition caused by cross-linking. However Bz-ATP maintains a higher inhibition of the current response. This may indicate the positioning of the covalent bond within the ATP binding pocket. It may also suggest that the photo-active efficiency of Bz-ATP is greater than that of ATP azidoanilide or ATP-benzophenone.

3.3.5 Receptor Protection

The competition for binding sites at the P2X₁ receptor can be used as another control experiment. In this series of experiments wild-type P2X₁ cells were plated onto 13mm coverslips and placed in an 8.8cm² petri dish. The growth media was removed
Figure 3.12. Is the block maintained over time or can it be attributed to desensitization?
A. Wild-type P2X<sub>i</sub> cells were plated onto 13mm coverslips and placed in a 8.8cm<sup>2</sup> petri dish. The media was replaced with either 2ml of 100μM ATP or 100μM ATP-AA and were left for 3min to equilibrate (green bar). Cells were then placed in the recording chamber and were continuously perfused with extracellular solution for 45min. Patch clamp recordings were continuously taken from different single cells every 2min during this 45min time period. Alternatively, wild-type P2X<sub>i</sub> cells were plated onto 13mm coverslips and placed in a 8.8cm<sup>2</sup> petri dish. The media was replaced with 2ml of 100μM ATP-AA and the cells were left to UV irradiate for 3min. The cells were recorded in the same manner as established previously. In both protocols single cells were whole cell patch clamped and their current amplitude response to 1μM ATP application was recorded. B. Patch clamp traces compare the recovery of P2X<sub>i</sub> receptors after treatment for 3min in 100μM ATP, 100μM ATP-AA and 100μM UV irradiated ATP-AA. Sample current traces compare the three scenarios between the wash-off time period of 16-30mins. C. The summary bar graph shows that cells recovered from 100μM ATP desensitization between 6-10mins. However when cells are left in the presence of 100μM ATP-AA it takes between 11-15mins for recovery. When the cells are exposed to UV irradiation in the presence of 100μM ATP-AA this recovery is reduced for the entire 45min period.
Figure 3.13. Is the block maintained over time or can it be attributed to desensitization?

A. Wild-type P2X, cells were plated onto 13mm coverslips and placed in a 8.8cm² petri dish. The media was replaced with either 2ml of 100μM ATP or 100μM ATP-BP and were left for 3min to equilibrate (green bar). Cells were then placed in the recording chamber and were continuously perfused with extracellular solution for 45min. Patch clamp recordings were continuously taken from different single cells every 2min during this 45min time period. Alternatively, wild-type P2X₂ cells were plated onto 13mm coverslips and placed in a 8.8cm² petri dish. The media was replaced with 2ml of 100μM ATP-BP and the cells were left to UV irradiate for 3min. The cells were recorded in the same manner as established previously. In both protocols single cells were whole cell patch clamped and their current amplitude response to 1μM ATP application was recorded. B. Whole cell patch clamp traces compare the recovery of P2X₁ receptors after 3min treatment in 100μM ATP, 100μM ATP-BP and 100μM UV irradiated ATP-BP. Sample current traces compare the three scenarios between the wash-off time period of 31-45mins. C. Summary bar graph shows that after pre-treatment in 100μM ATP, cells recover within 6-10mins. Recovery within 11-15mins was observed when cells were left to desensitize in 100μM ATP-BP. However significantly reduced currents were recorded throughout the 45min period from cells pre-treated in 100μM ATP-BP UV irradiated.
and was replaced with 1.5ml of excess ATP or suramin. 1.5ml of the established concentration of photoactive ATP analogue was immediately added to the petri dish and the mixture was allowed to equilibrate for 5min. The dish was placed inside the UV box and was UV irradiated for 3min at 312nm. As before, 1µM ATP applications were made thereafter and whole cell patch clamp recordings were noted. When P2X₁ cells were UV irradiated in the presence of 10µM Bz-ATP the subsequent mean current amplitude of ATP responses was -0.5 ±0.16nA (n = 52). Pre-treatment with either 300µM ATP or 100µM suramin successfully provided protection for the P2X₁ receptors. The mean peak amplitude measured was -1.42 ±0.32nA (n = 17) and -2.85 ±0.43nA respectively. These results were significantly different from the UV irradiated result and suggest protection of the P2X₁ receptor from the full inhibitory effects of cross-linking (figure 3.14).

When the protection experiment was conducted with UV irradiated 100µM ATP azidoanilide, a similar result was found. P2X₁ cells UV irradiated in the presence of 100µM ATP azidoanilide produced mean currents of -1.2 ±0.43nA (n = 30) when 1µM ATP was applied. When the cells were pre-treated with 300µM ATP the mean peak amplitude was -2.75 ±0.5nA (n = 17). When 100µM suramin was used the subsequent recorded currents in response to 1µM ATP application were -2.6 ±0.29nA (n = 16) (figure 3.15).

UV irradiation of P2X₁ cells treated with 100µM ATP-benzophenone caused a significant reduction in the response to 1µM ATP application. The mean current amplitude recorded was -1.19 ±0.17nA (n = 35). When P2X₁ cells were pre-treated in 300µM ATP or 100µM suramin the mean current amplitudes were -2.36 ±0.43nA (n = 15) and -3.03 ±0.29nA (n = 25) respectively (figure 3.16). Both results were significantly different from the responses recorded from P2X₁ cells UV irradiated in 100µM ATP-benzophenone only.

These results suggest that the inhibition of current amplitude caused by UV irradiation of the P2X₁ receptors in the presence of Bz-ATP, ATP azidoanilide or ATP-benzophenone can be significantly reduced with pre-treatment with excess ATP or suramin.
Figure 3.14. Can P2X₁ receptors be protected from the inhibition of current observed after UV irradiation in the presence of Bz-ATP? A. Wild-type P2X₁ cells were plated onto 13mm coverslips and placed in a 8.8cm² petri dish. The media was replaced with 1.5ml of 100μM suramin or 300μM ATP which were dissolved in extracellular solution (pre-treatment with 300μM ATP is indicated by green bar). 1.5ml of 30μM Bz-ATP was added (indicated by grey bar) and the cells were left to equilibrate for 5min. After 5min the cells were UV irradiated for 3min using the established protocol (UV irradiation indicated by yellow vertical bar). Cells were then placed in the recording chamber and were continuously perfused with extracellular solution for 5min before patch clamp recordings were made. The peak amplitude responses to 1μM ATP application were recorded (ATP application indicated by open bar). B. Current traces show that when cells were UV irradiated in the presence of Bz-ATP, currents were reduced. However these currents were recovered if cells were pre-treated in 300μM ATP or 100μM suramin prior to UV irradiation. C. Summary bar graph shows that pre-treating cells with either 300μM ATP or 100μM suramin prevented current inhibition. (* * * = P≤0.001 and show significant difference from UV irradiation with 10μM Bz-ATP.)
Figure 3.15. Can P2X$_1$ receptors be protected from the inhibition of current observed after UV irradiation in the presence of ATP-AA? A. Wild-type P2X$_1$ cells were plated onto 13mm coverslips and placed in a 8.8cm$^2$ petri dish. The media was replaced with 1.5ml of 100µM suramin or 300µM ATP which were dissolved in extracellular solution (pre-treatment with 300µM ATP is indicated by green bar). 1.5ml of 100µM ATP-AA was added (indicated by blue bar) and the cells were left to equilibrate for 5min. After 5min the cells were UV irradiated for 3min using the established protocol (UV irradiation indicated by yellow vertical bar). Cells were then placed in the recording chamber and were continuously perfused with extracellular solution for 5min before patch clamp recordings were made. The peak amplitude responses to 1µM ATP application were recorded (ATP application indicated by open bar).

B. Whole cell patch clamp traces show that like 2-azido ATP and Bz-ATP, the P2X$_1$ receptor was protected from the inhibition brought about by UV exposure (in the presence ATP-AA) by 300µM ATP or 100µM suramin. C. Currents of $-2.75 \pm 0.5 \, \text{nA} \, (n = 12)$ and $-2.6 \pm 0.29 \, \text{nA} \, (n = 23)$ illustrated the protective nature of both ATP and suramin respectively. These results were significantly different from cells which were UV irradiated with only 100µM ATP-AA as currents of only $-1.2 \pm 0.43 \, \text{nA} \, (n = 30)$ were witnessed. (*\* = P \leq 0.01 and **\* = P \leq 0.001 and show significant difference from UV irradiation with each respective compound).
Figure 3.16. Can P2X<sub>1</sub> receptors be protected from the inhibition of current observed after UV irradiation in the presence of ATP-BP? A. Wild-type P2X<sub>1</sub> cells were plated onto 13mm coverslips and placed in a 8.8cm<sup>2</sup> petri dish. The media was replaced with 1.5ml of 100µM suramin or 300µM ATP which were dissolved in extracellular solution (pre-treatment with 300µM ATP is indicated by green bar). 1.5ml of 100µM ATP-BP was added (indicated by grey bar) and the cells were left to equilibrate for 5min. After 5min the cells were UV irradiated for 3min using the established protocol (UV irradiation indicated by yellow vertical bar). Cells were then placed in the recording chamber and were continuously perfused with extracellular solution for 5min before patch clamp recordings were made. The peak amplitude responses to 1µM ATP application were recorded (ATP application indicated by open bar). B. Whole cell patch clamp traces show that the P2X<sub>1</sub> receptor was protected from the reduction in current caused by UV irradiation of P2X<sub>1</sub> cells pre-treated in 100µM ATP-BP. C. Summarized bar graph shows currents achieved from pre-treatment with 300µM ATP and 100µM suramin were -2.36 ±0.43nA (n = 15) and -3.03 ±0.29nA (n = 25) respectively, both were significantly different from the current responses recorded from cells which had been UV irradiation in the presence of 100µM ATP-BP. (* * * = P≤ 0.001 and show significant difference from UV irradiation with 100µM ATP-BP).
3.3.6 Radio-labelling at the P2X<sub>1</sub> receptor

The successful association of a radio-labelled cross-linking compound with immunoprecipitated P2X<sub>1</sub> protein can provide direct evidence of cross-linking. Due to financial and safety restrictions, 1µM ATP [γ<sup>32</sup>P] azidoanilide was UV irradiated with the P2X<sub>1</sub> receptor as described in section 3.2.5. In addition P2X<sub>1</sub> cells were also pre-treated in a mixture of 1µM ATP [γ<sup>32</sup>P] azidoanilide and 10µM ATP. The cells were then UV irradiated for 3min and the protocol was carried out as described in section 3.2.5.

Intense bands which correlated with the size of the glycosylated P2X<sub>j</sub> monomer (~55kDa) and dimer (~110kDa) were observed after labelling in 1µM ATP [γ<sup>32</sup>P] azidoanilide (n = 3) (figure 3.17). As observed previously in section 2.3.7, the low background suggested that the immunoprecipitation step was very effective. The intensity of binding was also reduced after protection with 10µM ATP. These results suggest that ATP [γ<sup>32</sup>P] azidoanilide successfully cross-linked to the P2X<sub>j</sub> receptor. In addition the decrease in intensity after co-treatment with 10µM ATP suggests direct competition between ATP and ATP [γ<sup>32</sup>P] azidoanilide for occupancy of the binding site.

3.3.7 Cross-linking compounds at other P2X receptor subtypes

Previous studies suggest there are many differences between the various subtypes of the P2X receptor. Basic differences in pharmacology are the most prominent. The success and failure of photoactive compounds to reduce the ATP response effectively may highlight these differences.

In the presence of the respective cross-linking compounds, HEK293 cells expressing P2X<sub>2</sub>, P2X<sub>3</sub> and P2X<sub>4</sub> were all UV irradiated for 3min. Subsequent patch clamp recordings monitored changes in the responses to their approximate EC<sub>50</sub> ATP concentrations (figure 3.18). 1µM ATP was used for P2X<sub>1</sub> and P2X<sub>3</sub> cells and 10µM ATP was used for P2X<sub>2</sub> and P2X<sub>4</sub> cells. ATP (at EC<sub>50</sub> concentration of ATP) was applied to untreated P2X<sub>2</sub>, P2X<sub>3</sub> and P2X<sub>4</sub> cells as a control. At the P2X<sub>2</sub> receptor...
Figure 3.17. Radio-labeled binding studies with P2X₁ protein. Samples of non-transfected HEK293 cells and HEK293 cells stably expressing the P2X₁ receptor were pre-treated in 1μM (0.92MBq) ATP azidoanilide-[γ³²P]. In addition, cells stably expressing the P2X₁ receptor were treated with 10μM ATP and IgM azidoanilide. All samples were UV irradiated for 3mins. After lysis, the protein was immunoprecipitated with the P2X₁ antibody (Alomone) and ran on 10% SDS PAGE. After drying, the gel was exposed to autoradiography film for 4 days at -80°C. Both gels (same experimental protocol) show intense bands at a similar weight range for the glycosylated monomer (~55kDa) and dimer (~110kDa) for the P2X₁ protein and suggest successful labeling by ATP azidoanilide. 1μM ATP azidoanilide treatment shows strong binding to the P2X₁ receptor but, as predicted in the electrophysiological study, this binding was reduced when the receptor was protected with excess ATP.
10μM ATP application caused transient inward currents that slowly desensitized. The mean peak current was -2.3 ±0.45nA (n = 5). The application of 1μM ATP at P2X3 receptors caused transient inward currents that quickly desensitized. The mean peak current was -2.12 ±0.38nA (n = 5). The application of 10μM ATP to P2X4 receptors evoked transient inward currents that were slow to desensitize. The mean peak current was -1.06 ±0.24nA (n = 5).

There were some similarities in the effectiveness of each of the cross-linking compounds. It was found that 100μM 8-azido ATP did not work at any of the receptors, but 30μM 2-azido ATP was effective at every receptor with inhibition of ATP response ranging from 82% (P2X1) to 44% (P2X4) (figure 3.18). 10μM ATP application to 2-azido ATP cross-linked P2X2 receptors produced a mean peak current of -0.46 ±0.07nA (n = 18). 1μM ATP application to 2-azido ATP cross-linked P2X3 receptors produced a mean peak current of -0.35 ±0.09nA (n = 12) and 10μM ATP application to 2-azido ATP cross-linked P2X4 receptors produced mean peak currents of -0.58 ±0.13nA (n = 9).

UV irradiation of 100μM ATP azidoanilide also reduced all subsequent currents from the different receptor subtypes (figure 3.18). The P2X3 receptor was the most strongly affected - currents were reduced by 81%. The mean peak amplitude of the ATP response was -0.34 ±0.12nA (n = 10). The P2X4 receptor was the least affected. 10μM ATP application following cross-linking to ATP azidoanilide produced a mean peak current of -0.4 ±0.09nA (n = 10). This was a 45% reduction in the peak current amplitude.

Differences however, proved to be more common. 10μM Bz-ATP, which showed strong inhibition of currents at the P2X1 receptor (82% inhibition), produced no blocking effect at the P2X2 or P2X4 receptor. Following cross-linking, the mean peak current in response to 10μM ATP application was -2.36 ±0.58nA (n = 9) and -0.98 ±0.14nA (n = 10) respectively. In addition to the P2X1 receptor, only the P2X3 receptor was affected by UV irradiation of 10μM Bz-ATP in its presence – the mean peak current was 0.44 ±0.23nA (n = 13) (figure 3.18).
Figure 3.18. Photoactive compounds at other P2X subtypes. HEK293 cells stably expressing the human P2X4 receptor and cells transfected with rat P2X2 and rat P2X3 DNA were subjected to similar experiments as those conducted at the P2X1 receptor. As before, cells were treated in a photoactive compound and exposed to UV irradiation for 3mins. Subsequent whole cell patch clamp recording revealed whether the cells response to their respective EC50 concentration of ATP was significantly altered. A. Whole cell patch clamp traces show the current response to ATP at EC50 concentration of each respective P2X subtype. B. Whole cell patch clamp traces depict the change in the ATP response of cells UV irradiated in the presence of 10μM Bz-ATP. Traces show the response across the different P2X receptor subtypes. C. Bar graph summarises the data with all the photoactive compounds. The contrasting effectiveness across all subtypes may hint at the differences between the P2X receptors known to exist. (* = P ≤ 0.05, ** = P ≤ 0.01 and *** = P ≤ 0.001 and show significant difference from the ATP control).
100μM ATP-benzophenone, after 8-azido ATP, was the next most ineffective, photoactive cross-linking compound. Though it worked at the P2X₁ receptor to cause a 69% reduction in ATP response, it was ineffective at the P2X₂ and P2X₃ receptor. Currents were reduced by 2% and 22% respectively. ATP-benzophenone seemed to show more activity at the P2X₄ receptor with cross-linking causing a reduction of 59% in subsequent patch clamp recordings (figure 3.18).

The P2X receptor subtypes have differences in both their characteristics (e.g. desensitizing and non-desensitizing phenotypes) and their pharmacology (Torres, 1998b). These differences most likely hint at specific changes between the subtypes. These changes could range from the ATP binding site to translation of binding to gating or gating itself (besides many others). The success and failure of each of the compounds may refer to the subtle differences between each of the subtypes.
3.4 Discussion

The results described in this chapter have identified Bz-ATP, ATP azidoanilide and ATP-benzophenone as successful cross-linking compounds at the P2X₁ receptor. Applications of Bz-ATP, ATP azidoanilide (ATP-AA) and ATP benzophenone (ATP-BP) evoked concentration-dependent inward currents in the P2X₁ receptor that rapidly desensitized. When P2X₁ cells were treated in Bz-ATP, ATP-AA or ATP-BP and UV irradiated for 3 min, the amplitude of P2X₁ receptor current, following 1 μM ATP application, was significantly reduced. These reductions were maintained over time and were prevented by pre-treatment of the P2X₁ cells with either excess suramin or ATP. Finally, direct evidence of ATP-AA cross-linking to the P2X₁ receptor was provided with the use of ATP [γ³²P] azidoanilide. The cross-linking ability of some of the photoactive compounds changed when tested with P2X₂, P2X₃ and P2X₄ cells. The ability or inability to cross-link to different receptor subtypes may be an indication of the differences that exist between each receptor subtype.

The UV irradiation protocols developed for 2-azido ATP were used for a variety of other photoactive compounds which have reactive groups at different attachment points on the ATP molecule. Investigation of their agonist action showed that Bz-ATP produced currents that were about half of the 1 μM ATP control. Bz-ATP has previously been reported to be a partial agonist at the P2X₁ receptor (Evans et al, 1995; Bianchi et al, 1998). 1 μM applications of 8-azido ATP, ATP-AA and ATP-BP were ineffective at the P2X₁ receptor. ATP-AA and ATP-BP required an increase to 100 μM before comparable results were observed. 100 μM 8-azido ATP still produced a lowered effect than both these compounds. The order of agonist action was: 2-azido ATP > Bz-ATP > ATP-BP > ATP-AA > 8-azido ATP. The results recorded from 8-azido ATP suggested that the activity produced at this concentration was influenced by an ATP contamination. It was the results from UV irradiation that confirmed this suggestion.

The UV irradiation of the pre-treated P2X₁ cells showed which compounds were active as cross-linking agents. The contrast between the activity of 2-azido ATP and
the inactivity of 8-azido ATP was interesting. The lack of inhibition of the ATP response from UV irradiated P2X₁ cells, pre-treated in 8-azido ATP, suggested that 8-azido ATP did not successfully bind to the ATP binding site. This contradicted the earlier results which showed that it had agonist action at 100µM. This suggests that the result was influenced by a contamination of ATP and taken together with the results from the cross-linking experiments, indicates that the compound does not occupy the ATP binding domain. These results were in contrast to the results recorded in a study by Koshimizu et al (2002). They used single cell calcium measurements to show that GT1 cells, which expressed the P2X₃ receptor, showed a preference for 8-azido ATP over ATP when the cells were stimulated by an equimolar concentration. Additionally, they showed positive association of the P2X₃ receptor with [γ⁻³²p] 8-Azido-ATP and they described a reduction in photolabelling when the cells were pre-incubated with 100-fold excess concentration of ATP. They also described inactivity of the compound at the P2X₂ receptor. This suggested that 8-azido ATP can discriminate between different P2X subtypes, but as discussed below I found no cross-linking action at any P2X receptor subtype (P2X₁ – P2X₄).

How could such a discrepancy arise with only a small change at the adenine ring? Could this difference be reference to the specifics of the ATP binding domain? A similar lack of activity was reported in Zhang et al’s (2000) study of the nucleotide-binding site of 5-lipoxygenase (5-LO). This enzyme catalyses the first two steps in the biosynthesis of leukotrienes (inflammatory mediators) and is stimulated by ATP. Though 8-azido ATP had some activity, it was significantly lower than that of 2-azido ATP. They concluded that this could have been due to the shape or contour of the binding site (Zhang et al, 2000). The results presented in this chapter suggest a similar conclusion.

It is known that C-8 substitution on the adenine ring restricts the molecule to the syn conformation whereas the parent nucleotide, ATP, exists in the anti conformation (Colman, 1983). The syn conformation, which places the purine and sugar rings on the same side of the N-glycosidic bond, prevents any rotation. This is not seen with C-2 substitution, hence 2-azido allows rotation of the bond and exists as both anti and syn (60/40 ratio) conformations. The reduced activity of 8-Br ATP also seemed to support this idea.
Bz-ATP is a compound that has a substitution at the ribose moiety. This addition is composed primarily of bulky aromatic rings that do not seem to affect its agonist ability. UV irradiation of P2X₁ cells treated in 10μM Bz-ATP resulted in an 89% reduction in the 1μM ATP response. This was comparable to the reduction caused by the UV irradiation of cells treated in 30μM 2-azido ATP. This similarity was interesting because two different areas within the binding domain may have been covalently linked to the compounds.

The previous chapter has already described the inability of FSBA to cause any change in the ATP response. This suggested that an intact phosphate tail was necessary for successful binding to the ATP binding site. ATP-AA and ATP-BP contain large aromatic additions at the γ phosphate but unlike FSBA, maintain both α and β phosphates. Though not as effective as the 2-azido ATP and Bz-ATP, the UV irradiation of P2X₁ cells treated in ATP-AA or ATP-BP produced a significant reduction of the current evoked by ATP. ATP-AA caused 67% reduction and ATP-BP caused 69% reduction. When this result is taken in context with the result from FSBA, this again suggests the importance of the phosphate tail. FSBA contained no phosphates in the tail and was ineffective. The ineffective activity of ADP has been documented (Mahaut-Smith et al, 2000). ATP-AA and ATP-BP have their terminal phosphate excised yet maintain activity (although lowered and possibly affected by a low level contamination by ATP) at the P2X₁ receptor and adenosine 5’-tetraphosphate has also been shown to have activity at P2X receptors (Lewis et al, 2000; Gomez-Villafuertes et al, 2000). This suggests that in addition to the negative charge (Ennion et al, 2001; Jiang et al, 2001); the length of the tail may also play a key role in determining any agonist activity.

Further confirmation of cross-linking ability was shown when irradiation of the P2X₁ cells in the presence of 10μM Bz-ATP, 100μM ATP-AA or 100μM ATP-BP produced a constant reduction of the 1μM ATP response over time. Additionally, the effects of cross-linking could be prevented by pre-treatment of P2X₁ cells in excess ATP or suramin. Both results suggest competition between the successful photoactive agents and ATP for occupancy of the ATP binding site. Once the photoactive compound is within the binding site of the receptor, UV irradiation causes a
permanent association of the compound with the receptor. This maintains the receptor in its desensitized state and prevents receptor recovery.

Radio-labelling provided the final evidence of the competition between ATP and ATP-AA for association with the P2X₁ receptor. The band observed after cross-linking of the P2X₁ receptor to 1 μM ATP [γ³²P] azidoanilide corresponded with the expected size of the P2X₁ protein (~55 kDa). The intensity of this band decreased when the cells were protected with 10 μM ATP. The fainter bands again suggest competition for occupancy of the P2X₁ receptor. Tanabe et al (2000) showed through the use of radio-labelling, that ATP azidoanilide could permanently bind to a specific subunit of ATP-sensitive potassium channels. Their study, like this one, showed that azidoanilide and ATP directly competed for occupancy of the site.

The experiments described the effectiveness of the photoactive compounds at the P2X₁ receptor however different P2X subtypes demonstrate distinct pharmacological and biophysical properties (Gever et al, 2006). P2X₁ and P2X₃ receptors desensitize rapidly and are both sensitive to the agonist α,β-me-ATP and the antagonists PPADS and TNP-ATP. P2X₂ and P2X₅ receptors desensitize slowly upon ATP application and are antagonized by PPADS. However they are not activated by α,β-me-ATP. P2X₄, P2X₆ and P2X₇ also desensitize slowly, however the P2X₇ receptor is much less sensitive to ATP than the other subtypes. These distinct differences suggest changes in the agonist binding site (and/or associated structures). The effectiveness of each photoactive compound may refer to the similarities and differences between the P2X receptor subtypes.

When the compounds were tested at receptor subtypes P2X₂ - P2X₄, similarities and differences in effectiveness were observed. 2-azido ATP, probably the least altered ATP analogue, worked at all receptors P2X₁ – P2X₄, in addition ATP-AA worked at all receptors, but 8-azido ATP did not show activity at any receptor. These results could be reference to the innate similarities between receptor subtypes. P2X₁ and P2X₃ exhibit similar fast desensitizing phenotypes while P2X₂ and P2X₄ are much slower. P2X₁ and P2X₃ are both sensitive to αβ-methylene ATP, have EC₅₀ concentrations to ATP in the 1 μM region and are both sensitive to suramin inhibition.
(Gever et al, 2006). Though their results, after UV irradiation in the different photoactive compounds, are slightly different with regards to ATP-BP, there are enough similarities to suggest that the ATP binding domain at both subtypes may show great resemblance.

Conversely, P2X₂ and P2X₄ receptors have been shown to be less sensitive to suramin and ATP (Brake et al, 1994; Bo et al, 1995). Again they show similarities, most notably the ineffectiveness of cross-linking Bz-ATP at either receptor. Bz-ATP has been reported to be a less potent partial agonist at these receptors (Evans et al, 2002; North, 2002) and the lack of cross-linking reflects this. The reactive site of Bz-ATP is bound to a large aromatic structure which in turn is attached to the ribose sugar of the ATP analogue (figure 3.2.A). The results suggest that although there is an interaction between the ATP analogue and the binding pocket of each receptor which results in a response, the reactive site of Bz-ATP may not come into close contact with any amino acid residues within the ATP binding domain. However it should also be noted that an increase in the Bz-ATP concentration may offset the reduced potency and produce cross-linking at both receptors. Since Bz-ATP is a very effective cross-linking compound at the P2X₁ receptor, these results imply that the binding domains at these receptors must differ slightly for this range of diverse behaviour to become apparent.

The ATP binding domain of the P2X₁ receptor (and at other P2X subtypes) is so specific that it can distinguish between ATP and ADP. Indeed, when the oxygen atoms at the α, β and/or β, γ position are replaced with other moieties there is still no appearance of activity with various synthesised ADP analogues at the P2X₂ or P2X₂/₃ receptor (Spelta et al, 2003). When similar substitutions are made with ATP analogues, there is some agonist and antagonist activity at P2X₂/₃ receptors, but poorer activity at P2X₂ receptors. Not only does this draw attention to the importance of a complete triphosphate chain, it also seems to show that the P2X₂ receptor is much more inflexible when it comes to accepting changes to the ATP molecule (Spelta et al, 2003). To some extent this has been shown in the investigation. Both P2X₂ and P2X₄ (both similar in nature) appeared more stringent, and less flexible when it came to accepting substitutions at the ATP molecule. This lack of flexibility may imply
that the ATP binding site and/or the conformational changes that occur after ligand binding differ vastly between each receptor subtype.

In conclusion the experiments described in this chapter have aided in the identification of other photoactive compounds which not only have cross-linking ability at the P2X₁ receptor, but have ability at other P2X receptor subtypes. The different positions of their respective reactive sites may help identify amino acid residues that contribute to the binding of the different co-ordinates of the ATP molecule and may help to indicate the specific orientation of the ATP molecule when it occupies the ATP binding site.
3.5 Summary and Conclusions

Electrophysiological and radio-labelled investigation has provided some insight into the characteristics of the P2X\textsubscript{1} receptor. The evidence describing the effectiveness of Bz-ATP, ATP-AA and ATP-BP as cross-linking compounds at the P2X\textsubscript{1} receptor is as follows:

- 10μM Bz-ATP, 100μM ATP-AA and 100μM ATP-BP evoked concentration-dependent currents in HEK293 cells which stably expressed the P2X\textsubscript{1} receptor (P2X\textsubscript{1} cells).
- UV irradiation of P2X\textsubscript{1} cells treated in 10μM Bz-ATP, 100μM ATP-AA or 100μM ATP-BP caused a significant reduction to the normal 1μM ATP response.
- When P2X\textsubscript{1} cells were exposed to a corresponding concentration of ATP or photoactive compound, cells recovered within 15min. The reduction caused by UV irradiation in the presence of photoactive compound caused a stable reduction that remained over time. Therefore the reduction in the ATP response cannot be attributed to desensitization.
- Photoactive compounds appeared to compete directly with ATP for occupancy of the binding site. P2X\textsubscript{1} receptors were protected if cells were co-treated in ATP or suramin prior to UV irradiation in photoactive compound. This protocol allowed the mean current amplitude recorded from P2X\textsubscript{1} cells (in response to 1μM ATP application) to be recovered.
- Direct evidence was provided when ATP [\textgamma^{32}\text{P}] azidoanilide was cross-linked to the P2X\textsubscript{1} receptor. Specificity of binding to the P2X\textsubscript{1} receptor was observed, as was the decrease in photo-insertion brought about by co-treatment of cells in micro-molar levels of ATP. This was in agreement with the electrophysiological study.
- The ability of some of the cross-linking compounds to work at other receptor subtypes of P2X showed some of the similarities between the receptors. Conversely, the inability of some of the compounds to work at other subtypes could refer to the subtle differences between these receptors.
In this project the use of photoactive affinity compounds will be the cornerstone to determining the binding site of ATP at the P2X\textsubscript{1} receptor. In this chapter, experiments have successfully identified compounds that can be used to cross-link at the ATP binding site of the P2X\textsubscript{1} receptor. The next chapter will describe experiments which will allow the protein to be purified.
CHAPTER 4
CONSTRUCTION OF THE RECOMBINANT P2X₁ RECEPTOR AND
PURIFICATION OF THE PROTEIN

4.1 Introduction

I have identified ATP analogues which can successfully cross-link to different P2X subtypes at different positions within the ATP binding pocket. In this chapter I will investigate methods which will allow the P2X₁ protein to be purified. This would enable subsequent investigation of the identity of the amino acid residues at which the ATP analogues cross-link to the P2X receptors.

A common approach used for purification of many proteins is affinity chromatography. This involves modifying the receptor by adding known epitopes to the amino and/or carboxy termini. Tagging the receptor should prove to be a highly efficient tool that will allow the purification of the P2X₁ protein from the crude cell extracts. It however has to be determined whether the addition of tags interferes with protein production, trafficking to the cell surface or its activity.

Two of the most commonly used tags are Flag and Hexahistidine (His). The Flag tag consists of 8 amino acids (Asp, Tyr, Lys, Asp, Asp, Asp, Lys), whilst the His tag is composed of 6 histidine residues. Previous studies such as Christoffers et al (2003) used both tags to purify and analyse the mouse μ opioid receptor. Using a polymerase chain reaction (PCR) protocol, they were able to generate a plasmid encoding the receptor and containing the Flag epitope on the amino terminus and the His tag at the carboxy terminus.

This chapter will describe experiments which detail the creation of a plasmid incorporating the modified P2X₁ receptor, the selection of colonies which exhibit the highest expression levels and the generation of a cell line which stably expresses the P2X₁ receptor construct. Using affinity chromatography the P2X₁ receptor will be purified to such a level that it successfully reveals a band after coomassie staining. The subsequent analysis of this band by mass spectrometry will reveal the degree of purity of the P2X₁ protein.
4.2 Materials and Methods

4.2.1 Creating the N-terminal and C-terminal Flag-His\textsubscript{6} Tag
The N- and C-terminal constructs, the P2X\textsubscript{1} constructs and the P2X\textsubscript{1} stable cell lines were generated by Catherine Vial.

To generate the respective N- and C-terminal constructs, a native pcDNA3.0 (Amersham) vector was digested with the following:

- Hind III and Kpn I to create an N-terminal tag
- Apa I and Xba I to create a C-terminal tag

Digestions were run on a 1% agarose gel, the bands were excised and the DNA was removed with a gel extraction kit (Qiagen). The linearized vectors were dephosphorylated with alkaline phosphatase to prevent re-ligation of the vector. To create the Flag-His\textsubscript{6} construct, specific primers were used:

- **NH\textsubscript{2} Tag**
  - Forward: 5' P-AGC TTA TGG GCG ACT ACA AGG ACG ACG ATG ACA AGG GTC ATC ATC ATC ATC ATC ATG GTA C 3'
  - Reverse: 5' P- CAT GAT GAT GAT GAT GAT GAC CCT TGT CAT CGT CGT CCT TGT CAT CGT CGT CCT TGT AGT CGC CCA TA 3'

- **COOH Tag**
  - Forward: 5' P-CTA GAG ACT ACA AGG ACG ACG ATG ACA AGG GTC ATC ATC ATC ATC ATC ATT GAG GGC C 3'
  - Reverse: 5' P-CTC AAT GAT GAT GAT GAT GAT GAC CCT TGT CAT CGT CGT CCT TGT AGT CT 3'

To form either tag, 9μl of the forward (100μM) and reverse primer (100μM) were mixed with 2μl of 10X ligase buffer. This mixture was heated to 95°C for 2mins, cooled down to 50°C for 5mins and then left to cool to room temperature. This
allowed the primers to split apart and then anneal, thus forming each respective fusion
tag. The tag was ligated into the linearized vectors using the following protocol:-

- Linearized vector              25ng
- Flag-His6 tag                  1.5ng
- Ligase buffer 10X              1μl
- T4 DNA ligase                  1μl
- H2O                            Final Volume = 10μl

The ligation mixture was left overnight at 14°C and 3μl of the mixture was used for
transformation of E.Coli bacteria using the standard heat shock method. Resultant
bacterial colonies were grown, harvested and lysed to isolate the plasmid of interest
(Qiagen Miniprep Kit). The plasmid samples were stored at -20°C.

4.2.2 Strategy for the creation of the P2X1 constructs

The addition of fusion tags to a known protein can cause adverse affects in many
different facets of protein function. Changes in folding, the inability of trafficking to
the membrane surface or alterations in receptor activity can often occur after the
addition of tags to a particular end of a nucleotide sequence. Unfortunately, the
precise nature of any changes cannot be predicted. In the case of the P2X1 receptor,
both N- and C-terminal tag vectors were generated and tested for function (section
4.3.2 and 4.3.3). The N-terminal tag was created by adding Kpn I and Xba I
restriction sites to the P2X1 fragment by PCR. The primers used are shown below.
The PCR product was run on a 1% agarose gel, the predicted band was removed and
the DNA was extracted with a gel extraction kit (Qiagen). The NH2 tag vector was
digested with the restriction enzymes Kpn I and Xba I, run on a 1% agarose gel and
the extracted DNA was treated with alkaline phosphatase to prevent re-ligation. The
modified P2X1 fragment was sub-cloned into the open NH2 tag vector and the ligation
mixture was left overnight at 14°C. 3μl of the ligation mixture was used for
transformation of E.Coli bacteria using the standard heat shock method. Resultant
bacterial colonies were grown in large amounts, harvested and lysed to isolate the
plasmid of interest (Qiagen Miniprep Kit). Creation of the C-terminal tagged P2X1
construct was similar. Initially the P2X1 fragment was modified with the addition of
Bam HI and Xba I restriction sites via PCR. Primers are shown below. The COOH-tagged vector was digested with Bam HI and Xba I and the modified P2X₁ fragment was ligated into the open vector using the established methods noted previously. All PCR protocols were carried out with the AccuPrime pfx Supermix (Invitrogen), which contains DNA polymerase (isolated from the extreme thermophile *Thermococcus kodakaraensis*), dNTPs and MgSO₄. Each PCR required the addition of 22.5μl of supermix combined with 1.5μl of DNA template (50ng) and 0.5μl of both the forward (200nM) and reverse primer (200nM). The PCR thermal cycler (MJ Research PTC-200) was programmed to heat the mixture to 95°C for 5mins and then complete 30 cycles of 95°C for 15secs, 60°C for 30secs and 68°C for 2mins. After cycling, the reaction was maintained at 4°C. The plasmid samples were stored at -20°C. Production of the tagged vectors was verified by DNA sequencing (Automated ABI Sequencing Service, University of Leicester). The plasmid maps for both tagged constructs are shown in figure 4.1.

**N-Terminal P2X₁ Construct Primers**

Forward:  
5' CCC GGT ACC GTG CTG GTG CGT AAT 3'

Reverse:  
5' GGC TCT AGA TGA GGA TGT CCT CAT 3'

**C-Terminal P2X₁ Construct Primers**

Forward:  
5' CCC GGA TCC ATG GTG CTG GTG CGT 3'

Reverse:  
5' GGC TCT AGA GGA TGT CCT CAT GTT 3'
Figure 4.1. Maps of the human P2X\textsubscript{1} receptor tagged with Flag-His\textsubscript{6} on either the N-terminal or C-terminal. \textbf{A.} N-terminal attached Flag-His\textsubscript{6} tag. Base pair length from Hind III to Xba I is 1266bp. \textbf{B.} C-terminal attached Flag-His\textsubscript{6} tag. Base pair length from Bam HI to Apa I is 1263bp. Construction of both vectors is detailed in section 4.2.2. Evaluation of both vectors is shown in sections 4.3.2 and 4.3.3.

4.2.3 Strategy for the creation of the P2X\textsubscript{2} construct

The success of the C-terminal P2X\textsubscript{1}-Flag-His construct (section 4.3.2) demonstrated that addition of the fusion tag was tolerated without disruption of receptor expression and/or function. This allowed me to use Catherine's tagged vector in the generation of the P2X\textsubscript{2} counter-part. Restriction sites for Hind III and Xba I were attached to the P2X\textsubscript{2} fragment by PCR. The primers used are shown below. To potentially increase expression, the Kozak sequence, CACC, was added to the beginning of the P2X\textsubscript{2} sequence. The PCR product was run on a 1\% agarose gel, the predicted band was removed and the DNA was extracted with a gel extraction kit (Qiagen). The COOH-tagged vector was digested with Hind III and Xba I and the modified P2X\textsubscript{2} fragment was ligated into the open vector using established methods. As before, the PCR protocol was carried out with the AccuPrime pfx Supermix (Invitrogen) and the previous conditions (section 4.2.2) were maintained. The plasmid samples were stored at -20°C. Production of the tagged vector was verified by DNA sequencing (DNA Sequencing Europe, GSK). A diagram of both tagged constructs is shown in figure 4.2.
Figure 4.2. Diagram of the completed P2X-Flag-His vectors. A. The P2X1-Flag-His construct. The P2X1 fragment contains restriction sites for Bam HI and Xba I. It was ligated into the backbone vector pcDNA3, which already contained the Flag and His tag (section 4.2.1). B. The P2X2-Flag-His construct with additional Hind III and Xba I restriction sites. Apa I is marked on both vectors and shows the end of the Flag-His tag. Both vectors include the Cytomegalovirus promoter (P-CMV) and an ampicillin resistant gene (AmpR). Both vectors were investigated for receptor functionality (electrophysiology) and expression (western blot) of all three epitopes.
C-Terminal P2X2 Construct Primers

Forward: \[5'\text{CCC AAG CTT CACC ATG GTC CGG CGC CTG}3'\]

Reverse: \[5'\text{GGC TCT AGA GAG TTG AGC CAA ACC TTT}3'\]

4.2.4 Cell Culture

Cell culture was performed as described previously in chapter 2 (section 2.2.1) and chapter 3 (section 3.2.1).

4.2.5 Cross-linking Assay

The cross-linking protocol was performed as previously described in chapter 2 (section 2.2.4).

4.2.6 Electrophysiology

Electrophysiological recordings were performed as previously described in chapter 2 (section 2.2.2).

4.2.7 Western Blot Analysis

Western blot analysis was used, in addition to patch clamp experiments, to detect total cellular protein levels of tagged construct. HEK293 cells transfected with P2X construct were allowed to grow to 90-100% confluence (six-well dish) before being washed in 1ml PBS and solubilized in 500µl lysis buffer. The lysis buffer was composed of 150mM NaCl, 40mM Tris HCl, 8mM Tris Base, Protease Inhibitor Cocktail (10µl/ml) and 1% Triton X (pH 7.5). The solubilized cells were spun at 13,000 rpm for 20mins (4°C) in a bench top centrifuge, the supernatant was stored on ice and the pellet was discarded. A 20µl aliquot of supernatant was mixed 50:50 with SDS-PAGE sample buffer (with 5% beta-mercaptoethanol) and spun down for 2mins. The protein samples were heated for 5min at 95°C and separated on a 10% SDS-PAGE. The gel was transferred to nitrocellulose and probed for immuno-reactivity with the corresponding anti-P2X antibody (1:500 – 1:1000) (Alomone), anti-Flag antibody (1:5000) (in-house GSK) or anti-Flag antibody (1:500) (Sigma).
4.2.8 Creating the stable cell lines

HEK293 cell lines (70% confluent) which stably express the tagged receptors were generated by initially transfecting one well of a six well plate with 1μg of DNA. In addition, another well, of equal confluence, did not undergo transfection. This was used as a control. After 48hrs incubation at 37°C in humidified 5% CO₂/95% air, cells in both wells were split into 80cm³ flasks and grown in media composed of Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) containing Earle’s salts, glutamax 1, 10% foetal calf serum, 1% non-essential amino acids. Cells were also subject to G-418 selection (1mg/ml). Over time both cell types were observed and the media was changed every 48hrs. The complete death of all non-transfected HEK293 cells implied that any surviving HEK293 cells successfully received the DNA and thus incorporated G-418 resistance and therefore must also express the modified receptor. Surviving cells were allowed to grow to 50-60% confluence before being subject to an extreme dilution. This resulted in only one cell being present per well on a 96-well plate. Again, cells were grown and placed under G-418 selection (1mg/ml). Cellular growth originating from one cell implied the creation of a monoclonal cell line; these cells were allowed to grow to confluence before being successively split into larger culture dishes. Whole cell patch clamp investigation allowed the identification of successful clones.

4.2.9 Protein purification of the P2X₁ construct – Flag and His Purification

Both protocols are based on separating proteins based on affinity and both protocols need to be optimized for the purification of any given protein. Using T-175 cell culture flasks, cell lysate stocks of both P2X₁-Flag-His and HEK293 were produced. Cells were grown to 90-100% confluence, washed twice with 10ml PBS and lysed in 3ml of the standard lysis buffer used for western blot analysis. The solubilized cells were spun at 13,000 rpm for 20mins (4°C), the supernatant was stored at -20°C and the pellet was discarded.

As a preliminary start, 200μl of either agarose beads (Flag) or Nickel-bound beads (His) were added to an eppendorf tube and mixed with 1ml of lysis buffer. The beads were mixed on a rotator for 5mins until they were fully suspended. The mixture was spun at 13,000rpm for 2mins and the supernatant was discarded. This process was
repeated 3 times and allowed the beads to be washed. Finally they were suspended in 100μl lysis buffer and 1.5ml lysate (250μg/ml). The mixture was rolled overnight at 4°C. The following describes washing and elution techniques for the His purification:

- The flow-through (unbound protein) was removed and stored on ice.
- The beads were washed in 500μl lysis buffer which included 20mM imidazole. After addition of the lysis buffer the mixture was rotated for 10min and then spun at 13,000rpm for 2mins. The supernatant was removed and stored on ice. This was repeated 3 times to ensure minimal non-specific binding.
- To elute the bound protein, the imidazole concentration was increased to 250mM (pH7.5) and the incubation time was increased to 15min (4°C). For the last two elutions the imidazole concentration was increased to 500mM (pH7.5). The resultant supernatant was removed and stored on ice.

The Flag purification technique was slightly different:

- The flow-through was removed and stored on ice
- The beads were washed in 500μl lysis buffer (no imidazole) and the resultant supernatant after centrifugation was removed and stored on ice. Again this occurred 3 times to ensure minimal non-specific binding.
- To elute the bound protein, 500μl of 0.1mg/ml 3 x Flag peptide (dissolved in lysis buffer) was added to the beads. The mixture was rotated for 15mins at 4°C and spun at 13,000rpm for 2mins. The supernatant was removed and stored on ice.

After determination of protein concentration, an aliquot of the protein sample was mixed with SDS-PAGE sample buffer (with 5% beta-mercaptoethanol) and spun down for 2mins. The samples were then heated for 5min at 95°C and separated on a 10% SDS-PAGE. Coomassie (Blue Stain Reagent, Pierce) or silver staining (Amersham Biosciences) protocols were carried out according to manufacturer’s guidelines. In addition a western blot was carried out to determine if P2X1 protein
was present in the eluate. Single coomassie bands observed in the eluate were sent for further analysis by Peptide Mass Fingerprinting (PMF). This will allow positive identification of the band and its relative purity. Minor alterations in both protocols can cause increased protein binding to the beads, reduction in non-specific binding, more effective wash and elution stages and produce a greater yield of final product. The optimization of either technique (sections 4.4.5 and 4.4.6) will allow a clean end-product.

4.2.10 Peptide Mass Fingerprinting (PMF)

Similarly to a human fingerprint, the peptide mass fingerprint (PMF) of a protein is unique to that molecule. PMF is an analytical technique which allows the identification of an unknown protein. The method relies on the enzymatic digest of the protein by a specific protease. The mass of each resultant peptide will be the sum of the amino acids present; this includes any modifications that those amino acids might have undergone (addition of a fusion tag). Since different proteases cleave at different amino acid residues, the resultant set of peptides (of varying mass) will be characteristic of a particular protein, thus, the PMF of the protein will depend on the protease used, but will always be unique for each one. Since the modified protein sequence is on a database (for comparison), the technique allows the purified protein bands (observed after coomassie staining of the SDS-gel) to be successfully analysed for presence of the P2X1 protein. PMF analysis was completed at The Protein Nucleic Acid Chemistry Laboratory (PNACL, Leicester) and at Gene Expression & Protein Biochemistry (GEBP, Stevenage). Both labs used the following protocol to digest the protein with trypsin prior to analysis by PMF:

In brief, the gel pieces which showed positive staining with coomassie-blue were destained in 50% methanol in 100mM ammonium bicarbonate for 15min, washed with water, dehydrated with 50% acetonitrile in 25mM ammonium bicarbonate for 10min and then rehydrated in 100mM dithiothreitol in 25 mM ammonium bicarbonate (30 min at 56°C). The gel pieces were alkylated with 55mM iodoacetamide in 25mM ammonium bicarbonate (30 min in dark) and the reaction was stopped by incubating in 25mM ammonium bicarbonate for 20min. The extracted protein was dried in a vacuum centrifuge (Eppendorf concentrator 5301) for 10min and rehydrated in 50mM
ammonium bicarbonate. The protein was then digested with sequencing grade trypsin (20 ng/mL; Promega, USA) for 16hrs at 37°C.

PMF analysis of the P2X1 peptide fragments produced peaks at particular masses; these were compared with the predicted peptide fragment masses generated in the protein database at www.matrixscience.com. The protein which had the most predicted peptide fragments after trypsin digest was identified as the best matched protein. Often 5 or 6 accurate peptide matches are enough to confidently identify a given protein.

4.2.11 Data Analysis

The patch clamp results are expressed as mean ± standard error of mean and analyzed using the appropriate Student’s T-test with P values ≤ 0.05, when compared to the control considered as significant (*). P values ≤ 0.01 were considered very significant (**) and P values ≤ 0.001 were considered highly significant (***). Data analysis was carried out with the computer package, “Excel” and the graphics package “Origin 6” was used to construct the graphs.
4.3 Results

4.3.1 The comparison of adherent and suspension cells

Adherent cells are left encumbered with the limitation of contact inhibition. This refers to the cessation of cell division in neighbouring cells which come into contact. The use of adherent cells means there will be a monolayer expressing a maximum number of cells (thus protein) which can possibly grow in any given area. Suspension cells are not limited by this and can grow freely in solution therefore generating high yields of mammalian protein. Like adherent cells, suspension cells can be transfected with P2X₁ DNA. The application of 1μM ATP evoked significantly different peak amplitude responses from the two cell types. The mean peak response from adherent HEK293 cells (transiently transfected with P2X₁ DNA) was -1.48 ±0.41nA (n = 7). When 1μM ATP was similarly applied to suspension HEK293 cells (transiently transfected with P2X₁ DNA) the mean peak response was -0.45 ±0.17nA (n = 9) (figure 4.3). Additionally, the results of the western blot showed that there was a significant reduction in the total cellular expression of P2X₁ protein in the suspension HEK293 cells (figure 4.3). This reduction in P2X₁ cellular expression and the reduced response to ATP application suggested that the suspension cells were not a good alternative for the expression of the P2X₁ tagged construct. All subsequent experiments were conducted with adherent HEK293 cells.

4.3.2 Evaluating the N- and C-terminal Tags

The addition of the Flag-His construct to the N-terminal yielded two different cloned plasmids - N1 and N2. Control responses were recorded from transiently expressed wild-type P2X₁ receptors. The mean peak amplitude in response to 1μM ATP application was -2.94 ±0.64nA (n = 13). HEK293 cells were transfected with either the N1 or N2 plasmid and the resultant cells were tested for P2X₁ receptor activity in response to 1μM ATP. Recorded currents from plasmid N1 and N2 were -2.41 ±0.83nA (n = 4) and -0.81 ±0.18nA (n = 3) respectively (figure 4.4).

The addition of the Flag-His construct to the C-terminal yielded five different cloned plasmids (C1-C5). The application of 1μM ATP produced no differences in the time course of any of the clones when compared to the wild-type P2X₁ receptor control.
Figure 4.3. Comparing the activity of the P2X<sub>1</sub> receptor after transfection into both adherent and suspension HEK293 backgrounds. Adherent and suspension HEK293 cells were transfected with 1µg of human P2X<sub>1</sub> DNA as described in section 4.2.4. Cells were mounted on 13mm cover slips and whole cell patch clamp recordings were conducted 48hrs after transfection. A. Sample patch clamp recording data from P2X<sub>1</sub> transfected HEK293 adherent cell line and P2X<sub>1</sub> transfected HEK293 suspension cell line after 1µM ATP application (application indicated by clear bar). B. Pooled data shows that there is a significant difference in recorded currents between both cell types. 1µM ATP evoked a significantly higher peak response in adherent HEK293 cells. C. Western blot analysis of HEK293 cells transfected with P2X<sub>1</sub> DNA. Bands at the 55kDa mark suggest the presence of the P2X<sub>1</sub> protein in both cell types, although the presence in suspension cells is markedly less.
Figure 4.4. Patch clamp evaluation of the different plasmids of the N-terminal P2X<sub>1</sub>-Flag-His construct. HEK293 cells were co-transfected with P2X<sub>1</sub> construct and GFP. After 48hrs electrophysiological recordings were made. Cells that fluoresced were considered to express the receptor and were selected for recording. A. Whole cell patch clamp traces show that both the constructs (N1 and N2) retain their P2X<sub>1</sub>-mediated response to 1µM ATP (application indicated by clear bar). B. Summary bar graph describes the effect on the peak amplitude of attaching flag and his tags to the amino termini. The response from the N2 plasmid was significantly lower than the responses from the wild-type P2X<sub>1</sub> receptor. The application of 1µM ATP caused mean currents of -2.41 ±0.83nA (n = 4) in plasmid N1 and -0.81 ±0.18nA (n=3) in plasmid N2. This was significantly different from control responses produced by HEK293 cells transiently transfected with the wild-type P2X<sub>1</sub> receptor. The average response for these cells were -2.94 ±0.64nA (n = 13). (* = P ≤ 0.05 and ** = P ≤ 0.01 and show significant difference from 1µM ATP control).
The recorded control response (wild-type transient P2X<sub>1</sub>) was -2.94 ±0.64nA (<i>n</i> = 13). 1μM ATP evoked mean peak currents of -3.78 ±0.41nA (<i>n</i> = 3) and -2.86 ±1.1nA (<i>n</i> = 3) from HEK293 cells transfected with plasmids C1 and C2 respectively. Plasmids C3 and C5 produced currents of -4.54±1.4nA (<i>n</i> = 3) and -3.07 ±0.55nA (<i>n</i> = 3) and 1μM ATP evoked a mean peak current response of -5.71 ±0.35nA in cells transfected with the C4 plasmid (figure 4.5). Since the response was largest from HEK293 cells transfected with the C4 plasmid, this plasmid was chosen for further study.

Whole cell lysate from C4 plasmid transfected cells were probed with each respective antibody (P2X<sub>1</sub>, Flag, His) to confirm the presence of all three functional epitopes. Western blot analysis successfully identified a band at around 55kDa. This corresponds with the size of the P2X<sub>1</sub> protein (figure 4.6).

Although the C4 plasmid had successfully retained its ATP response and the three epitopes (P2X<sub>1</sub>, Flag and His) had been successfully detected by western blot, it was still important to confirm that the ATP response could still be blocked by 2-azido ATP. To investigate, the cross-linking assay developed in chapter 2 was again put into practice. P2X<sub>1</sub>-Flag-His cells treated in 30μM 2-azido ATP and UV irradiated for 3min showed an 87% reduction in response following 1μM ATP application (figure 4.7). Prior to treatment, mean responses were -4.9 ±0.77nA (<i>n</i> = 6), after cross-linking, responses were decreased to -0.66 ±0.25nA (<i>n</i> = 9).

4.3.3 The P2X<sub>2</sub>-Flag-His Construct

The successful generation of a tagged P2X<sub>1</sub> construct may allow the cross-linked receptor to be purified and the amino acid residues which contribute to the ATP binding site may be revealed. The results from chapter 3 revealed that the cross-linking abilities of various ATP analogues were altered in different P2X receptor subtypes. Hence the generation of a P2X<sub>2</sub> receptor construct could directly show the differing amino acid residues involved in agonist binding and allow a comparison between receptor subtypes.

The addition of the Flag-His construct to the C-terminal of the P2X<sub>2</sub> receptor yielded two different cloned plasmids (1-2). The plasmid map is shown in figure 4.2. Whole
Figure 4.5. Patch clamp evaluation of the different plasmids of the C-terminal P2X₁-Flag-His construct. HEK293 cells were co-transfected with P2X₁ construct (C1 to C5) and GFP. After 48hrs electrophysiological recordings were made. Cells that fluoresced were considered to express the receptor and were selected for recording. A. Whole cell patch clamp traces show that all of the constructs retain their P2X₁-mediated response to 1 μM ATP (application indicated by clear bar). B. Summary bar graph shows the pooled response of each plasmid after 1 μM ATP application. The mean responses demonstrate the continued viability of the P2X₁ receptor even with a Flag and His tag attached to the carboxy termini. (* = P ≤ 0.05 and show significant difference from 1 μM ATP control).
Figure 4.6. P2X$_i$-Flag-His verification with western blot. Western blots show analysis of total cellular protein for the C-terminal tagged P2X$_i$-Flag-His receptor. The construct is detected with the A. P2X$_i$ antibody (1:1000) B. His antibody (1:5000) and C. Flag antibody (1:1500). Positive detection of all three epitopes is indicative of the successful addition of the fusion tags. Intense bands appeared at a similar weight range for the glycosylated monomer (~55kD), dimer (~110kD) and trimer (~165kDa) of P2X$_i$ protein. Size markers are shown in kilo Daltons.
Figure 4.7. Are currents from the P2X₁-Flag-His receptor effected after UV irradiation in the presence of 2-azido ATP? A. Whole cell patch clamp traces show that when cells, expressing the P2X₁-Flag-His receptor, were UV irradiated in the presence of 30μM 2-azido ATP, the subsequent response to 1μM ATP application is markedly reduced (application indicated by clear bar). B. Summary bar graph showing that currents are reduced by 87% after cells are UV irradiated in the presence of 30μM 2-azido ATP. The control response produced from P2X₁-Flag-His cells after 1μM ATP application was -4.9 ±0.77nA (n = 6). After cells were cross-linked in 30μM 2-azido ATP, these responses were reduced to -0.66 ±0.25nA (n = 9). (* * * = P< 0.001 and show significant difference between the control cells and those UV irradiated in the presence of 2-azido ATP).
cell patch clamp investigation showed no differences in the time course of any of the plasmids when compared to HEK293 cells transfected with human P2X2 DNA (figure 4.8). When 10μM ATP (EC_{50}) was applied, cells responded with peak currents which were not significantly different to the transfected P2X2 receptor control. The mean control response was -2.3 ±0.15nA (n = 4), Plasmid 1's mean primary peak response was slightly higher at -3.11 ±0.4nA (n = 11) and Plasmid 2's response was slightly lower than the control. Recordings averaged at -1.91 ±0.3nA (n = 12). When the cross-linking assay was applied, the familiar reduction in current was again observed.

Mean currents for HEK293 cells transfected with Plasmid 1 or 2 and UV irradiated in the presence of 30μM 2-azido ATP were reduced by 74% and 68% respectively. Pooled data from HEK293 cells, transfected with Plasmid 1, showed a decrease in the response to 10μM ATP. The recorded response was -0.81 ±0.22nA (n = 13). Plasmid 2 cell responses were reduced to -0.61 ±0.13nA (n = 13) (figure 4.8). These results allowed Plasmid 1 to be chosen for further study. Western blot analysis was used to probe all three epitopes on HEK293 cells transfected with Plasmid 1. Antibodies which probed P2X2, Flag and His epitopes showed intense bands at a mass of ~70kDa. This suggested the positive presence of all three epitopes (figure 4.9).

Attempts were made to generate tagged P2X3 and P2X4 plasmids. However, the P2X3-Flag-His plasmid failed to ligate and HEK293 cells transfected with the P2X4-Flag-His plasmid were non-functional. A western blot analysis of the total cellular protein revealed faint bands at the weight range associated with the P2X4 monomer (~70kDa) and intense bands associated with the dimer (~140kDa) and trimer (~210kDa). The results may suggest that the protein was sufficiently translated but was not trafficked to the cell surface. This may have resulted from interference due to the addition of the epitope tags (figure 4.9).

4.3.4 Generating the Stable Cell Lines

The level of protein expression following transient transfection can vary from one transfection to another. To help in the maintenance of high levels of protein expression, stable cell lines which incorporated the P2X constructs were generated.

The P2X1-Flag-His vector expressed in Plasmid C4 was transfected into HEK293 cells as described in section 4.2.8. A mock transfection was also carried out on other
Figure 4.8. Are currents from the P2X2-Flag-His receptor effected after UV irradiation in the presence of 2-azido ATP? A. Whole cell patch clamp traces show that both P2X2-Flag-His plasmids produce similar responses as those shown by HEK293 cells after transfection with P2X2 DNA. When cells, expressing the P2X2-Flag-His receptor were UV irradiated in the presence of 30µM 2-azido ATP, the subsequent response to 1µM ATP application was markedly reduced in cells transfected with either plasmid (application indicated by clear bar). B. Summary bar graph shows that currents from plasmid 1 and plasmid 2 are reduced by 74% and 68% respectively. (* * * = P≤ 0.001 and show significant difference between the control cells and those UV irradiated in the presence of 2-azido ATP).
Figure 4.9. **P2X₂-Flag-His verification with western blot.** Western blots show analysis of total cellular protein for the C-terminal tagged P2X₂-Flag-His receptor and the potential P2X₄ construct. The P2X₂ construct was detected with the A. P2X₂ antibody (1:500) B. His antibody ([1:5000] inside box)) and C. Flag antibody (1:1500). Positive detection of all three epitopes is indicative of the successful addition of the fusion tags. An attempt was made to generate a P2X₄ construct to allow comparisons between different P2X subtypes. HEK293 cells transfected with the P2X₄ construct were non-functional. Western blot analysis detected faint bands in the weight range associated with the P2X₄ monomer (≈70kDa) and intense bands associated with P2X₄ dimer (≈140kDa) and trimer (≈210kDa). The results suggest that although P2X₄ protein was produced, the receptors may not have been adequately trafficked to the cell surface.
HEK293 cells of equal confluence. This control acted as an indication of non-transfection, as cells which did not receive the DNA would die due to 1mg/ml G418 selection. After complete cell death of the non-transfected cells it was assumed that all remaining cells encompassed the transfected DNA. Monoclonal growth was established after infinite dilution (section 4.2.8) and several clones were successfully grown and were ready for testing. Eleven such stable clones (1-11) potentially expressed the P2X1 tagged receptor. The response to the application of 1μM ATP was used to discriminate between the clones; figure 4.10 shows the differences between them. Four of the clones (2, 5, 7 and 11) were non-functional with currents ranging from -0.03 ±0.02nA (n = 4) to -0.2 ±0.12nA (n = 3). Clone 4 and Clone 10 showed a significantly reduced pooled response when compared to the P2X1 stable control; mean control currents, after 1μM ATP application, were measured at -4.57 ±0.45nA (n = 5), whilst mean Clone 4 responses were -2.74 ±0.87 (n =3) and mean Clone 10 responses were -2.55 ±0.74nA (n = 5). Nonetheless, four of the clones (1, 3, 8 and 9) responded with average primary peak currents that were similar to those expressed by the stable P2X1 control. Clone 1 responses after ATP application were measured at -4.73 ±1.17nA (n = 4). Clone 3 responses were the lowest of this group; 1μM ATP application elicited responses of -3.32 ±0.51nA (n = 4). Responses from Clone 8 were the highest of all the measured currents. The mean peak amplitude was -4.79 ±0.86nA (n = 5). Clone 9 responses were measured at - 4.05 ±0.93nA (n = 5).

Total cellular expression was investigated with western blot analysis. Results shown in figure 4.10 show equally loaded samples of all the clones compared to expression from HEK293 cells transfected with human wild-type P2X1 DNA. As alluded to in the electrophysiological study, Clone 8 is one of the clones which express the P2X1 receptor construct at the highest levels. Since this is a study of the total cellular protein expression and not the surface expression, the intensity of the band observed in the western blot does not necessarily predict the magnitude of response to ATP. The result of clone 10 is one such example. In the western blot the intensity of the band is similar to that of clone 8, however the mean peak amplitude in response to ATP application was ~50% of that recorded from clone 8. This could suggest the reduced amount of receptor trafficking to the cell surface, possibly due to an incorrect insertion into the genome. Conversely the result for Clone 3 could be reference to the overwhelmingly large number of receptors trafficked solely to the membrane. In
Figure 4.10. Evaluation of the various stable cell line clones incorporating the P2X₁-Flag-His construct. HEK293 cells were transfected with P2X₁ construct (plasmid C4) and left in the presence of 1mg/ml G418 selection. Surviving cells were plated onto 96-well plates and diluted in such a manner as to allow one cell to be present per well. After growth in the presence of 1mg/ml G418 selection, the different clones were prepared for electrophysiological study. A. Sample traces from whole cell patch clamp study show that only a few clones retain the P2X₁-mediated response to 1μM ATP (application indicated by clear bar). B. Summary bar graph shows the pooled response of each clone after 1μM ATP application (n = 5). The mean responses demonstrate the viability of clones 1, 3, 8 and 9. (* = P ≤ 0.05 and *** = P ≤ 0.001 and show significant difference from 1μM ATP control. C. Total cellular expression of P2X₁ was analysed with western blot. HEK293 cells were transfected with 1μg DNA from each respective clone and from wild-type P2X₁ DNA. Cells were lysed, equally loaded and separated on 10% SDS PAGE. The gel was transferred to nitrocellulose and probed for immuno-reactivity with the P2X₁ antibody (1:2000) (Alomone). Results show similarities and differences in the expression of each of the clones. The bands shown above correspond to the approximate weight of P2X₁ protein (~55kDa).
conclusion, the results showed that Clone 8 was the most suitable stable cell line which expressed the P2X₁ receptor construct. Further work (e.g. protein purification) was conducted with this clone.

Attempts to generate a stable HEK293 cell line expressing the P2X₂ receptor construct were unsuccessful on two separate occasions. In total 31 clones were raised and all were found to be non-functional. The non-functionality of the clones suggests an inability of the plasmid to incorporate into the genomic DNA. The failure to generate a stably expressed P2X₂-tagged receptor meant that the purification protocol would be developed with the P2X₁-tagged receptor only.

4.3.5 Optimizing the His Purification Protocol
The successful generation of a tagged construct would allow the P2X₁ receptor to be extracted from the total cell lysate by affinity chromatography. The concept for metal chelate affinity chromatography is quite simple. Using the presence of naturally occurring metal binding sites in many proteins, an agarose bead is covalently modified to the chelating adsorbent nitritotriacetic acid (NTA). NTA occupies four of the six ligand binding sites on the nickel ion, leaving two sites free for interaction with the His-tag (see figure 4.11). The high specificity and bonding for the His protein allows one-step purification from the crude cell lysate. Purification of HEK293 cells transfected with P2X₁-Flag-His consists of four steps: -

1. Cell lysis
2. Binding
3. Washing
4. Elution

When the protein mixture is added to the top of the column the sample flows downwards due to gravity (see figure 4.12). During the downward flow the proteins adsorb to the beads with varying degrees of affinity. In addition to the P2X₁-Flag-His protein there will be non-specific binding. This can be removed by washing the matrix with low concentrations of imidazole. Imidazole has a high affinity for nickel,
Figure 4.11. Metal Chelate Affinity Chromatography. Diagram shows the method with which the Flag-His tagged P2X₁ receptor can be purified by exploiting the addition of the His epitope. A. Ni-NTA beads form a matrix when packed into a flow column. When the total cell lysate is added to the column, gravity forces the liquid through the matrix. The column can be rolled overnight at 4°C to maintain maximum exposure between the lysate and the His-binding matrix. B. Despite centrifugation, the total cell lysate contains many proteins and cellular debris as well as the protein of interest. C. In addition to an affinity to the hexahistidine tag, Ni-NTA beads can bind to proteins with exposed patches of histidine, cysteine and tryptophan. This non-specific binding can be washed away with low concentration imidazole. Imidazole will competitively displace lower affinity binding to the nickel ion. D. The P2X₁-Flag-His protein is left attached to the Ni-NTA beads. The protein can be eluted by adding a higher concentration of imidazole. E. The purified sample can be run on a 10% SDS gel and stained with coomassie solution to confirm whether enough protein has been harvested.
and through competitive displacement, non-specific binding can be removed. To elute the His-tagged P2X1 protein, a higher concentration of imidazole is required.

Figure 4.12. Diagram shows the experimental setup for His purification. A gravity-flow column allows the cell lysate to come into contact with the NTA-Ni beads. The beads are composed of the tetradentate chelating adsorbent NTA which is covalently bound to agarose beads. This metal chelating arm binds to four of the six binding sites on the Nickel ion, leaving two free binding sites. It is these binding sites on the nickel ion which bind to the protein contained in the cell lysate.

Proteins can vary from each other in size, shape, hydrophobicity, charge, solubility, binding affinity and biological activity, thus the purification protocol has to be optimized depending on the protein of interest. All four steps can be altered to produce a higher concentration and greater purity of the P2X1 receptor.

To gauge whether the His purification protocol was possible with the P2X1 construct, initial investigation was conducted with the stably expressed P2X1-Flag-His cell line and non-transfected HEK293 cells. Cells from 1xT-175 flask were lysed into 3ml of lysis buffer (composition described in section 4.2.7) and spun down at 13,000rpm for 10mins on a bench-top centrifuge. Cells are composed of complex biological machinery and many other proteins which are considered as contamination of the purified P2X1 protein. To ensure a reduction in contamination, the supernatant was
centrifuged again at 100,000g for 1 hour (4°C) and the cell debris was removed. Previous control experiments showed that there was no significant change in the total cellular expression of the P2X<sub>1</sub> receptor following 100,000g spin (figure 4.13). The supernatant was combined with 1.5ml Ni-NTA beads (which had been previously washed in lysis buffer) and were rolled overnight at 4°C. Adsorbed to the surface of the beads was P2X<sub>1</sub> protein as well as other contaminating protein due to non-specific binding. Hence the beads were washed twice with 5ml 50mM imidazole (dissolved in lysis buffer) in an attempt to remove any contaminating compounds. Preliminary experiments (not shown) showed that the bound P2X<sub>1</sub> protein eluted more efficiently with a graded increase of imidazole concentration. Therefore elution was completed by initially by adding 100μl of 250mM imidazole. The fraction was collected and the elution step was repeated by adding 100μl of 500mM imidazole – this produced another eluted fraction. The elution step was again repeated with another addition of 100μl of 500mM imidazole. The fractions were separated by 10% SDS-PAGE, transferred to nitro-cellulose paper and probed with either a P2X<sub>1</sub> antibody (1:1000) or a his antibody (1:5000) (figure 4.14). The results showed a successful separation of the P2X<sub>1</sub> protein from the crude cell lysate. However the appearance of P2X<sub>1</sub> protein in the wash fraction suggested that the wash concentration of imidazole (50mM) was too high. A coomassie-stained gel of the separated fractions (not shown) did not show any stained bands. This initial investigation showed that the protocol successfully isolated the P2X<sub>1</sub> protein from the crude cell extract. However the protocol had to be refined to remove P2X<sub>1</sub> protein from the wash fraction and produce visible bands on a coomassie-stained gel. To achieve this, a decrease in the concentration of the imidazole wash was required as well as an increase in the protein concentration of the eluted fractions.

To increase the protein concentration, centrifugal filter devices (Amicon Ultra, Millipore) with 10kDa pore sizes were used to concentrate the eluted fraction. The filter device excludes water, protein and other contaminating compounds under 10kDa thus increasing the final protein concentration. The eluted fraction was 100μl and the filter devices were used to decrease the volume to 20μl. The protocol was repeated as before, but the imidazole wash concentration was decreased to 25mM. The resultant coomassie-stained gel and western blot (figure 4.15) show the appearance of coomassie-stained bands (of various sizes) throughout the gel. This
Figure 4.13. The effects of high speed centrifugation on protein expression. P2X₁-Flag-His cells from 1xT-175 flasks were lysed into 3ml of lysis buffer and spun down at 13,000rpm for 10mins on a bench-top centrifuge. To measure the effects of high speed centrifugation, the crude cell lysate was centrifuged at 100,000g or 50,000g for 1hr (4°C). The supernatant was removed and the pellet was discarded. The supernatant was equally loaded and separated on 10% SDS PAGE. As a control, crude cell extract which had not been subjected to high speed centrifugation was also loaded on the same gel. The gel was transferred to nitrocellulose and probed for immuno-reactivity with the P2X₁ antibody (1:2000) (Alomone). Results showed that there was no significant loss in the expression of P2X₁ following 100,000g spin. This extra spin step was incorporated into the purification protocol as a method of removing excess contamination from other protein sources in the crude cell lysate. (Size markers are shown in kilo Daltons).
Figure 4.14. Protein Purification with the His Protocol. P2X₁,Flag-His stably expressed in HEK293 cells and wt HEK293 cells were lysed from 1xT-175 flask and purified using the His protocol. W refers to the wash fraction, Bl is blank and E refers to the elution fraction. A. Western blot probed with the P2X₁ antibody (1:1000) indicates that the purification successfully isolated the P2X₁ protein in all the elution volumes. However the appearance of P2X₁ protein in the wash fraction suggests that the imidazole concentration used for washing the beads is too high. B. Western blot probed with His antibody (1:5000). Again shows detection of His tagged protein in elution volumes. The presence of P2X₁ protein in the wash fractions of both western blots suggested that a refinement of the protocol was required.
Figure 4.15. The secondary His purification protocol. P2X$_1$-Flag-His stably expressed in HEK293 cells and wt HEK293 cells were lysed from 1xT-175 flask and purified using the His protocol. Centrifugal filter devices were used to concentrate the elution sample. A. The appearance of bands throughout the gel suggests the level of contamination in the final product. The smeared pattern was caused by the increased concentration of the detergent triton X. B. Western blot probed with the P2X$_1$ antibody (1:1000) indicates that the purification successfully isolated the P2X$_1$ protein in all the elution volumes. The results suggest that a stricter wash regimen is required to remove the background contamination. In addition, the detergent was changed to n-octyl-β-D-glucopyranoside.
indicated the presence of contamination from other proteins. In addition the smear pattern observed was due to the increased concentration of the detergent triton X. Similarly to the other protein contaminants, the detergent's large molecule size rendered it unable to pass through the filter's pores and its concentration increased along with the protein concentration. To remedy this, Triton X was replaced with n-octyl-β-D-glucopyranoside (Calibochem). This detergent has a lower molecular size and can pass through the filter's pores. The results from figure 4.15 also showed that although P2X1 protein had been removed from the wash fraction, the increase in the protein concentration had caused an increase in the concentration of other proteins. The results suggested that the wash regimen had to be developed further to remove these contaminants.

The protocol was again repeated but the wash regimen was changed. Previous experiments had used a 5min incubation time for the protein-bound Ni-NTA beads to be in contact with 25mM imidazole. The incubation time was increased to 15min and the mixture was continually rolled during this time period. Centrifugal filter devices were again used to increase the protein concentration. 100μl eluted fractions were filtered down to 20μl. The 20μl sample was loaded onto the SDS-gel and stained with coomassie blue. The results showed a successful reduction in the background contamination that had been present in the previous experiment and the presence of faint bands ~60kDa (figure 4.16). The associated western blot again showed the successful isolation of P2X1 protein from the crude cell lysate. The intense bands of ~55kDa appeared to correspond with those observed in the coomassie-stained gel. However PMF analysis indicated that the bands observed in the coomassie-stained gel were actually bovine serum albumin (BSA).

The Ni-NTA protocol is prone to a higher degree of non-specific binding than other affinity chromatography matrices. Ni-NTA beads can also bind proteins with exposed patches of histidine, cysteine or tryptophan residues and despite the rigorous wash regimen developed, BSA was detected in the eluted fractions. Since non-specific binding was still a significant problem, the Flag protocol was developed instead and the His protocol was used in an auxiliary capacity.
Figure 4.16. Tertiary Protein purification with the His protocol. P2X₁-Flag-His stably expressed in HEK293 cells and wt HEK293 cells were lysed from 1xT-175 flask and purified using the His protocol. Centrifugal filter devices were again used to increase the protein concentration and a stricter wash regimen was implemented. The samples were washed with 25mM imidazole and an incubation time of 15min was allowed in between wash steps. A. The coomassie-stained gel showed faint bands (shown by box) in the region corresponding to the weight of the P2X₁ protein. These regions were excised and analysed for P2X₁ presence. B. Western blot probed with the P2X₁ antibody (1:1000) indicates that the purification successfully isolated the P2X₁ protein in the first two elution volumes. PMF analysis indicated that the bands observed in the coomassie-stained gel were actually bovine serum albumin (BSA).
4.3.6 Optimizing the Flag Purification Protocol

Protein purification using the Flag epitope is similar to the His method. However instead of NTA-Ni beads there is an anti-Flag affinity gel. This is composed of agarose beads bound to a purified monoclonal antibody. Similarly to the NTA-Ni beads, the affinity gel can be placed into a gravity-flow column and the resultant matrix can bind to Flag-tagged protein. The protein-bound beads are washed with lysis buffer and a triple Flag peptide is used to competitively displace the Flag-tagged protein.

Preliminary investigation used 2ml of Flag agarose beads to 3ml of lysate from 1xT-175 flask. The protein was eluted with 0.1mg/ml triple flag peptide. Centrifugal filter devices were again used to increase the protein concentration by decreasing the volume of the eluted fractions from 100μl to 20μl. The resultant western blot showed isolation of P2X_1 protein, and the corresponding coomassie-stained gel showed bands in the area of interest which may have referred to the presence of P2X_1 protein. This band also appeared in the crude P2X_1-Flag-His lysate, but was absent from the HEK293 lysate. This suggested that it was unique to all three and may indicate the successful purification of P2X_1 protein (figure 4.17). The bands were excised and subjected to PMF analysis. PMF analysis showed that the bands observed around the 64kDa region in each case were the proteins keratin and BSA. The most likely source of the BSA was from the culture media and the keratin source was most likely from environmental contamination (e.g. skin, hair etc). To reduce each contaminating source subsequent experiments used fresh gloves to remove keratin and extra wash steps with PBS during cell lysis to remove BSA. The western blot in figure 4.17 showed that P2X_1 protein was successfully isolated but at levels which were too low for successful identification via PMF. The results suggested that a scale up of the process may yield a positive result.

The P2X_1-Flag-His cell lysate was increased to 24ml (8xT-175 flask). The lysate was combined with 5ml flag agarose beads and after binding, were washed three times in 10ml lysis buffer and eluted three times in 4ml. The eluted fractions were concentrated as previously described and a 20μl aliquot was run on a gel. Western blot analysis showed the succesful isolation of P2X_1 protein in the eluted fractions and the coomassie-stained gel showed bands which corresponded with the expected
A. The coomassie-stained gel shows bands (shown by boxes) that appear to be unique to P2X₄ protein and do not appear in the HEK 293 lysate sample. These bands were subjected to PMF analysis.

B. Western blot probed with the P2X₄ antibody (1:1000) indicates that the purification successfully isolated the P2X₄ protein in all the elution volumes. PMF analysis indicated that the bands were keratin and BSA.

Figure 4.17. Protein Purification with the Flag Protocol. P2X₄-Flag-His stably expressed in HEK293 cells and wt HEK293 cells were lysed from 1×T-175 flask and purified using the Flag protocol. Centrifugal filter devices were again used to increase the protein concentration by decreasing the volume of the eluted fractions from 100µl to 20µl. A. The coomassie-stained gel shows bands (shown by boxes) that appear to be unique to P2X₄ protein and do not appear in the HEK 293 lysate sample. These bands were subjected to PMF analysis. B. Western blot probed with the P2X₄ antibody (1:1000) indicates that the purification successfully isolated the P2X₄ protein in all the elution volumes. PMF analysis indicated that the bands were keratin and BSA.
weight of P2X₁ protein. These bands were not observed in the HEK293 lysate sample (figure 4.18). These bands were excised and PMF analysis showed positive detection of the P2X₁ protein with 90% purity. To further increase purity, an additional purification was made using the His protocol. The concentrated samples were pooled and combined with 400μl of Ni-NTA beads. The beads were washed at 25mM, and eluted at 250mM and 500mM imidazole. These samples were loaded onto a gel and a sample of the second elution fraction from the Flag purification was loaded for comparison. The coomassie-stained gel shown in figure 4.19 shows that though the intensity of the band is not greater, the amount of background contamination was significantly reduced. Figure 4.20 shows the PMF analysis data on the peptide fragments generated by the digest of P2X₁ by trypsin. The purity was measured at ~98%. The number of peptide fragments identified was enough to identify the protein as P2X₁, but the coverage of the amino acid sequence only amounted to ~30%. The areas which were identified are shown in figure 4.21. This low level of coverage is an indication that trypsin is not the optimal enzyme for digestion of the protein. The digestion pattern suggests that in some parts of the protein there is a lack of digestion and in other parts there is an over-digestion. Both scenarios produce peptide fragments which are hard to identify. Trypsin cleaves peptides at arginine and lysine residues. The results may be an indication that the 3-dimensional structure of P2X₁ protein allows easy access of trypsin to some areas (over-digestion), but trypsin is inaccessible in other areas (lack of digestion).

4.3.7 Final Protein Purification Protocol (Dual Approach)

HEK293 cells which stably express the P2X₁-Flag-His receptor were grown to 90% confluence in 8xT-175 flasks (Corning Life Sciences). The cells were washed five times in 5ml PBS and solubilised in lysis buffer (3ml per flask). The lysis buffer is composed of 150mM NaCl, 40mM Tris HCl, 8mM Tris base, Protease Inhibitor Cocktail (10μl/ml) and 1% n-octyl-β-D-glucopyranoside (Calibochem) (pH 7.5). The solubilised cells were spun at 13,000 rpm for 20mins (4°C) on bench-top centrifuge and the pellet was discarded. The supernatant was centrifuged at 100,000g for 1hr (4°C) and the pellet was again discarded. The lysate was combined with 5ml Flag agarose beads (50ml Falcon tube) and rolled overnight at 4°C. The protein-bound Flag agarose beads were poured into the gravity-flow column and 10ml lysis buffer was added. The beads were incubated in the lysis buffer for 15min before the buffer
Figure 4.18. Secondary protein purification with the Flag protocol. P2X₁-Flag-His stably expressed in HEK293 cells were lysed from 8xT-175 flask and purified using the modified Flag protocol. **A.** Coomassie-stained gel shows distinct bands (shown in box) in the P2X₁ region. **B.** Western blot probed with the P2X₁ antibody (1:1000) indicates that the purification successfully isolated the P2X₁ protein in all the elution volumes. These bands (shown in box) corresponded with those in the coomassie-stained gel. The bands were removed and subjected to PMF analysis. Analysis showed positive detection with a purity of 90%. (MM refers to the molecular marker).
Figure 4.19. The combination of both the Flag and His protocol. Following scale-up to 8xT-175 flasks, the flag purification produced a band that was later identified as P2X₁. To clean-up the eluted fraction, the His protocol was used. A. Coomassie-stained gel shows that while the intensity of the band (shown in box) has not increased, the background has been severely diminished. PMF analysis indicated ~ 98% purity following the extra His clean-up. B. Western blot probed with the P2X₁ antibody (1:1000) indicates that the purification successfully isolated the P2X₁ protein in all the elution volumes. There is additional P2X₁ detection in the flow-through. This is indicative of an insufficient amount of Ni-NTA beads.
Figure 4.20. A spectra from the PMF analysis of P2X$_1$ protein. The purification protocol was used to isolate the P2X$_1$ protein. The protein sample was separated by SDS-PAGE and stained with coomassie-blue. The band which corresponded to the size of P2X$_1$ protein was excised from the gel. The protein was extracted and subjected to proteolytic digestion with trypsin. Trypsin cleaves peptide bonds at the C-terminal of every arginine and lysine residue. This produced peptide fragments of different sizes. The masses of the resultant fragments were acquired by mass spectrometry and produced the spectra shown above. This “fingerprint” is diagnostic of the P2X$_1$ protein and when compared to the theoretical digest (www.matrixscience.com) was successfully identified. Often 5 or 6 accurate peptide matches are enough to confidently (P <0.05) identify a given protein.
**Figure 4.21. The percentage coverage of the P2X₁ protein.** The diagram shows the primary sequence of the P2X₁ protein and the amino acid residues which were successfully identified. The average sequence coverage was 30%.
was discarded. This allowed the beads to be washed. This was repeated three times so as to allow maximum removal of contaminating compounds. The protein was eluted from the beads with 4ml of triple Flag peptide (0.1mg/ml) (Sigma). This was repeated four times. The eluate was concentrated with 10kDa centrifugal filter tubes (Amicon Ultra, Millipore). The volume was reduced to 100μl and pooled together. 1ml Ni-NTA beads were added to this in an eppendorf and were rolled overnight at 4°C. The protein-bound NI-NTA beads were washed and eluted using a bench-top centrifuge. 500μl of 25mM imidazole was added to the eppendorf and the mixture was left to incubate for 15min whilst rolling. The eppendorf was spun down at 13,000rpm and the supernatant was removed and discarded. This was repeated three times. The protein was eluted once into 50μl of 250mM imidazole and twice into 50μl of 500mM imidazole. All the wash and elution steps were completed at 4°C. The final concentration of the eluted sample was ~ 0.75mg/ml and was determined with the BCA assay kit (Pierce). The eluted fractions (50μl samples) were run on a 10% SDS-PAGE, transferred to nitro-cellulose paper and probed for immuno-reactivity with the anti-P2X1 antibody (1:500 – 1:1000) (Alomone), anti-His antibody (1:5000) (in-house GSK) or anti-Flag antibody (1:500) (Sigma). A corresponding gel was stained with coomassie blue and bands which corresponded to the predicted weight of P2X1 protein were excised and subjected to PMF analysis.
4.4 Discussion

In this chapter I have described the successful creation of tagged P2X₁ and P2X₂ receptors. The attachment of the C-terminal Flag-His tag did not alter the response of either receptor when ATP was applied. Additionally, the ATP response was markedly reduced after UV cross-linking with 2-azido ATP. These characteristics were similar to those of their wild-type counterparts. A HEK293 cell line which stably expressed the P2X₁-Flag-His receptor was generated and a repeatable protocol which allowed the purification of P2X₁–Flag-His protein from crude cell lysate was also developed.

To separate proteins from each other and from other non-protein contaminants, particular differences between them have to be exploited. Proteins can vary from each other in size, shape, hydrophobicity, charge, solubility, binding affinity and biological activity. The use of a tag is one of the simplest techniques that can help differentiate protein and can allow characterisation, in vivo localisation and purification of the protein of interest. The attachment of the fusion tags Flag and His to the carboxy termini of the P2X₁ and P2X₂ receptor allowed a method of purification which could be completed in standard conditions rather than developing a protocol based on the protein’s unique physical characteristics. Previous studies have used Flag or His tags to ascertain various pieces of structural information about P2X receptors such as evidence which suggests the functional receptor is formed after the assembly of three P2X subunits (Nicke et al, 1998; Barrera et al, 2005) and the hetero-oligomeric assembly of different P2X subunits (Torres et al, 1999).

The addition of the Flag-His epitope to the carboxy terminal of the P2X₁ receptor generated functional P2X₁ currents (comparable to the wild-type) which were significantly larger than those produced by the amino tagged receptor. These results led to the development of both the C-terminal tagged P2X₁ and P2X₂ receptor. Both receptors were indistinguishable from their wild-type counterparts and both showed a marked reduction in the current following cross-linking with the ATP analogue 2-azido ATP. These results suggested that the epitope tag had no significant effects on the normal biological function of both receptors.
A HEK293 cell line which stably expressed the P2X1-Flag-His receptor was established through the use of aggressive antibiotic selection with G-418. HEK293 cells were transfected with the C4 plasmid and were grown in a high concentration of G-418. Since the plasmids contained G-418 resistance, HEK293 cells which were successfully transfected grew to confluence and the remaining non-transfected cells died. Monoclonal cell growth was established and the cell line which expressed the strongest peak response to ATP application was chosen as the cell line to conduct the protein purification protocol. Tandem experiments with the P2X2 construct could not successfully produce a stable P2X2-Flag-His cell line. Since the transfection of HEK293 cells with the P2X2 construct produced cells which were not significantly different from the P2X2 wild-type, this suggests that there wasn’t any change in the receptor architecture. The results imply that either the plasmid could not incorporate into the cell genome or that it was a simple case of serendipity and with more transfections I would have eventually been able to create the stable line.

A stable cell line was generated using adherent HEK293 cells. Adherent cells are encumbered with the limitation of contact inhibition and therefore maximum confluence is expressed as a monolayer of cell growth. This could have been a limiting factor in the purification of the P2X1 protein, thus suspension HEK cells were considered as an alternative. These cells are not limited by contact inhibition, grow freely in solution and can generate high-yields of mammalian protein. Stable cell lines have been established previously in this cell line and high levels of expression have been reported (Berg et al, 1993; Reeves et al, 1996). However the ATP responses recorded from suspension HEK293 cells which expressed the wild-type P2X1 receptor were significantly lower than wild-type P2X1 receptors expressed in adherent HEK293 cells.

The Murine Erythroleukemia (MEL) expression system also offered another method of high level expression of recombinant protein. MEL cells have the advantage of an increase in the amount of protein per cell due to an inducible β–globin promoter. In addition, MEL cells can grow to extremely high densities in large scale culture. In a previous study by Needham et al (1995), the MEL expression system was used to express the human calcitonin receptor. The generated stable cell line expressed an overwhelmingly large amount of total cellular protein which, when subjected to
ligand binding studies, were shown to be indistinguishable from the native receptor. Unfortunately the limited availability of the cell line made it an unviable alternative to adherent HEK293 cells.

Isolation and purification of the P2X$_1$ protein was conducted with affinity chromatography. Affinity chromatography exploits the inherent interaction (affinity) of two biomolecules (Bauer & Kuster, 2003). Commonly it involves the attachment of one of the molecules on a solid support (beads) and the extraction of its affinity partner (along with associated proteins) from the crude cell lysate.

In this study both the anti-Flag beads (affinity partner: Flag) and the Ni-NTA beads (affinity partner: His) were used to maximise the level of discrimination between the P2X$_1$ protein and the rest of the cellular background. Although both methods isolated the P2X$_1$ protein, they both needed extensive optimization to minimise the level of contamination and maximise the purity of the final yield. The development of the protocol encompassed the optimization of the cell lysis and washing steps prior to the final scale-up. Utilizing both the flag and the his epitopes, a dual approach was used which efficiently extracted the P2X$_1$ protein and produced a recognizable band after coomassie staining. Following separation on SDS-PAGE, the band which appeared at the corresponding size to P2X$_1$ protein was excised and cleaved into peptides with trypsin. Trypsin cleaves peptides at arginine and lysine residues. The mass of the peptide fragments were compared against the known peptide masses in the online genomic database www.matrixscience.com and the identification was made. The purification of the P2X$_1$ protein was calculated to be ~98% and is a testament to the stringent protocol steps which were developed. However sequence coverage was only ~30%.

The lack of coverage means that there is a greater chance that the area to which each ATP analogue attaches to the P2X$_1$ receptor may not be able to be successfully identified. One of the reasons that the coverage was lower than expected was that tryptic digestion was inefficient in some areas and excessive in others. High sequence coverage of integral membrane proteins is usually hampered by the lack of accessibility to proteolytic cleavage sites which results in large, hydrophobic fragments which can’t be analysed by mass spectrometry. Conversely, an excessive
digest produces additional cleavages at leucine residues. This is in addition to the predicted cleavages made at arginine and lysine residues. This produced peptides which were too small to be detected by mass spectrometry. Further work is necessary if a higher coverage is to be found. Van Montfort et al (2002) and Fischer and Poestch (2006) have shown that different enzymes and different combinations of enzymes can produce better coverage of the protein. Van Montfort and his colleagues (2002) studied the lactose transporter of the *Streptococcus thermophilus* and found that they were able to double the coverage of the protein by digesting the protein with trypsin and then adding an additional cyanogen bromide digest to the peptide fragments. Fischer and Poestch (2006) also found that a combination of enzymes, namely chymotrypsin and staphylcoccal peptidase I, gave better coverage results than trypsin by itself. A recent digest of purified P2X₁ protein by Anda Tsironis (in this lab) showed a similar level of coverage of P2X₁, but different peptide fragments were identified. A combination of both enzymes may very well increase the level of protein coverage to a level that allows confident spectral analysis.

Trypsin is the most commonly used digestive enzyme and both analysis labs (PNACL and GEPB) were well equipped to analyse tryptic fragments. Other enzymes such as chymotyrpsin, cyanogen bromide, casapse-2 or BNPS skatole could result in greater coverage of the P2X₁ protein, however due to time constraints this could not be investigated any further. Though the coverage was less than expected, the development of a repeatable purification protocol can reveal more details about the structure of the P2X₁ protein. Further study can confirm the positions of the disulphide bridges and can aid in crystallization studies. The development of a successful crystallization protocol can produce crystals which can analysed by X-ray diffraction, this in turn can lead to the development of a 3-dimensional structure for the P2X₁ receptor.
4.5 Summary and Conclusion

The work conducted in this chapter can be summarized as follows: -

- P2X₁ and P2X₂ constructs, which express both the flag and his epitope, were successfully generated. These constructs behaved similarly to their wild-type counterparts in response to ATP.

- UV irradiation of the modified receptors in the presence of 2-azido ATP caused an inhibition in the peak amplitude response to ATP. This suggests successful cross-linking of the compound to the receptor.

- The P2X₁ construct was successfully introduced to the HEK293 cell line and a cell line stably expressing the P2X₁-flag-his receptor was generated. Generation of a stable P2X₂-flag-his cell line was unsuccessful.

- A combination of both Flag and His purification methods allowed a final purification method to be developed. Analysis of the final purified sample showed positive detection of the P2X₁ protein. The purity was over 90% and the coverage was ~30%.

- The reduced level of coverage suggests an excessive digestion of the P2X₁ protein by trypsin.

This chapter has described the successful generation of a HEK293 cell line which stably expressed the P2X₁-flag-his receptor. The purification protocol successfully isolated the P2X₁ protein with a purity > 90%, but analysis with mass spectrometry revealed that only 30% coverage of the protein was obtainable. This low level of coverage requires the development of a different technique for the successful identification of cross-linked P2X₁ peptide fragments.
CHAPTER 5

DIGESTION ANALYSIS OF PEPTIDE FRAGMENTS INVOLVED IN THE ATP BINDING SITE OF THE P2X₁ RECEPTOR

5.1 Introduction

The aim of this investigation was to identify the amino acid residues that contribute to the ATP binding site of P2X receptors. This was to be carried out by cross-linking the receptor to an ATP analogue and digesting the complex with trypsin. The resulting peptide fragments were to be analyzed by mass spectrometry. The amino acid residues which interacted with the reactive site of the ATP analogue would be identified and this would give the first direct evidence of the contributing amino acid residues in and around the ATP binding site. The results from the previous chapter showed that the protein coverage of P2X₁, following trypsin digestion, only amounted to ~30%. This low amount of coverage necessitated a slight change in the methodology.

Previous work conducted in chapter 2 has shown that the P2X₁ receptor can be permanently labelled with 2-azido [γ²³P] ATP. The labelled protein can then be digested with proteolytic enzymes and an SDS gel can be used to separate the products of enzymatic cleavage. Since the glycosylated P2X₁ receptor migrates to ~55kDa, the shift in the gel pattern (caused by smaller peptide fragments still associated with the radio-label) can indicate the amino acid residues involved in the binding of ATP.

Trypsin and cyanogen bromide were used to cleave the proteins in this study. Trypsin cleaves proteins at lysine and arginine residues and cyanogen bromide cleaves proteins solely at methionine residues. This produces peptide fragments of different sizes. This gel based methodology has been used previously. A combination of photo-reactive cross-linking and CNBr digestion was used to identify the different ligand interaction sites at the parathyroid hormone (PTH) parathyroid hormone-related protein (PTHrP) receptor (Maanstadt et al, 1998; Behar et al, 2000). The receptor (PTH1R) is a G-protein
coupled receptor which mediates the biological actions of the two distinct hormones, PTH and PTHrP. Maanstadt and his group were able to localise the binding of a radio-labelled photo-reactive analogue to the amino terminal of the extracellular domain. After labelling the 80kDa receptor, they digested it with CNBr and observed a peptide fragment of ~ 9kDa which maintained association with the radio-label. The strategic introduction or replacement of methionine residues in the receptor allowed them to localise the binding of the ligand to the amino terminal of the extracellular loop (Maanstadt et al, 1998). Behar et al (2000) used a similar approach to suggest different points of interaction within the receptor between a photo-reactive agonist and antagonist.

In this chapter I used the method established in section 2.2.5 to permanently label the P2X₁ receptor with 2-azido [γ³²P] ATP. I then digested the protein with trypsin and separated the peptide fragments using high performance liquid chromatography (HPLC). Any peptide fragment which expresses radiation suggests the binding of the radio-labelled ATP analogue and mass spectrometry can be used to identify the amino acid residues contained therein. Alternatively, the radio-labelled P2X₁ protein was digested with either cyanogen bromide (CNBr) or trypsin, separated by SDS-PAGE and exposed to autoradiography film. A shifted band pattern again suggests the binding of the radio-labelled ATP analogue to a smaller sized fragment that can be identified with mass spectrometry.
5.2 Materials and Methods

5.2.1 Radio-labelled Cross-Linking
Radio-labelled cross-linking was carried out using the same methods as described in chapter 2 (section 2.2.5).

5.2.2 Protein Purification
Protein purification of the P2X\textsubscript{1} protein was carried out using the protocol developed in chapter 4 (section 4.4.7).

5.2.3 Trypsin Digest
100\,\mu l of BSA (1\,\mu g/\mu l) were lyophilised in a speed vacuum (Eppendorf concentrator 5301) and re-suspended in 50\,\mu l of 50mM ammonium bicarbonate. 6\,\mu l of acetonitrile was added to this and was mixed with a bench-top vortex for 5\,min. 2.8\,\mu l of 100\,\mu M DTT was added and the mixture was allowed to incubate at 60°C for 30\,min. It was then removed from the heat and allowed to cool for 30\,min. Following this an addition of 5.9\,\mu l of iodoacetamide was made and the mixture was incubated in the dark and at room temperature for 30\,min. 10\,\mu l of Promega modified porcine trypsin was added and the sample was incubated at 37°C overnight. The following day 1\,\mu l of 100% formic acid was added to terminate the digestion. When digesting the purified P2X\textsubscript{1} protein, 100\,\mu l (0.75mg/ml) was initially dialysed into 50mM ammonium bicarbonate using a standard dialyser kit (Novagen). This was done to remove the excess salts which would be a hindrance to spectral analysis. The purified P2X\textsubscript{1} protein was then subjected to the same method used for BSA digestion.

5.2.4 High Performance Liquid Chromatography (HPLC)
HPLC is a process which can allow the separation of compounds based on their specific physical or chemical properties. In this chapter I used this technique to separate peptide fragments based on their hydrophobicity. The chromatography column is the area at which the mixture of compounds is separated. It is packed with silica beads (~50\,\mu m
diameter) which have hydrophobic alkyl chains bound to them (figure 5.1). In this investigation the silica beads had 18 carbon long alkyl chains (C18) bound to their surface. The sample is injected into the column and the peptide fragments bind to the C18 alkyl chains. Mixtures of two solvents (solvent A and B) of differing compositions are then pumped into the column at a constant rate. Solvent A is composed of 0.1% trifluoroacetic acid (TFA) and HPLC grade water. Solvent B is composed of 80% acetonitrile (ACN), 0.1% TFA and HPLC grade water. The process is programmed to allow an exchange between solvent A and solvent B. This exchange occurs over a linear time gradient. Solvent A is an aqueous solvent, thus peptide fragments which are more hydrophilic tend to resolubilize and elute back into the solution early in the process. Conversely, solvent B contains the organic solvent ACN, this causes peptide fragments which are more hydrophobic to elute off the column at a later time. The linear exchange gradient governs the percentage mixture of solvent A to solvent B, and as time continues, a larger percentage of the solution passing through the column is composed of solvent B. Hence, the more hydrophobic a peptide fragment is, the longer it will take to elute from the column. This allows a separation of the digested fragments. The eluted fragments run through the HPLC and can be collected at regular time intervals. Prior to collection, the eluted fragments pass through a detector. This detector contains a UV emitter set at a particular wavelength. The amount of UV absorption is indicated as a peak on the resulting chromatograph (figure 5.1).

For this investigation, the fragmented $^{32}$P-labelled P2X$_1$ protein (following trypsin digest) was concentrated to 50µl with a speed vacuum and a 20µl sample was injected into the Vydac C18 column. The sample was fractionated using the following conditions: Solvent A was composed of 0.1% trifluoroacetic acid (TFA) and HPLC grade water, solvent B was composed of 80% acetonitrile (ACN), 0.1% TFA and HPLC grade water; the gradient was from 0% - 100% solvent B in 50min at a flow rate of 60µl/min. Fractions of ~500µl were collected every 2min and absorption was monitored simultaneously with a diode array detector set at 215nm. To detect the fraction which expressed radioactivity, the fractionated samples were concentrated to 10µl using a speed vacuum and were spotted onto grid-referenced Whatman filter paper with a pipette. The
Figure 5.1. The HPLC chromatography Column. HPLC is a process which allows the separation of compounds based on their unique physical or chemical characteristics. For this series of experiments P2X₁ protein was radio-labelled with 2-azido [γ32P] ATP and digested with trypsin. The resulting peptide fragments were separated according to their hydrophobicity. A. The chromatography column is packed with silica beads which have 18 carbon long alkyl chains (C18) bound to their surface. The sample is injected into the column and the peptide fragments bind to the C18 chains. Mixtures of two solvents (A and B) are pumped into the column at a steady flow rate. In the beginning, solvent A is the predominant solvent. It is composed of 0.1% trifluoroacetic acid (TFA) and HPLC grade water. Peptide fragments which are more hydrophilic dissolve in the solvent and elute from the C18 chains and exit the column where they can be collected. Peptide fragments which are more hydrophobic stay bound to the C18 chains. B. Over time the composition of the entering solvent is changed to favour solvent B. It is composed of 80% acetonitrile (ACN), 0.1% TFA and HPLC grade water. This is an organic solvent and it allows the elution of peptide fragments which are more hydrophobic. These peptide fragments can be collected. This process allows the separation of the different peptide fragments. The detection of radiation from a specific peptide fragment suggests the successful binding of the ATP analogue and it can be analysed with mass spectrometry to reveal the amino acid residues involved in the binding of 2-azido ATP.
filter paper was exposed to a phospho-imager screen for 72hrs at room temperature and the appearance of a blot in a particular grid suggested the elution of the radio-labelled peptide fragment in a specific eluted fraction.

5.2.5 CNBr Digest

Cyanogen bromide (CNBr) cleaves peptides at their methionine residues. It has been used previously in similar experiments (Taniuchi and Anfinsen, 1966; Doyen and Lapresle, 1979) to cleave proteins to produce smaller peptide fragments which can be identified at a later stage via mass spectrometry. For this investigation, protein was lyophilised using a speed vacuum and reconstituted in 50μl water, 50μl ammonium bicarbonate and 5μl β-mercaptoethanol. The mixture was transferred to an eppendorf and the oxygen contained within the tube was displaced with argon. The tube was sealed and incubated at 37°C for 2hrs. The mixture was lyophilised again and re-suspended in 100μl 70% formic acid. One crystal of CNBr (Sigma) was added to the tube and it was sealed and allowed to incubate at room temperature overnight. The mixture was lyophilised and re-suspended in 20μl trifluoroacetic acid and allowed to incubate for 30min at room temperature. It was lyophilised again and re-suspended in 400mM ammonium bicarbonate and allowed to stand for 30min. The mixture was lyophilised and re-suspended in 20μl loading buffer and separated on a 12% SDS-PAGE. The SDS-PAGE was stained with coomassie-blue (Blue Stain Reagent, Pierce) according to manufacturer’s guidelines. The entire CNBr digest was conducted within a fume cupboard due to the toxicity of CNBr.

5.2.6 Cell Culture

Cell culture was carried out using the same methods used in chapter 2 (section 2.2.1)
5.3 Results

5.3.1 Trypsin Digest and HPLC Analysis
Radio-labelled P2X₁ was digested with trypsin and run on the HPLC as described in section 5.2.3. The peptide fragments collected from the digested radio-labelled P2X₁ sample were spotted onto filter paper and exposed to a phospho-imager screen for 72hrs. The phospho-imager is more sensitive than autoradiography film and was able to detect radioactivity from the fragment eluted between 5-8min. The radiation detected in this time frame corresponded with the detection of a peak absorbance (figure 5.2). This would suggest the presence of peptide fragments. However the peak was near the beginning of the chromatograph and would be composed of salts and other contaminating material which would hinder spectral analysis. As a control, a 20μl sample of 1μM 2-azido ATP (dissolved in lysis buffer) was also run on the HPLC machine. The 2-azido ATP sample produced an exact peak absorbance at the start of the run (5-8min). The fractionate from this time period was collected and analyzed with a scanning spectrophotometer and was compared with a scan taken from a 2-azido ATP sample (figure 5.3). The scanning spectrophotometer was set to scan between 200nm - 600nm at a speed of 240nm/min. The scans were identical and suggested that either the peptide fragment which was bound to the radio-label was only a few amino acid residues in length or that the 2-azido ATP was cleaved from the peptide by either the experimental conditions or trypsin.

5.3.2 CNBr Digestion
The tryptic digestion and separation by HPLC proved to be unsuccessful. An alternative approach involved digestion of purified P2X₁ protein with CNBr. CNBr only cleaves proteins at methionine residues and so produces larger peptide fragments. The predicted fragments following the digest of P2X₁ protein after CNBr or trypsin digest are shown in figure 5.4. There are only 7 methionine residues contained in the P2X₁ sequence, thus the radio-label should be attached to a fragment that can be identified after separation by SDS-PAGE and exposure to autoradiography film. Prior to labelling experiments, the
Figure 5.2. HPLC separation of trypsin-digested $^{32}$P 2-azido ATP-labelled P2X$_1$. $^{32}$P 2-azido ATP labelled P2X$_1$ was digested with trypsin as described under methods 5.2. A. Samples were concentrated to 50μl with a speed vac and a 20μl sample was injected to a Vydac C18 column and was fractionated using the following conditions. Solvent A was 0.1% trifluoroacetic acid and 5% acetonitrile, Solvent B was 80% acetonitrile and 0.1% trifluoroacetic acid. A linear gradient of 0 – 100% of solvent B in 50min at a flow rate of 60μl/min was used. A diode array detector was used to measure absorbance at 215nm. At 2min intervals fractions were collected and stored for radioactivity detection. B. Fractionated samples were concentrated to 10μl with speed vac and spotted onto Whatman filter paper. The Filter paper was exposed to a phospho-imager screen for 72hrs at room temperature. Result shows the presence of radioactivity at fraction 3. This correlates to an elution time between 5-8min (highlighted area on trace).
Figure 5.3. Control HPLC trace for 2-azido ATP. A 20μl sample of 1μM 2-azido ATP was injected onto the vydac C18 column and was fractionated using the same conditions. The diode array detector was used to measure absorbance at 271nm. A. The trace shows a peak (highlighted) between 4 – 6min. B. The fractionated sample was analysed on a scanning spectrophotometer (200nm – 600nm) at a speed of 240nm/min. The fractionated sample (red trace) shows strong absorbance at ~272nm which corresponds with the strong absorption shown from a sample of 1μM 2-azido ATP (blue trace).
<table>
<thead>
<tr>
<th>Mass (kDa)</th>
<th>Position</th>
<th>Peptide Sequence</th>
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<td>104 – 214</td>
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<td>22 - 103</td>
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</tr>
<tr>
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<td>2 – 21</td>
<td>ARRFQAEELAAFLFEYDTPRM</td>
</tr>
<tr>
<td>2.1</td>
<td>375 - 396</td>
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</tr>
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</table>

**CNBr**

<table>
<thead>
<tr>
<th>Mass (kDa)</th>
<th>Position</th>
<th>Peptide Sequence</th>
</tr>
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<tr>
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<td>71 – 111</td>
<td>GLAVTQLPLGPGPQWDVADYVFPAQGDSFVMNTNVTPK</td>
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<td>3.6</td>
<td>326 – 359</td>
<td>FDIIPTMTTIGSAGIFGAVTVLCDDLWHLIPK</td>
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<td>222 – 249</td>
<td>TLHPLCPVFQGLYVQESGQFSTLAEK</td>
</tr>
<tr>
<td>2.6</td>
<td>158 - 180</td>
<td>TCEFIFCWCPVEVDDIRPRAILLR</td>
</tr>
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</table>

Figure 5.4. The predicted peptide fragments following digestion with CNBr or trypsin. CNBr cleaves proteins at methionine residues. The primary sequence of P2X1 contains 7 methionine residues and should produce 7 peptide fragments. The first table describes the expected sequences of these fragments. Trypsin digests at Arginine (A) and Lysine (K). Digestion with trypsin is predicted to produce up to 48 potential cleavage products, the largest of which is only 4kDa. Residues emboldened indicate those which contribute to the extracellular loop.
P2X₁ protein was purified, dialyzed into 50mM ammonium bicarbonate and digested with CNBr. The resulting sample was run on a 12% SDS-PAGE with CNBr-digested BSA and stained with coomassie-blue. The results showed a clean digest of the BSA sample and the production of peptide fragments which were similar in weight to those predicted previously. These fragments were excised from the gel and successfully identified by mass spectrometry. However no banding pattern was observed from the P2X₁ sample. This result suggests the loss of P2X₁ protein at some stage of the experiment, most likely the dialysis stage. A western blot was conducted on samples gathered from both before and after the dialysis. The results shown in figure 5.5 suggest that there was a significant loss of protein following dialysis into 50mM ammonium bicarbonate. This loss in protein was due to the detergent. The detergent was changed from triton X to octylglucoside during the development of the purification protocol. This was done to take advantage of the smaller molecule size of octylglucoside. However this small molecule size proved to be a disadvantage in the dialysis step. Due to its size, the detergent exited the membrane along with the other contaminating material and caused precipitation of the P2X₁ protein. To counter this, 1% octylglucoside was added to 50mM ammonium bicarbonate. A repeat of the experiment showed identical results which suggested that another factor - possibly the salt content, could be the limiting factor in causing the protein to drop out of the solution. The addition of salts would be in conflict with the conditions required for the successful spectral analysis of the sample.

The gel based technique was also conducted with trypsin, but a similar result occurred after the dialysis of the P2X₁ sample. Hence the dialysis step was not further considered and a protocol which allowed either the CNBr or tryptic digest to be conducted directly in the lysis buffer was used.

5.3.3 Modified Digestion Protocol

11.2μl of 100μM DTT was added to 25μg/ml BSA (dissolved in lysis buffer) and incubated at 60°C for 30min. The mixture was then removed from the heat source and allowed to cool. Once the mixture had cooled to room temperature, 23.6μl of iodoacetamide was added. The mixture was incubated in the dark at room temperature.
Figure 5.5. Dialysis of the purified P2X₁ sample causes protein loss. The loss of protein experienced in the CNBr digest was examined with a western blot of the P2X₁ samples before and after dialysis. 100mg/ml samples were taken and probed with P2X₁ antibody (1:1000). The results showed the significant loss of protein caused by the dialysis stage.
for 30 min. 10 μl of trypsin was then added and the sample was incubated at 37°C overnight. To stop the digestion, 1 μl of formic acid was added. The digested sample was then separated on a 12% SDS-PAGE and stained with coomassie-blue. The CNBr digest was similar to that described in section 5.2.5. The only difference was that the starting BSA sample (dissolved in lysis buffer) was not lyophilised and reconstituted in 50 μl water, 50 μl ammonium bicarbonate and 5 μl β-mercaptoethanol. The BSA sample was simply transferred to an Eppendorf tube and the oxygen was displaced with argon. The rest of the protocol was unchanged.

The digest of BSA using these new protocols produced stained bands of a particular weight (kDa). As previously described, these bands were excised from the gel and were successfully identified as BSA peptide fragments. The new digestion protocol for CNBr digest was used on radio-labelled P2X₁ and the resultant sample was separated on 12% SDS-PAGE and exposed to auto-radiography film for 72 hrs. The results showed that although a control sample was visible, there were no observed bands present in the predicted areas of weight (figure 5.6). The digest of radio-labelled P2X₁ protein with trypsin also produced similar results (figure 5.6). Taken together these results again suggest that either the peptide fragment attached to 2-azido [γ³²P] ATP was too small (<10 kDa) or that the radio-label was cleaved from the protein (by either enzyme) during the digest. It is important to note that radioactivity was still detected from the sample prior to loading on the 12% SDS-PAGE.

5.3.4 Partial Digestion
Since the full digestion of labelled P2X₁ protein produced no discernible result, the protein was subjected to a partial digestion. This involved specific changes in the digestion protocol. The previous results in figure 5.6 could be a reflection of over-digestion of the protein which produced smaller, less detectable peptide fragments. A partial digestion would produce larger peptide fragments and would reveal whether the previous results were an indication of the radio-label attaching itself to a peptide fragment composed of only a few amino acid residues.
Figure 5.6. Digest of radio-labelled P2X1 with CNBr or trypsin. P2X1 protein was radio-labelled with 2-azido $[^{32}\text{P}]$ ATP and digested with either CNBr or trypsin. Samples were separated on 12% SDS-PAGE and were exposed to autoradiography film for 72hrs. The diagram shows the control band appearing at ~55kDa, however no bands were observed in either of the digested samples. The marked boxes show the sizes at which the largest peptide fragments for the CNBr or trypsin digest should have appeared. The gel was not run to completion and unbound 2-azido $[^{32}\text{P}]$ ATP appeared at the bottom of the control sample. However there is no unbound compound on either digested sample. These results may suggest that the radio-label was cleaved from the protein by both enzymes. The radio-label may have dispersed into the surrounding SDS buffer.
As a control, BSA was again used to test the difference in the banding pattern exhibited after 10%, 25%, 50% or 100% trypsin digestion (figure 5.7). The differences in the banding pattern were also compared between BSA digested with 5%, 12.5%, 25%, 50% or 100% CNBr. The stained gels showed the production of larger peptide fragments when less trypsin or CNBr was used in the digestion protocol (figure 5.7).

Radio-labelled P2X₁ was digested with either 10% or 25% trypsin, or 12.5% or 25% CNBr. The samples were separated with a 12% SDS PAGE, but the gel was not run to completion. This was to show any unbound radioactive product. As before, the gel was exposed to autoradiography film for 72hrs. The results again showed an absence of any radioactivity in any of the digested lanes (figure 5.8). Unbound radioactive material was observed at the bottom of the control sample but was absent from the digested samples. This suggests that the radio-label was indeed removed by trypsin and CNBr and most likely dissipated into the surrounding SDS running buffer.
Figure 5.7. Partial digest of BSA with CNBr and trypsin. The complete digestion of radio-labelled P2X1 failed to show a radio-labelled peptide fragment of any particular size. A partial digest would produce larger peptide fragments and show a shift in the band size which would indicate the binding of 2-azido [γ32P] ATP. Different digest conditions were tested with 500 μg/ml BSA. A. CNBr digest of 500μg/ml in varying concentrations of CNBr. Results show that the production of more lower weight peptide fragments with higher concentrations of CNBr. The protein is barely digested with 5% CNBr. B. Trypsin digest of BSA (500μg/ml) at varying concentrations of trypsin. Successful digest of BSA with all concentrations of trypsin suggest the over-efficiency of the enzyme.
Figure 5.8. Partial Digest of radio-labelled P2X<sub>1</sub> with CNBr or trypsin. P2X<sub>1</sub> protein was radio-labelled with 2-azido [γ<sup>32</sup>P] ATP and digested with either 12.5%, 25% CNBr or 10%, 25% trypsin. Samples were separated on 12% SDS-PAGE and were exposed to autoradiography film for 72hrs. The diagram shows the control band appearing at ~55kDa. As previously, there are no observable bands in the digested samples. These results suggest that the 2-azido [γ<sup>32</sup>P] ATP label has been removed during the digest and was lost in the running buffer.
5.4 Discussion

This chapter has described attempts to localise the binding of radio-labelled 2-azido ATP to a peptide group within the P2X1 receptor. Digestion of radio-labelled P2X1 with either trypsin or cyanogen bromide appeared to cause the disassociation of the compound from the peptide fragments. This was possibly caused by enzymatic cleavage or the conditions used. Further experiments encompassing the rest of the identified ATP analogues (chapter 3) may conclusively reveal the identity of some of the amino acids which reside within the ATP binding site of the P2X1 receptor.

The basic premise of this concluding chapter was to analyse gel shift caused by digestion of radio-labelled P2X1 protein. The digested protein was separated by SDS-PAGE and exposed to autoradiography film. This exposure should have revealed peptide/s of smaller mass which were still associated with 2-azido [\(\gamma^{32}P\)] ATP. Trypsin and cyanogen bromide were chosen to digest the protein. Trypsin, which has already been discussed in section 4.5, is the most commonly used digestive enzyme. Cyanogen bromide (CNBr) distinctively cleaves proteins at methionine residues. Similar experiments have used CNBr to produce smaller peptide fragments which can be identified by mass spectrometry. Taniuchi and Anfinsen (1966) digested an extracellular nuclease of *Staphylococcus aureus*. Digestion of the nuclease, which is produced during the growth of the bacterium, produced five peptide fragments whose amino acid composition was analysed using Edman sequencing. Winkler and Klingenberg (1992) used CNBr to digest the uncoupling protein from hamster brown adipose tissue which was radio-labelled with 8-azido [\(\gamma^{32}P\)] ATP. They separated the peptide fragments by SDS-PAGE and were able to localise the binding of the radio-label to a single fragment. This fragment was sequenced and they were able to identify some of the amino acid residues that contributed to the nucleotide binding site. More recently, Van Montfort et al (2002) used a combination of trypsin and CNBr digestion to produce peptide maps for the lactose transporter of the bacterium *Streptococcus thermophilus*.
A similar approach was carried out with the P2X\textsubscript{1} receptor. The initial results from the HPLC experiments suggested that either the radio-label may have associated with a peptide fragment composed of only a few amino acid residues or that the radio-label had been cleaved from the protein. Similar problems have been described by Salvucci et al (1992). In this study he described the inherent lability of the covalent bonds which are formed during photo-insertion of the azido analogue. A similar problem was also reported by Tran et al (1994). In their study of the ATP binding site of Na,K-ATPase they found that the radio-label (2-azido ATP) eluted from the chromatography column after 1-2 min. They tried several HPLC solvent systems but they kept getting the same result.

Since separation of the tryptic fragments by HPLC did not work, a different digestion enzyme and a gel based methodology were undertaken.

The receptor was radio-labelled with 2-azido $[^{32}\text{P}]$ ATP, immunoprecipitated with the P2X\textsubscript{1} antibody and digested with CNBr. CNBr cleaves proteins at the methionine residue. The P2X\textsubscript{1} receptor contains 7 methionine residues in its primary sequence and digestion will result in the production of 7 smaller peptide fragments (figure 5.4). The peptide fragments were separated using SDS-PAGE. This should have given a shift in the band pattern since the radio-label should have maintained association with one or more of these peptides. The results showed no presence of the radio-label in either the CNBr digest or the trypsin digest. When the SDS-PAGE was conducted and the gel front was allowed to remain visible, no radiation was detected in the digested samples. Similarly, when the time of exposure to the autoradiography film was increased to 2 weeks there were still no observable bands on the resultant film. Since radiation was detected (with a Geiger counter) prior to gel loading, this suggests that cross-linked 2-azido ATP may have been cleaved from the peptide fragments during the digestion process (due to the enzymes or the digestion conditions) and dissipated into the SDS running buffer.
The most likely cause of the cleavage of 2-azido ATP was the conditions of the buffers used to conduct each of the digestions. The amino acid residue with which the covalent linkage was formed may have been compromised by the experimental conditions used. Salvucci et al (1992) noted in their study that photo-insertion which involved the tyrosine residue formed a bond which was stable enough to preserve the radioactive tag throughout the HPLC procedure. The results from this chapter appear to show that the amino acid residue with which 2-azido ATP forms a bond may not have been stable enough to persist through the experimental conditions, thus the radioactive tag was lost. It is also possible that some radioactivity remained bound to the P2X1 protein, but at levels which were undetectable. This reduction of efficiency due to the majority of the label being removed from the protein may be resolved by increasing the amount of radio-label used in the protocol. Only a small detectable amount is necessary to direct the investigation to the specific peptide fragment which bound to the photo-active compound and further research may still yield an answer.

The photo-reactive compounds which display activity at the P2X1 receptor contain reactive sites which are attached at different coordinates around the ATP molecule. This suggests that covalent bonds will be formed at different amino acid residues within the binding pocket. These bonds may offer more stability during the HPLC separation and finally allow identification of the amino acid residues involved. Due to time and financial constraints I could not investigate this further but this would be an interesting avenue for future research.
5.5 Summary and Conclusion

The work conducted in this chapter can be summarized as follows:-

- Radio-labelled P2X₁ which was digested with trypsin was separated by HPLC. Its early elution from the chromatography column suggested that either the radio-label was removed during digestion or that the eluted peptide was composed of only a few amino acid residues.

- Although CNBr digestion of BSA was successful, digestion of P2X₁ protein was not observed due to protein loss during dialysis into ammonium bicarbonate. Dialysis was necessary to remove contaminating compounds and improve the fidelity of the mass spectrometry.

- The adoption of a modified digestion protocol proved successful with BSA; however the partial digestion of radio-labelled P2X₁ did not reveal larger, radio-labelled peptide fragments as initially predicted.

- The results suggested that 2-azido ATP was removed from the protein during digestion.

This chapter has described the attempt to identify the P2X₁ peptide fragments which were associated with ³²P labelled 2-azido ATP. Although different methods of protein digestion were tried, the amino acid residues which constitute the ATP binding site of the P2X₁ receptor could not be elucidated. Further work is necessary to develop a working protocol that implements the cross-linking of the ATP analogues to the P2X receptor and combines this with a suitable digestive enzyme which can efficiently fragment the bound protein so that the bound amino acid residues can be identified.
CHAPTER 6

Final Discussion

The systematic mutagenesis of conserved amino acids within the extracellular loop has led to the generation of a model of the ATP binding site of the P2X\textsubscript{1} receptor (Vial \textit{et al}, 2004). Although mutagenesis has provided the majority of information as to the key residues involved in coordinating the binding of ATP, direct binding evidence is also required. The aim of this thesis was to provide corroborating evidence by using various biochemical techniques to directly identify the amino acid residues that contribute to the ATP binding site of the P2X receptors.

One of the primary aims of this thesis was to identify ATP analogues which could not only mimic the actions of ATP, but under certain conditions would cross-link to the P2X\textsubscript{1} receptor and permanently reside within its binding pocket. A number of experiments were used to prove that the photo-reactive ATP analogue 2-azido ATP permanently labelled the P2X\textsubscript{1} receptor from within the ATP binding pocket.

The photo-reactive compound 2-azido ATP was tested against the P2X\textsubscript{1} receptor. 2-azido ATP contains an azido (N\textsubscript{3}) group at the 2-position within the adenine ring. Upon UV irradiation, this azido group is converted into a nitrene and covalently attaches itself to the protein. Attachment within the binding domain prevents further activation of the receptor by ATP. 2-azido ATP mimicked the actions of ATP and once UV irradiated in the presence of wild-type P2X\textsubscript{1} cells, caused a significant inhibition of the ATP response. ATP protection experiments were conducted to verify whether the binding of the reagent was specific to the ATP binding site. The results showed that the presence of excess ATP caused a prevention of the inhibition caused by 2-azido ATP. Further experiments showed that the binding of the compound was permanent and definitive evidence was recorded from the successful radio-labelling of P2X\textsubscript{1} protein by 2-azido [\textgamma\textsuperscript{32}P] ATP. The development of the radio-labelling binding assay can be used as a predictive tool to directly allow the investigation of the ATP binding site of mutant P2X\textsubscript{1} receptors.
Previously shown mutations of the NFR region (290-292) and the positive lysine at the 309 position to a cysteine residue revealed a significantly reduced ATP potency when compared to the wild-type (Roberts & Evans, 2004; Roberts & Evans, 2006). It has been recently shown that binding of the radio-labelled 2-azido ATP was significantly decreased in these mutants when compared to the wild-type (Roberts & Evans, 2007). This decrease is indicative of a reduction in direct binding to the P2X₁ receptor and provides further evidence that the NFR region and the lysine residue at 309 contribute to the binding site of the P2X₁ receptor. Additionally, since the binding of 2-azido [γ³²P] ATP has been shown to be reduced after pre-treatment with ATP, a repeat of the experiment with P2X₁ antagonists may reveal whether they are competitively binding to the ATP binding site.

The successful identification of 2-azido ATP as a cross-linking compound at the P2X₁ receptor established a criteria which other photo-reactive ATP analogues were tested against to investigate their cross-linking abilities. Various ATP analogues were identified as having varying degrees of success at binding to the ATP binding site of the P2X₁ receptor. Importantly each analogue contained its reactive site at different coordinates around the ATP analogue. This indicated that different amino acid residues at different positions within the binding domain were permanently labelled.

The photo-active reagents 8-azido ATP, ATP azidoanilide, Bz-ATP and ATP benzophenone were tested for their ability to cross-link to the ATP binding domain of the P2X₁ receptor. Bz-ATP, which contains a benzophenone group attached to the ribose moiety, produced a similar level of inhibition as 2-azido ATP. ATP benzophenone contains a benzophenone group attached to the terminal phosphate. It significantly inhibited the ATP response of P2X₁ receptors but not at a similar level as that produced by 2-azido ATP or Bz-ATP. ATP azidoanilide contains an azido group at the terminal end of the phosphate tail; it produced the same level of inhibition of ATP response as that of ATP benzophenone. The results were verified with cell recovery and cell protection experiments and ATP azidoanilide [γ³²P] was found to successfully radio-label P2X₁ protein. The results indicated that a number of different coordinates within the binding
domain had successfully been probed by the photo-active compounds. Although both compounds contain bulky substitutions at the phosphate tail, the results from ATP benzophenone and ATP azidoanilide suggest that an intact phosphate tail is necessary for activity at the P2X₁ receptor. FSBA and ADP do not have a triphosphate tail and neither compound is active at the P2X₁ receptor. 8-azido ATP had a lower potency at the P2X₁ receptor and it did not inhibit the ATP response after UV irradiation. It contains an azido group at the 8 position of the adenine ring. This result was in contrast to that of 2-azido ATP. The subtle structural change in the reagents suggests that the ATP binding site of the P2X₁ receptor is highly specific and that the 8-position of the adenine ring may interact with a key amino acid residue(s) which reside(s) within the ATP binding domain.

Figure 6.1. Photoactive ATP analogues. Substitutions within the ATP molecule can yield compounds with various cross-linking ability across all P2X subtypes. Reactive groups attached to the diverse areas of the adenine ring, ribose sugar or phosphate tail can prove to be useful in finding different attachment points at the ATP binding site.

The molecular cloning of each P2X subtype has allowed each receptor to be studied in various expression systems and successfully characterized. The pharmacological differences between each subtype suggest subtle differences in each subtype’s ATP binding domain (amongst other possibilities). The ability of each photo-reactive
compound to cross-link to the P2X₂, P2X₃ and P2X₄ receptor was tested using the same conditions established for the P2X₁ receptor. It was found that there were similarities and differences in the ability of each photo-reactive compound to cross-link to each subtype.

In addition to their different distribution patterns, P2X receptor subtypes have distinctive properties and pharmacological characteristics (reviewed in Gever et al, 2006). This may suggest that each ATP binding site is slightly different in its architecture. The abilities of each photo-active reagent varied when tested against each P2X receptor subtype (P2X₂ - P2X₄), however there were also some similarities. 2-azido ATP successfully cross-linked to each receptor with varying degrees of success. At the P2X₁ receptor the inhibition of the ATP response was 82%, P2X₂ receptors were inhibited by 77%, P2X₃ receptors were inhibited by 86% and P2X₄ receptors were inhibited by 44%. These significant reductions in the ATP response suggested a structural commonality existed between each receptor. This suggestion was further substantiated by the continued ineffectiveness of 8-azido ATP. ATP azidoanilide also caused a reduction in the ATP response in all of the subtypes. Differences in the characteristics of each P2X receptor subtype were observed when Bz-ATP was tested. It effectively reduced the ATP response in the P2X₁ (82%) and P2X₃ (84%) receptor, but had no activity at the P2X₂ and P2X₄ receptor. Differences were also visible during the testing of ATP benzophenone. It successfully reduced the responses of the P2X₁ (69%) and P2X₄ receptor (59%), but was ineffective at the P2X₂ and P2X₃ receptor. Taken together these results again suggest that slight differences exist in the ATP binding domains of each receptor.

Further research which utilises the radio-labelling protocol can be used to test each P2X sub-type and substantiate the results from the electrophysiological study. Photoactive compounds can also be labelled with biotin (Ali et al, 2004). The cross-linked P2X protein can then be digested and isolated with streptavidin. The development of a repeatable protocol may be a viable option instead of radio-labelling the receptor. Furthermore, photo-active compounds with two or more active sites attached to the ATP molecule can be generated. Compounds which include substitutions at both ends of the molecule may act differently when tested against the P2X subtypes. This may provide
more direct evidence concerning the differing architecture of the ATP binding domains of the different P2X subtypes.

In an attempt to purify the P2X₁ receptor, a P2X₁ receptor construct was generated by Catherine Vial (in a joint project). The receptor was tagged with both flag and his epitopes and a stable cell line was established in HEK293 cells. A repeatable purification protocol was developed which exploited the addition of both epitope tags. This allowed the modified P2X₁ receptor to be successfully purified. PMF analysis showed that the P2X₁ protein was ~98% pure. However digestion of the P2X₁ protein with the enzyme trypsin provided only ~30% protein coverage.

In the original methodology the P2X receptor was to be cross-linked to the ATP analogue, purified and enzymatically digested. The amino acid residues still associated with the ATP analogue were then to be identified by mass spectrometry. The isolation and high purity of the P2X protein by various purification methods was required for analysis by mass spectrometry. The P2X₁ receptor construct showed identical properties to the wild-type P2X₁ receptor and thus was successfully cross-linked with 2-azido ATP. The P2X₂ receptor construct was similarly generated and it too possessed identical properties to the wild-type and cross-linked to 2-azido ATP. However attempts to create a stable cell line were unsuccessful, possibly indicating an incorrect insertion into the cell genome. The addition of the two epitope tags allowed the development of two strategies in the purification of the P2X₁ protein. Affinity chromatography has been previously used in similar experiments (Olcott et al, 1994; Bauer & Kuster, 2003) and this thesis described the development of a dual purification protocol which utilized both epitope tags to isolate P2X₁ protein. PMF analysis confirmed the identity of the protein and showed that it was of high purity. During PMF analysis the protein was digested by trypsin and the peptide fragments produced were compared to those which were predicted. Although the protein was correctly identified as P2X₁, the sequence coverage was below that which was necessary for confident analysis by mass spectrometry. Further research is necessary to find an enzyme or combination of enzymes which can allow the protein coverage to be increased. However the development of a purification protocol may be used as the basis
for further investigation into crystallizing the P2X<sub>1</sub> receptor. The development of a successful crystallization protocol can produce protein crystals which can be used for X-ray diffraction. The X-ray diffraction of the P2X<sub>1</sub> protein crystals can finally reveal the three-dimensional structure of the P2X<sub>1</sub> receptor and aid in the study of the binding domain of the P2X<sub>1</sub> receptor.

The absence of coverage of some of the key amino acid residues which are thought to play a crucial role in ATP binding necessitated a change in the methodology. The P2X<sub>1</sub> receptor was labelled with 2-azido [γ<sup>32</sup>P] ATP and digested with either trypsin or cyanogen bromide (CNBr). The peptide fragments were then separated by SDS PAGE and exposed to autoradiography film in an attempt to localise the binding of the radio-label to a particular fragment. The results appeared to show that the experimental conditions or the digestive enzymes themselves caused the cleavage of the bond between 2-azido ATP and the amino acid residue to which it was bound. The lability of this bond may be an inherent feature of 2-azido ATP and may be overcome with the use of the other identified photo-active ATP analogues. Since they have reactive sites which are attached to different coordinates around the ATP molecule, they may be able to form bonds which are not susceptible to degradation during the enzymatic digest. If the CNBr digest can be modified to prevent removal of the radio-label, further experiments could then be conducted with mutant P2X<sub>1</sub> receptors. In a similar study to Maanstadt <i>et al</i> (1998), methionine residues could be introduced or removed from the P2X<sub>1</sub> primary sequence. The changes in the peptide fragments produced and the gel shift caused by the association of the radio-label with different fragments can help localise the binding of the photo-reactive compound to the amino or carboxy end of the extracellular domain.

The elucidation of the ATP binding site of P2X receptors would allow the rational design of a number of P2X-affecting drugs with applications in a number of maladies. Hopefully the work conducted in this thesis can provide the basis towards the full realization of the ligand binding site of P2X receptors.
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