Purinoceptors and the control of eicosanoid release from cultured rat astrocytes

by

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Abstract

CNS astrocytes in culture are known to be capable of synthesising and releasing a variety of eicosanoids. However, we are far from understanding both the identity of the signal molecules which elicit this response and the intracellular pathways which regulate it. ATP is one of the very few biologically relevant signals known to promote eicosanoid release from cultured astrocytes. A range of purine and pyrimidine nucleotides were examined for their ability to promote eicosanoid release from astrocytes. In terms of TXA$_2$ release, the specific P2Y purinoceptor agonist 2-MeSATP was found to be the most potent agent examined, with ATP, ADP, UTP and UDP all having similar potencies. UTP and UDP were found to be more effective than ATP or other nucleotides in their ability to promote TXA$_2$ release. A range of other nucleotide di- and triphosphates also elicited TXA$_2$ release from astrocytes. The effect of the removal from cultures of microglia and serum depletion was investigated, and both these regimens were found to reduce TXA$_2$ release for all nucleotides tested. A range of nucleotides were found to be able to evoke changes in [Ca$^{2+}$]$_i$ and stimulate the production of inositol phosphates. The reason why agents such as noradrenaline (NA) fail to evoke eicosanoid release from astrocytes was examined. An increase in [Ca$^{2+}$]$_i$ is essential for eicosanoid release, however, no differences in the Ca$^{2+}$ transients evoked by ATP or NA were detected. NA was found to inhibit the ability of a range of nucleotides to stimulate TXA$_2$ release from these cells. Astrocytes were previously thought to possess only IP$_3$-sensitive Ca$^{2+}$ pools, however, the presence of IP$_3$-insensitive ryanodine receptors was detected, but these are not involved in TXA$_2$ release. These results may have significance in terms of P2-purinoceptor classification and astrocyte function within the CNS.
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"If at first you do succeed, then try to hide your astonishment...."

Anon
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<td>AMP</td>
<td>adenosine 5'-monophosphate</td>
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<td>ADP</td>
<td>adenosine 5'-diphosphate</td>
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<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>[Ca^{2+}]_i</td>
<td>concentration of free intracellular Ca^{2+}</td>
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<td>cADPR</td>
<td>cyclic adenosine diphosphate ribose</td>
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<td>caff</td>
<td>caffeine</td>
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<td>cAMP</td>
<td>cyclic adenosine 5'-monophosphate</td>
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<td>CICR</td>
<td>Ca^{2+}-induced Ca^{2+}-release</td>
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<td>CNS</td>
<td>central nervous system</td>
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<td>COX</td>
<td>cyclooxygenase</td>
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<td>CTP</td>
<td>cytidine 5'-triphosphate</td>
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<td>DAG</td>
<td>diacylglycerol</td>
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<tr>
<td>DM</td>
<td>disaggregation medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease I</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>EGTA</td>
<td>ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf system</td>
</tr>
<tr>
<td>Fura-2 AM</td>
<td>Fura-2 acetoxy-methylester</td>
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<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GDNF</td>
<td>glial-cell-line derived neurotrophic factor</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
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<tr>
<td>GM</td>
<td>growth medium</td>
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<td>GTP</td>
<td>guanosine 5'-triphosphate</td>
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<td>5-hydroxytryptamine</td>
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<td>IP</td>
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<td>inosine 5'-triphosphate</td>
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<td>LME</td>
<td>L-leucine methyl ester</td>
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<td>LTP</td>
<td>long-term potentiation</td>
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<td>2-MeSATP</td>
<td>2-methylthioadenosine 5'-triphosphate</td>
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<tr>
<td>Mv1Lu</td>
<td>mink lung epithelial cells</td>
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<td>NA</td>
<td>noradrenaline</td>
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<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<tr>
<td>ONO-RS-082</td>
<td>2-(p-amylcinnamoyl) amino-4-chlorobenzoic acid</td>
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<tr>
<td>PGG/H</td>
<td>prostaglandin G/H</td>
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<tr>
<td>PI</td>
<td>phosphoinositol</td>
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<td>phospholipase D</td>
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<td>TGF-β</td>
<td>transforming growth factor-β</td>
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<td>UTP</td>
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<td>xanthosine 5'-triphosphate</td>
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Chapter 1
Introduction
1.1. Introduction.

CNS astrocytes in culture are known to be capable of synthesising and releasing a variety of eicosanoids (Murphy et al, 1988). However, we are far from understanding both the identity of the signal molecules which elicit this response and the intracellular pathways which regulate it. Adenosine 5' triphosphate (ATP) is one of the very few biologically relevant signals known to promote eicosanoid release from cultured astrocytes (Bruner and Murphy, 1990b; Gebicke-Haerter et al, 1988, 1991; Pearce et al, 1989). However, the question arises as to why other signal molecules, whose receptors are often found on the same cells as those that evoke eicosanoid release, fail to turnover eicosanoids, despite an apparent common signal transduction pathway involving phosphoinositide (PI) turnover and Ca^{2+} mobilisation. This discrimination with respect to ATP-induced eicosanoid release appears unique to astrocytes in culture, and is not observed with other types of eicosanoid producing cells. In thyroid FRTL-5 cells, for example, both purinergic and adrenergic agonists are capable of evoking eicosanoid release (Tornquist et al, 1994). This problem has formed the basis of the work presented in this thesis. Accordingly, in this introduction, I shall examine what is known about the role of astrocytes in the CNS, their ability to release eicosanoids, and the potential importance of this phenomenon. I shall proceed to review the pharmacology of the purinergic receptors at which ATP acts, and discuss the possible significance of this to astrocyte function in vivo.

1.2. Overview of astrocytes as cells of the CNS.

Traditionally, attempts to explain brain function have been formulated in terms of the electrical activity of neurons. It is perhaps surprising, therefore, that as many as nine-
tenths of the cells in the brain are electrically non-excitable, the majority of these being termed neuroglia (Young, 1994). There are two major types of glia, astrocytes and oligodendrocytes, which between them make up over half of the brain’s volume (Travis, 1994). In addition, macrophage-like cells known as microglia are present, notably at sites of CNS damage and neuropathy (Streit, 1993; Svensson et al, 1993).

Named for their starry shape, astrocytes have been known since the late 19th century. For most of that time it has been assumed that they serve as little more than passive physical support elements for the neurons. However, improved methods for identifying and studying astrocytes has made it possible to attempt an increased understanding of their role in the development, physiology and pathology of the CNS. An overview of some of the functions ascribed to astrocytes is illustrated in Figure 1.1.

Astrocytes appear to function significantly in the maintenance of normal brain physiology, during both development and adulthood. For example, it is postulated that cell-cell contact between astrocytes and neurons in the prenatal brain controls the number of different cell types and determines their correct proportions (Aschner and LoPachin, 1993). In addition, the astrocyte forms distinctive, highly specialised contacts with a variety of neural cell types and other brain structures (Smith, S., 1992). They elaborate lamellar expansions that line both the subpial and ependymal surfaces of the brain. Neighbouring astrocytic expansions overlap in such a way that both the exterior and ventricular surfaces of the brain are composed almost entirely of astrocytic lamellae. Neural tissue is also lined by astrocytic projections where it is invaded by blood vessels; neighbouring astrocytic expansions again appear to form an essentially continuous lining. They also make a variety of contacts with specific neuronal structures, such as cell bodies, synapses and nodes of Ranvier (Smith, S., 1992). In addition, astrocytes make numerous contact with other astrocytes, resulting in elaborate networks.
Figure 1.1. Overview of the main functions of astrocytes in the CNS.

- Phagocytic functions
- Neuronal migration
- Modulation of immune/inflammatory responses
- Nutrient support of neurons
- Water, ion and neurotransmitter metabolism
- Blood brain barrier
- Neurite outgrowth
- Synaptic plasticity
It is, therefore, becoming increasingly clear that astrocytes have the potential to perform important and varied roles in the CNS, especially since they are intimately located around neuronal cell bodies, their processes and (via end feet) with the cerebral vasculature (Landis, 1994). This intimacy with neurons puts them in an ideal position to respond to and modify events at synapses, and it is now known that astrocytes are capable of influencing the passage of information through their ability to control local ion and neurotransmitter concentration (Barres, 1991; Kimelberg and Norenberg, 1989). In recent years it has become apparent that astrocytes are themselves potential targets for neurotransmitters released from neurons, and it now seems they possess functional receptors for a wide variety of neurotransmitters and growth factors (McCarthy and Salm, 1991; Murphy and Pearce, 1987). Indeed, Salm and McCarthy (1989) have used autoradiography and immunohistochemistry to show that astrocytes in situ possess β-adrenergic receptors, which correlates to receptor localisation in culture. Further, glia are now known to synthesise or release neurotransmitters or potentially neuroactive compounds, such as eicosanoids, growth factors, nitric oxide, peptides, glutamate, steroids and adenine nucleotides (Martin, 1992). This ability is important, because, in general, cells that use a compound for signalling also synthesise it. In the absence of synthesis, the presence of a compound in a cell may indicate that the compound is merely being accumulated as part of catabolism (Martin, 1992). Recently, a glial-cell-line-derived neurotrophic factor (GDNF) has been reported (Oppenheim et al, 1995). It appears GDNF is capable of enhancing the survival of dopaminergic neurons in vitro, rescue developing avian motor neurons from natural programmed cell death in vivo and promote the survival of enriched populations of cultured motor neurons (Oppenheim et al, 1995). This may well be indicative of the importance of astrocytes with regard to neuronal function in vivo.
Recent evidence has supported the view that astrocytes in culture are capable of signalling to other astrocytes and neurons, and it seems that intercellular communication may well be achieved through gap junction-mediated Ca\(^{2+}\) fluxes (Nedergaard, 1994; Parpura et al, 1994). Ca\(^{2+}\) plays an important role in a number of signal transduction-regulated functions in many cells, including astrocytes. Consequently, the increase in intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), in glial cells observed with many different stimuli has received much attention (Finkbeiner, 1992, 1993; Smith, S., 1992). Indeed, disturbances in glial Ca\(^{2+}\) homeostasis may have a role in certain pathological conditions such as ischaemia and epileptic brain damage (Finkbeiner, 1993). Further, Ca\(^{2+}\) deposits are found in dead astrocytes bordering encephalomalasic lesions and in glia from brains subject to hypoxia or epilepsy (Finkbeiner, 1993). The discovery of complex Ca\(^{2+}\)-based, gap junction-mediated glial signalling systems, capable of sensing and influencing neural activity, suggest a more integrated neuro-glial model of information processing in the CNS (Smith, S., 1992).

1.3. The use of culture as a model for studying astrocyte function.

There can be little doubt that primary cultures of astrocytes derived from neonates have provided a model from which these cells can be examined. Indeed, it could be said that without the use of culture, much of the current understanding of astrocyte function would still be poorly understood. In general, astrocyte cultures are easy to prepare and maintain in vitro, are more likely to resemble its corresponding cell type in situ than are passaged cells or cell lines, and provide a homogeneity of cells that is not afforded by other methods such as explant cultures (Aschner and Kimelberg, 1991).
While *in situ* astrocytes may be identified by their location, shape and association with neighbouring cells, their morphological appearance upon isolation, and even after some time in culture, bears only a distant resemblance (Murphy and Pearce, 1987). *In vitro* the term astrocyte is synonymous with a CNS cell that labels with antibodies to glial fibrillary acidic protein (GFAP) (Bignami *et al*, 1972).

It is now apparent that astrocytes are a heterogeneous class of cells, not just in terms of morphology but in surface properties and also in distribution within the CNS (Dave *et al*, 1991; McCarthy and Salm, 1991; Wilkin *et al*, 1990). Cultures prepared from the immature cerebral cortex yield cells which have a fibroblast-like morphology, referred to as protoplasmic astrocytes, whereas cultures prepared from white matter display two types of GFAP-positive cells. One is protoplasmic, while the other is process-bearing to the point of being stellate in appearance and is often referred to as a fibrous astrocyte. Miller and Raff (1984) have shown using frozen sections of rat optic nerve, that these two populations are distinct in origin, and are referred to as type 1 (protoplasmic) or type 2 (fibrous) astrocytes (Raff *et al*, 1983). However, it has proved difficult to correlate astroglial forms in culture with those observed *in vivo*. Moreover, there is no firm evidence that type 2 astroglia even have a counterpart *in vivo* (Levison and Goldman, 1993), and the lack of specific surface markers only confounds the uncertainty. Indeed, Miller *et al* (1989) no longer hold the hypothesis that type 2 is a separate class of astroglia, and it remains a possibility that type 2 astrocytes are cells that reflect the plasticity of glial development *in vitro*.

Contrary to this view, however, there are reports of responses that are specifically attributed to type 2 astrocytes *in vitro*. Jensen and Chiu (1991) have demonstrated differences between the two types of astrocyte in respect of their intracellular Ca\(^{2+}\) responses to glutamate. They conclude that differences in response between the two types are indicative of two classes of glutamate ion channel, ionotropic on type 1, and
metabotropic on type 2. To this end, Stephens et al (1993) have demonstrated differences between type 1 and 2 astrocytes in the effect of bradykinin on Ca\(^{2+}\) mobilisation and electrophysiology. Moreover, Inagaki et al (1991) have shown that type 2 astrocytes respond with an increase in [Ca\(^{2+}\)]\(_i\) when challenged with a wide range of neuroactive substances, including carbachol, histamine, noradrenaline, substance P, vasopressin and glutamate. Further, Marriott and Wilkin (1993) found substance P-induced inositol phosphate (IP) accumulation was restricted to oligodendrocytes and type 2 astrocytes, whereas type 1 astrocytes were unresponsive to the peptide. Clearly, further elucidation of the identity of astrocytes both in culture and in situ is required.

It appears that membrane properties of glia differ in culture and in situ as well. Optic nerve type 1 astrocytes in culture express fewer voltage-operated ion channels, such as Ca\(^{2+}\) and K\(^+\), than type 1 astrocytes that have been acutely isolated (Barres et al, 1990). Further to this, Duffy et al, 1990 have found that acutely isolated type 1 hippocampal astrocytes are insensitive to Ca\(^{2+}\) channel antagonists, whereas those cells in culture are not. Moreover, these differences may be accounted for by the absence of neurons, since nerve transection causes astrocytes in situ to revert to the simpler channel phenotype, whereas the addition of neurons to the cultures induces the more complex phenotype (Minturn et al, 1990). Barres (1991) reported that the neurons specifically affect type 1 astrocytes; the channel phenotypes of the other optic nerve glial cells are not affected.

Subpopulations of astrocytes, distinguished by morphology, antigen expression or brain region, exhibit regional and developmental differences in respect to their Ca\(^{2+}\) responses to various ligands (McCarthy and Salm, 1991; Shao and McCarthy, 1993). Moreover, Wilkin et al (1983) have described differences between protoplasmic and fibrous astrocytes in their ability to accumulate \(\gamma\)-aminobutyric acid (GABA).
It seems certain that there are likely to be even more subclasses of astrocytes in culture. For example, cells that undergo reactive gliosis are process bearing and larger than type 2 astrocytes (Miller et al, 1985, 1986). It may be that culture method and conditions are capable of inducing type 1 astrocytes to express the phenotype and characteristics associated with reactive gliosis and CNS injury (McMillan et al, 1994). Generally, it must be remembered that not all astrocyte cultures are the same. The degree and nature of contaminating cell types (like macrophages); the age, species and brain region; the growth conditions (like presence or absence of serum) must all be taken into account when interpreting results obtained from astrocyte cultures. This is a valid concern since much of the knowledge of glial ion transport, pH regulation, neurotransmitter uptake, energy metabolism, and responses to neurotransmitters derives from studies of astrocytes in neuron-free cultures (Barres, 1991). The presence of neurons may improve the validity of astrocyte cultures as a model. In culture, type 1 astrocytes lack processes; in situ they bear many processes. This difference appears to be accounted for by an influence in situ of neurons on the astrocytes, whereby addition of neurons to astrocyte cultures induces the formation of processes by the astrocytes (Barres, 1991).

1.4. Astrocytes as eicosanoid-producing cells.

The eicosanoids are a highly active group of substances, comprising the prostaglandins, thromboxanes, leukotrienes and lipoxins, which play an important role in a variety of biological functions including feeding, water balance, body temperature, synaptic transmission, regulation of the vasculature and in the response to invading pathogens (Chiu and Richardson, 1985). Until quite recently, it was assumed that the vasculature, choroid plexus and neurons were the major sources of eicosanoids in the
CNS. It is now a distinct possibility that glial cells contribute greatly to the eicosanoid synthesising capacity of the brain (Murphy et al, 1988). Studies have shown that eicosanoid synthesis in astrocytes is 20-fold higher than that in neurons and that the developmental expression of this synthetic capacity closely follows that in the whole brain (Keller et al, 1985; Seregi et al, 1987). However, relatively little is known about the cellular mechanisms which control the synthesis of eicosanoids and the nature of the signal molecules which promote their release.

1.4.1. Eicosanoid synthesis and release.

The term 'eicosanoid' has evolved to denote a large, and still growing, family of oxygenated C20 unsaturated fatty acids (Figure 1.2). The eicosanoids consist of the prostanoids (prostaglandins and thromboxanes) which are synthesised via the cyclooxygenase pathway, the leukotrienes and certain mono-, di- and tri-hydroxy acids which are formed via lipoxygenase pathways, and the epoxides which are formed by a cytochrome P-450 epoxygenase pathway (Smith, 1989). The major precursor of eicosanoids in most mammalian systems is assumed to be arachidonic acid (AA). However, there are at least three series (1-, 2-, and 3-) of eicosanoids, and other precursors such as eicosapentaenoic acid and γ-homolinolenic acid could also be involved in the synthetic pathway (Coleman et al, 1990). Indeed, Moore et al (1991) have shown docosahexaenoic acid, another substrate for the production of prostanoids, is present in astrocytes, but not neurons. The functional significance of this remains unclear.

In this thesis, I have paid particular attention to the release of prostanoids, since very little information is available on the role of leukotrienes released from astrocytes. It is generally thought that, in astrocytes, AA is principally metabolised via the
Figure 1.2. The arachidonate cascade (adapted from Smith, 1989).

neurotransmitter

membrane phospholipid

\textit{phospholipases}

\textbf{Arachidonic acid}

\textit{cytochrome p-450 exoxygenases} \hspace{1cm} \textit{cyclooxygenase} \hspace{1cm} \textit{5-, 12-, or 15-lipoxygenase}

Epoxyeicosatrienoic acids (EETs)

prostaglandin endoperoxide G2 (PGG2)

Hydroperoxyeicosatetraenoic acids (HPETEs)

\textit{peroxidase}

Prostaglandin endoperoxide H2 (PGH2)

\textit{synthases}

Prostanoids (prostaglandins and thromboxanes)

Leukotrienes and lipoxins
cyclooxygenase pathway. In contrast, Ishizaki and Murota (1991) report that the lipoxygenase pathway is the major metabolic pathway for arachidonate metabolism in astrocytes since the principal AA metabolite obtained after stimulation with Ca^{2+} ionophore was 12-hydroxyeicosatetraenoic acid (12-HETE); however, the majority of work suggests that lipoxygenase activity in astrocytes is orders of magnitude lower than those of prostanoids (Murphy et al, 1988).

Prostanoids are not stored by cells, but rather are synthesised *de novo* in response to cell-specific proteolytic or hormonal stimuli (Smith, 1989). An immediate effect of these stimuli is to increase the concentration of free arachidonate in the vicinity of PGG/H synthase. The biosynthesis of prostanoids is carried out in a stepwise fashion by membrane-bound enzymes, cyclooxygenase and endoperoxide isomerase, and by the soluble enzyme peroxidase.

After membrane phospholipids are released from their esterified state via phospholipase(s) to form, presumably, the stable prostanoid precursor AA, molecular oxygen is added to this polyunsaturated fatty acid by the enzyme cyclooxygenase to form PGG\(_2\). Arachidonate release happens quickly, in the order of 5-60 s. According to Smith (1989), some of the arachidonate used for prostanoid formation may be derived from the sequential hydrolysis of membrane phosphoinositides by phospholipase C to yield diacylglycerol (DAG), and hence AA; however, it seems unlikely that this pathway is involved to any major extent in astrocytes (Pearce et al, 1987). The major sources of released arachidonate are probably the most abundant phospholipids, phosphatidylethanolamine and phosphatidylycholine, with the key enzyme most likely being phospholipase A\(_2\) (PLA\(_2\)). It has been shown previously that in astrocytes, AA is mobilised from phospholipids (but not phosphoinositides) by the action of PLA\(_2\) (Bruner and Murphy, 1990a; Keller et al, 1987; Pearce et al, 1987). However, it is by no means certain that PLA\(_2\) is the only phospholipase involved in
eicosanoid release from astrocytes. Moreover, PLA₂ is known to exist in two forms, secretory and cytosolic. Oka and Arita (1991) have reported the presence of the secretory form in cultured rat astrocytes. The cytosolic form, which is thought to control receptor-mediated eicosanoid production and to participate in intracellular signal transduction processes, has been detected in GFAP-positive protoplasmic astrocytes in sections of human cerebral cortex using a monoclonal antibody (Stephenson et al., 1994). Astrocytes of the white matter were not immunoreactive.

Phospholipase D (PLD) is capable of facilitating arachidonate synthesis (Burgoyne and Morgan, 1990). PLD hydrolyses phosphatidylcholine producing free choline and phosphatidic acid. The latter species is hydrolysed via phosphatidic acid phosphohydrolase to DAG, and hence to AA via DAG lipase. However, although present in astrocytes, PLD does not appear to be involved in eicosanoid production (Bruner and Murphy, 1990a).

Petroni et al. (1990) have shown that phospholipase C (PLC) is responsible for platelet activating factor-induced AA release from astrocytes. The regulation of PLC and its isoforms is extremely complex, but it seems that each PLC isoform has different selectivity in terms of the hydrolysis of inositol containing phospholipids, and thus produces different ratios of cyclic to non-cyclic products (Mitchell and Trautman, 1993). It appears that astrocytes in culture possess a different form of PLC to neurons or oligodendrocytes (Mizuguchi et al., 1991). PLC-δ has been reported to be characteristic of astrocytes and it has been suggested this may be due to its involvement in specialised astrocytic functions (Mizuguchi et al., 1991). Exactly how the various PLC isoforms contribute to cell function has yet to be elucidated.

PGG₂ is rapidly converted to PGH₂ by reduction of the peroxide to a hydroxyl group by the enzyme peroxidase. PGG₂ and PGH₂ are unstable, biologically active
molecules, called endoperoxidases, which are intermediates in the transformation of AA to prostanoids (Smith, 1989). The two enzymes, cyclooxygenase and peroxidase, are collectively known as PGG/H synthase (or, confusingly, also as cyclooxygenase, COX), of which there appear to be two different isozymes (Smith, W., 1992). These two isozymes are referred to as PGG/H synthase I (PGGHS-I) and II (PGGHS-II), or COX-1 and COX-II respectively. The reason for the existence of the two separate synthases is unknown. However, the most dramatic difference between the two isozymes is their pattern of expression, and it is likely that the two enzymes serve the same biosynthetic function but under different cell physiological settings (Smith et al, 1994). COX-1 is known to be constitutively expressed in most cells, and appears to be maintained at constant levels, although small increases of about two- to four-fold can occur after stimulation with hormones and growth factors (DeWitt, 1991). In contrast, COX-II is undetectable in most cells, but its expression can be dramatically induced during inflammation, and can be stimulated by phorbol esters, growth factors, cytokines and serum (O'Banion et al, 1992). Typically, COX-II expression increases as much as 10-80 fold (Smith et al, 1994).

Astrocytes are capable of releasing a variety of prostanoids (Seregi et al, 1984a) and leukotrienes (Ishizaki et al, 1989), with the major eicosanoid in the CNS being PGD$_2$ (Keller et al, 1985). In this thesis, thromboxane A$_2$ (TXA$_2$) was measured (assayed as its stable metabolite thromboxane B$_2$, TXB$_2$), since it is known to be released specifically from astrocytes in culture, and not from cells of the cerebral vasculature, unlike PGD$_2$ (Murphy et al, 1985). TXA$_2$ is formed from PGH$_2$ by the action of thromboxane synthase, but it is unstable at physiological pH, and is rapidly hydrolysed to TXB$_2$. The enzyme thromboxane synthase is also capable of synthesising TXA$_1$ and TXA$_3$, albeit to a limited extent, however the physiological properties of these 1- and 3-series eicosanoids remain largely unknown (Kennedy, 1982).
The biochemical details of the events involved in arachidonate release have not yet been fully resolved, and it is important to realise that many aspects of the biochemical processes relating to eicosanoid release, and their interrelationships to other mediators, remain largely unknown (Fletcher, 1993). It does seem, however, that the process is usually receptor-mediated and that many types of neurotransmitters, hormones growth factors and tumour promoters can elicit eicosanoid release (Smith, 1989).

1.4.2. Prostanoid receptors.

Prostanoids, like other local hormones, produce their effects by interacting with specific receptors on cell membranes (Coleman et al, 1990). Even in the absence of direct evidence, the presence of such receptors may be inferred from the properties of prostanoids. Thus, for example, they display high potency, sometimes being active at concentrations as low as $10^{-11}$ M, and small chemical modifications can have profound effects on their potency and profile of biological activity, which implies the existence of specific recognition sites (Coleman et al, 1990).

The role of prostanoid receptors on astrocytes is poorly understood, and most of the literature examining the role of astrocyte-derived eicosanoids have ignored the presence of them entirely. However, Nakahata et al (1989; 1992) have demonstrated the presence of a single, high affinity binding site for TXA$_2$ on both human 1321N1 astrocytoma and primary cultures of adult rabbit cerebral cortex. Stimulation of these receptors with a stable TXA$_2$ analogue resulted in the accumulation of intracellular IPs in both cell types (Nakahata et al, 1989; 1992). Similarly, Kitanaka et al (1991) found that the prostanoid, PGF$_{2\alpha}$, stimulated PI turnover in rat cortical astrocytes. The receptors for TXA$_2$ and PGF$_{2\alpha}$ appear to be coupled to PLC, the enzyme responsible for catalysing PI metabolism, via a regulatory G-protein which is not sensitive to
pertussis toxin (Kitanaka et al, 1991; Nakahata et al, 1989; 1992). The fact that there appear to be prostanoid receptors on astrocytes suggests eicosanoids may be involved in both paracrine and autocrine functions. As yet, there is no direct evidence to suggest the presence of receptors for the leukotrienes on astrocytes.

1.4.3. Role and significance of eicosanoids in the CNS.

It is clear that the eicosanoids are involved in crucial physiological processes in vivo such as blood clotting, inflammation, control of vascular tone, renal function, behaviour, and reproductive system (Shimizu and Wolfe, 1990). However, their role within the CNS are less well understood, and despite the evidence of eicosanoid receptors on glia in culture, there are few indications of either their functions or whether they exist on cells in situ.

Prostanoids, particularly of the E series, can exert potent inhibitory effects on autonomic transmitter release in a range of tissues, and have been reported as having anticonvulsice properties (Hertting and Seregi, 1989). Evidence also points to them having a neuromodulatory role. Thus, prostanoids alone have little effect on, for example, noradrenaline release but modify evoked release from brain slices, probably by restricting Ca^{2+} fluxes (Chiu and Richardson, 1985). Further, it has been demonstrated that prostaglandin E2 (PGE2) potentiates dopamine (D2) receptor-mediated potentiation of AA release in Chinese hamster ovary cells, suggesting that it is acting as a paracrine messenger (Di Marzo and Piomelli, 1992). The excitatory actions of prostanoids on neuronal activity take a number of forms; for example, they have been shown to enhance neurotransmitter release, to elicit neuronal depolarisation and induce hyperalgesia through a lowering of the excitation threshold of peripheral nociceptors (Coleman et al, 1990). Additionally, prostanoids induce upper airways
irritancy and cough, as well as gastric irritancy, nausea and vomiting, and have been implicated in hyperthermia, fever and sleep (Hertting and Seregi, 1989). Whether all these actions are manifestations of the same effect is not clear, but it seems clear that prostanoids can profoundly affect neuronal function in many tissues.

Arachidonic acid and its metabolites have been shown to be released by cerebral tissues as a result of ischaemia (Haun et al, 1993; Kunievsky et al, 1992), and may participate in either damaging or protecting neural tissues, depending on the particular AA metabolite produced. Prostacyclin has been shown to protect rat cortical neurons in culture against hypoxia and glutamate-induced injury (Cazevieille et al, 1993). In addition, Renkawek et al (1986) have reported that prostacyclin exerted a cytoprotective effect on neurons via glial cell activity, however, the mechanism involved is unknown. Moreover, hypothermia has been reported to reduce both cellular injury and the release of $[^3H]$-labelled AA metabolites during anoxic conditions in vitro, the two events presumably being linked (Haun et al, 1993). In addition, when the human astroglial cell line UC-11MG was treated with adverse conditions, such as ATP depletion, membrane phospholipids were degraded, with the release of AA metabolites (Sun et al, 1993). The severity of cell injury could be blocked by inhibitors of PLA$_2$ and PLC.

There is increasing evidence that AA itself may serve as a signal molecule. For example, Murphy and Welk (1989) have shown that AA stimulates PI breakdown in cortical astrocytes. One interesting possibility is that AA is involved in long-term potentiation (LTP). LTP is a model of synaptic plasticity and information storage, which can be produced in the hippocampus by high frequency stimulation of the perforant pathway, and is generally believed to consist of two phases: induction and maintenance (Piomelli and Greengard, 1990). AA has been shown to potentiate the current through N-methyl-D-aspartate (NMDA) receptors, probably due to an increase in channel open probability (Miller et al, 1992). It is released when glutamate raises the
[Ca$^{2+}$]; the induction of LTP is triggered by the postsynaptic entry of Ca$^{2+}$ through the NMDA receptor channel (Williams et al, 1989). These authors suggest that AA increases synaptic efficacy both *in vivo* and *in vitro*, and may act as a retrograde messenger in the late (maintenance) stages of LTP. Further to this, there have been reports of AA release from cortical and hippocampal neurons in culture when challenged with NMDA, and the suggestion is that AA modulates the function of NMDA receptors during synaptic transmission and plasticity of the CNS (Sanfeliu et al, 1990; Tapia-Arancibia et al, 1992).

Given the intimacy of astrocytes with the cerebral vasculature, the effect of eicosanoid release on the cerebral circulation is an area of great potential importance. Eicosanoids have been implicated in cerebral vasospasm, which is the major cause of death after subarachnoid haemorrhage, and to a lesser extent, head trauma (Shimizu and Wolfe, 1990). The impairment of prostanoid metabolism during convulsion and their anticonvulsive effects have been demonstrated (Seregi et al, 1984b). After freezing injury to the brain, several prostanoids are formed in the lesion area, and AA release has been observed in brain adjacent to the injury (Shimizu and Wolfe, 1990).

Although astrocytes have demonstrated their ability to synthesise eicosanoids, little is known about the triggers for synthesis and release. Attempts to examine this question have concentrated on the stimulation of receptors coupled to phosphatidylinositol 4,5-bisphosphate (PIP$_2$) breakdown and Ca$^{2+}$ mobilisation. PIP$_2$ breakdown yields DAG and inositol 1,4,5-trisphosphate (IP$_3$). The DAG formed may provide a source of AA following metabolism via DAG lipase, while IP$_3$-induced Ca$^{2+}$ mobilisation could activate a Ca$^{2+}$-dependent phospholipase, such as PLA$_2$, and the newly available AA from phospholipid breakdown is then subject to cyclooxygenase/lipoxygenase action (Abdel-Latif, 1986). Stimulation of such receptors in a variety of tissues promotes
eicosanoid release. Despite the fact that astrocytes possess an array of receptors coupled to the PI/Ca^{2+} second messenger release, activation of the vast majority of them fails to evoke eicosanoid release (Pearce and Murphy, 1988). Stimuli which either increase [Ca^{2+}]_{i} (Ca^{2+} ionophores), directly stimulate PLA_{2} (melittin) or activate protein kinase C (phorbol esters), have been shown to elicit eicosanoid release from astrocytes (Bruner and Murphy, 1990b; Hartung and Toyka, 1987; Jeremy et al., 1987; Keller et al., 1985, 1987; Murphy et al., 1985, 1988). However, only a few physiologically relevant signal molecules have been shown to evoke eicosanoid release from these cells, and these include ATP, substance P and bradykinin (Keller et al., 1987; Marriott et al., 1991; Marriott and Wilkin, 1993; Pearce et al., 1989). Substance P-induced eicosanoid release could only be demonstrated in astrocytes derived from spinal cord; astrocytes cultured from cerebral cortex and cerebellum were insensitive to substance P in terms of eicosanoid release and IP accumulation, suggesting regional heterogeneity in astrocyte function (Marriott et al., 1991). ATP and bradykinin, on the other hand, appear to evoke eicosanoid release from astrocytes derived from all regions of the CNS. In the next section, I shall proceed to discuss what is known about ATP and the receptors at which it acts.

1.4.4. Purinergic receptor-activation and eicosanoid release from astrocytes.

It is now well established that endogenous ATP can be released extracellularly, and following activation of specific extracellular receptors, modulates the function of many biological systems (Burnstock and Kennedy, 1985; Dubyak, 1991; Dubyak and El-Moatassim, 1993; El-Moatassim et al., 1992; Gordon, 1986; Harden et al., 1995; Williams and Cusack, 1990). It appears that ATP has physiological, and perhaps pathological, roles in the nervous system (both central and peripheral), muscle
contraction, cardiac function, platelet aggregation, vascular tone, secretory, endocrine and immune responses, as well as spermatocytes, osteoblasts, fibroblasts and tumour cells (Burnstock, 1993).

ATP is rapidly metabolised by ectonucleotidases (Ceballos et al, 1994; Gordon, 1986), providing a mechanism for regulation of its actions. Through metabolism ATP becomes a source of extracellular adenosine that is capable of exerting its own effects. However, adenosine does not stimulate eicosanoid release from astrocytes (Pearce et al, 1989). Binding studies with cultured cerebellar and spinal cord astrocytes have shown the presence of P1 purinoceptors (Hosli and Hosli, 1988). P1 purinoceptors have been found to produce cellular actions through a number of different effector systems, including modulation of adenylyl cyclase activity, PI turnover, activation of various ion channels and reducing Ca²⁺ release from intracellular Ca²⁺ stores (Linden, 1991; Olah and Stiles, 1992). The potential interplay between the purine nucleoside and nucleotide is a complex and elegant mechanism to regulate neurotransmission and other processes affecting the function of the CNS, such as inflammation.

ATP receptors, termed P2-purinoceptors to distinguish them from the P1-subtype which respond preferentially to adenosine, are not a homogenous population. Based almost entirely on evidence gained from using agonist analogues, it has been proposed that P2-purinoceptors can be subdivided into P2X and P2Y subclasses (Burnstock and Kennedy, 1985). The P2Y differs from P2X in that the former has a higher affinity for the synthetic ATP analogue, 2-methylthioadenosine 5'-triphosphate (2-MeSATP), than for α,β-methyleneadenosine 5'-triphosphate or β,γ-methyleneadenosine 5'-triphosphate (Burnstock and Kennedy, 1985). The P2X has a greater affinity for the latter two ATP analogues than for either ATP or 2-MeSATP. The P2Y subclassification has now been extended to include additional subclasses; P2T (on platelets), P2Z (on mast cells), P2S (on guinea pig ileum) and P2U (nucleotide
receptor, originally on neutrophils) (Dalziel and Westfall, 1994). Recently, subtypes of these subclasses have been proposed on the basis of cDNA cloning and the functional expression in *xenopus* oocytes, of a brain G protein-coupled ATP receptor (Webb *et al*, 1993).

ATP has been shown to be capable of evoking eicosanoid release from astrocytes in culture via P2-purinoceptor stimulation (Bruner and Murphy, 1990b; Gebicke-Haerter *et al*, 1988; Pearce *et al*, 1989). ATP also stimulates IP₃ production and Ca²⁺ mobilisation (Kastritsis *et al*, 1992; Pearce *et al*, 1989). In addition, adenosine 5'-diphosphate (ADP), but not adenosine 5'-monophosphate (AMP) or adenosine, is capable of stimulating the breakdown of PI's, mobilisation of Ca²⁺ and TXA₂ release (Pearce *et al*, 1989; Shao and McCarthy, 1995). Astrocyte-derived eicosanoids may play a particular role in vascular blood flow and in the brain's response to inflammation as a result of brain injury or neurotrauma (Sharma *et al*, 1993). Moreover, it has been suggested that ATP may be capable of stimulating astrocytes to adopt the reactive phenotype which is invariably found at the site of CNS damage (Norenberg, 1994). For example, it has been shown that chronic exposure to ATP results in marked stellation of astrocytes (Norenberg *et al*, 1990). Furthermore, chronic exposure to high concentrations of ATP (1 mM) stimulates [³H]-thymidine incorporation in rat astrocytes (Neary and Norenberg, 1992), suggesting that ATP plays a role in mitogenesis and perhaps the generation of reactive astrocytes. The concentration of ATP released by neurons into the synaptic cleft appears to range from low micromolar during normal transmission to high micromolar upon repetitive excitation, such as occurs during neurotrauma (Neary and Norenberg, 1992). It may be possible that astrocytes in the reactive state have an even greater potential to release eicosanoids and growth factors than normal. Moreover, because astrocytes maintain close contact with other cell types of the CNS, they are also likely targets for neuronally released purines. It is interesting
to note that astrocytes in culture adopt some of the characteristics of the reactive phenotype (McMillan et al, 1994).

This release of TXA$_2$ has been shown to be dependent on the presence of extracellular Ca$^{2+}$ (Bruner and Murphy, 1990b). This study confirmed that mobilisation of AA was a receptor-mediated event, and not due to non-specific cell permeabilisation as assessed by leakage of cytoplasmic lactate dehydrogenase. However, precisely why ATP, and not other PI-linked receptors which mobilise Ca$^{2+}$ such as $\alpha$-adrenoceptors, evoke eicosanoid release from cultured astrocytes remains to be elucidated. The possibility that only a subset of cells are capable of synthesising eicosanoids and responding to P2-purinoceptors can be largely discounted. Analysis of Ca$^{2+}$ transients evoked by P2-purinoceptor and $\alpha$-adrenoceptor agonists demonstrate considerable colocalisation of these receptors in cortical astrocytes (McCarthy and Salm, 1991). Although these receptors are coupled to separate PI pools, they appear to access a common pool of Ca$^{2+}$ (Pearce et al, 1989; Shao and McCarthy, 1995).

It appears that uridine 5'-triphosphate (UTP), a pyrimidine, may also have biological significance if released into the extracellular space (Seifert and Schultz, 1989). Tran-Thi et al (1988) have demonstrated that UTP is capable of evoking eicosanoid release from perfused rat liver. Furthermore, in certain tissues, UTP apparently produces its biological effects by interacting with a receptor which has equivalent sensitivity to ATP (O'Connor et al, 1991; O'Connor, 1992). Bruner and Murphy (1993a), however, have shown that astrocytes in culture possess a UTP-sensitive receptor which is distinct from the P2Y subtype. In addition, these authors have shown UTP to be capable of evoking TXA$_2$ release, stimulating PI turnover and increasing [Ca$^{2+}$].
1.5. Aims of this thesis.

The main aims of the work presented in this thesis were:

• to characterise pharmacologically the purinoceptors coupled to eicosanoid release and to determine whether pyrimidines act in a similar fashion at either the same or distinct receptor sites.

• to examine the relationship between P2 purinoceptor-second messenger coupling, intracellular \( \text{Ca}^{2+} \) mobilisation and eicosanoid release from astrocytes.

• to explore the possibility that specific intracellular \( \text{Ca}^{2+} \) stores are responsible for regulating eicosanoid release from these cells.
Chapter 2
Methods and Materials
2.1. Astrocyte cell culture.

Astrocyte-enriched primary cultures of neonatal rat cerebral cortex were prepared according to the method of Dutton et al. (1981). Following decapitation of 1 or 2 day old rat pups, the cerebral cortices were detached from the hind brain and removed. The cortices were thoroughly cleaned of meninges and vasculature with fine forceps, and placed in a few drops of sterile disaggregation medium (DM) of the following composition: glucose (14 mM), bovine serum albumin (BSA) (3 mg/ml) and MgSO$_4$.7H$_2$O (1.5 mM) in Ca$^{2+}$ and Mg$^{2+}$ free Earle's Balanced Salt Solution (Gibco). The tissue was then coarsely chopped with a sterile scalpel. The tissue suspension was transferred to a trypsinisation flask containing trypsin (250 μg/ml) in 10 ml of DM and placed in a shaking water bath at 37°C for 15 minutes. A 10 ml solution containing soybean trypsin inhibitor (192 μg/ml), DNase (6.4 μg/ml) and MgSO$_4$.7H$_2$O (240 μM) in DM was then added and the resulting suspension transferred to 50 ml sterile plastic tubes and centrifuged at 100x g for 5 seconds (Denley, BS 400) to sediment cell bodies. The supernatant was removed and the cell pellet resuspended in a solution containing a few drops of soybean trypsin inhibitor (1.2 mg/ml), DNase (40 μg/ml), and MgSO$_4$.7H$_2$O (1.5 mM) in DM.

The tissue was then mechanically dissociated by gentle trituration through a 1.5 mm diameter stainless steel cannula attached to a 2 ml sterile syringe, and the cells allowed to settle. The supernatant was removed by a plastic pipette and transferred to a sterile 15 ml plastic tube. This step was then repeated. The final cell suspension was underlaid with a 4% w/v BSA solution containing MgSO$_4$.7H$_2$O (1.2 mM) in DM, and the intact cells were pelleted through the BSA underlay by centrifugation at 100x g for 5 minutes. The supernatant and the BSA underlay, which contained cell debris, were removed and the cell pellet resuspended in a small volume of growth medium (GM) of the following composition: Minimum Essential Medium with Earle's Salts (glutamine-
free, Gibco) supplemented with foetal calf serum (10% v/v), glutamine (2 mM), glucose (33 mM) and gentamicin (65 μg/ml). The cell suspension was appropriately diluted in GM to give a seeding density of 100,000 cells per well or cover slip. Cells were then seeded onto poly-D-lysine (50 μg/ml) coated 32 mm² glass cover slips (BDH), 6 well (35 mm diameter) or 24 well (15 mm diameter) plates (Nunc). The cells were maintained for 21 days in vitro in a humidified atmosphere containing 5% CO₂ in air at 37°C.

All experiments were performed on cultures at approximately 21 days in vitro; when they were confluent. It has been established previously (Pearce et al, 1985) that cultures prepared by this method contain >95% protoplasmic astrocytes as revealed by immunofluorescence labelling with an antibody against GFAP. Illustrations of astrocyte cultures are shown in Figure 2.1.

2.1.1. Preparation of astrocyte cultures devoid of microglia.

Cultures were prepared as described above (2.1). However, the GM was supplemented with 5 mM L-leucine methyl ester (LME). LME has been shown to serve as a lysosomotrophic agent that destroys mononuclear phagocytic cells such as amoeboid microglia (Thiele et al, 1983; Giulian and Baker, 1986).

2.1.2. Treatment of cultures with serum-free medium.

Cultures were prepared and maintained as described above (2.1). At day 17, the GM was removed and replaced with Dulbecco’s Minimum Essential Medium/Nutrient mix Ham’s F12 (1:1) (without glutamine, Gibco) which had been supplemented with 33
Figure 2.1. Photographs of astrocytes after three weeks in culture. A. This photograph illustrates the "star-like" morphology of astrocytes in a region of non-confluence. B. When the cells are completely confluent, it is virtually impossible to identify individual cells. Cultures were irrigated with 1% Toluidine Blue (which stains positively-charged proteins), rinsed and photographed at 125 X magnification.
mM glucose, 2 mM glutamine and 65 µg/ml gentamicin. Cultures were maintained in vitro for a further 4 days before use (Marriott et al, 1995).


Cultures in 24 well plates were incubated for 48 hours in GM containing 1 µCi/ml [³H]myo-inositol (15 Ci/mmol, Amersham) to prelabel membrane phosphoinositides. This labelling regime has the advantage in that it enables the inositol lipids to be prelabelled prior to receptor activation and thus alleviates some of the problems which may be associated with experiments where simultaneous agonist-induced lipid breakdown and synthesis are involved (Pearce et al, 1986). Cultures were washed in buffer of the following composition: NaCl (116 mM), NaHCO₃ (26 mM), NaH₂PO₄ (1 mM), MgSO₄ (1.5 mM), KCl (5 mM), CaCl₂ (1.3 mM) and glucose (20 mM) pregassed with 5% CO₂ in O₂, then incubated in the same buffer containing 5 mM LiCl and an agonist. The LiCl acts to inhibit the hydrolysis of [³H]IPs by inhibition of inositol monophosphate phosphomonoesterase (Berridge et al, 1982). This effectively blocks the recycling of inositol by cells, which causes a build-up of IP, and thus provides a convenient experimental tool to study inositol polyphosphate generation. All incubations were for 60 minutes at 37°C in a 5% CO₂/air atmosphere. Reactions were terminated by removal of the incubation medium and the addition of 0.5 ml of ice-cold methanol to each well. The cells were harvested and the water soluble [³H]IPs extracted by the addition of 0.75 ml of water and 0.75 ml of chloroform to each sample. Aliquots (0.75 ml) of the aqueous phase were taken, and added to 0.5 ml of a 50% slurry of AG 1-X8 resin in the formate form (Biorad), then applied to ion exchange chromatography columns. Following five washes with 2 ml volumes of water to remove free [³H]inositol, the total [³H]IPs were eluted from the columns with 1 ml of 1 M ammonium formate in 0.1 M formic acid. Aliquots of the chloroform-soluble [³H]inositol lipids and the [³H]IPs eluted from the columns were taken and the
radioactivity in each determined by liquid scintillation counting (Pearce et al., 1985, 1986).

2.3. Measurement of thromboxane A₂ release.

Cultures grown on 6 well plates were washed twice in buffer of the following composition: NaCl (116 mM), NaHCO₃ (26 mM), NaH₂PO₄ (1 mM), MgSO₄ (1.5 mM), KCl (5 mM), CaCl₂ (1.3 mM) and glucose (20 mM) pregassed with 5% CO₂ in O₂, and then incubated for 30 minutes. Cultures were washed twice more with buffer, and incubated for a further 15 minutes in the same buffer plus a drug or combination of drugs. All incubations were carried out at 37°C in a 5% CO₂/air atmosphere. The incubation medium was collected and aliquots assayed in duplicate for TXB₂ (the stable metabolic product of TXA₂) using a specific "in-house" radioimmunoassay (RIA) (Jeremy et al 1985, 1986). The limit of detection was 0.1 ng/ml.

The RIA was prepared as shown in Table 2.1.

Table 2.1. TXB₂ radioimmunoassay protocol.

<table>
<thead>
<tr>
<th>Tube</th>
<th>vol of buffer (µl)</th>
<th>vol of TXB₂ As (µl)</th>
<th>vol of [³H]TXB₂ (µl)</th>
<th>vol of STD or sample (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2</td>
<td>NSB</td>
<td>300</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-4</td>
<td>TOTAL</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-6</td>
<td>B₀</td>
<td>200</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>7-</td>
<td>STD or Sample</td>
<td>100</td>
<td>100</td>
<td>50</td>
</tr>
</tbody>
</table>

The volume of appropriate reagent added to each polypropylene test tube is shown. NSB = non-specific binding of [³H]TXB₂ in the absence of antisera, B₀ = binding in the absence of unlabelled ligand, STD = TXB₂ standard of known concentration, Total = volume of [³H]TXB₂ added, As = antisera.
A stock solution of 1 μg/ml TXB$_2$ was appropriately diluted to 10 ng/ml with chilled RIA buffer of the following composition: Tris (6.9 g/l) and gelatin (500 μg/l), buffered to pH 7.4. This represents the highest TXB$_2$ concentration over the standard curve dose-response range. The stock was further diluted in RIA buffer to obtain standard solutions of the following concentrations: 5, 2.5, 1.25, 0.625, 0.312, and 0.156 ng/ml.

Routinely, 100 μl of incubation supernatant or TXB$_2$ standard were added to polypropylene LP3 test tubes (Denley), and were diluted with 100 μl of chilled RIA buffer. The contents of each tube were well mixed and equilibrated overnight (approximately 18 hours) at 4°C with 100 μl rabbit TXB$_2$ antisera (the gift of Dr. J.Y. Jeremy, Royal Free Hospital, London) and 50 μl of 0.1 μCi/ml [$^3$H]TXB$_2$ (205 Ci/mmol, Amersham). The cross reactivity of the antisera with some major prostaglandins is shown in Table 2.2.

The next morning, 500 μl of chilled dextran-coated charcoal solution comprised of dextran (1.0 g/l) and activated charcoal (8.0 g/l) in RIA buffer, was added to each tube (except Total’s), mixed and allowed to equilibrate for 10 minutes at 4°C. Tubes were then centrifuged at 1000x g at 4°C for 5 minutes (Beckman GS-6KR), the supernatant was decanted into scintillation vials, and the radioactivity determined by liquid scintillation counting. Standard curves were compiled and fitted using Microsoft Excel software on a Macintosh computer, and unknown concentrations of TXB$_2$ determined similarly from the following formula:

\[
\% \text{ Binding} = \frac{\text{Counts in sample (dpm)} - \text{Non-specific binding (NSB)}}{\text{Total counts (dpm)}} \times 100
\]

A representative standard curve is shown in Figure 2.2.
Table 2.2. Cross reactivities of some major prostaglandins with the TXB$_2$ antisera employed in this thesis (data kindly supplied by Dr. J. Y. Jeremy, Royal Free Hospital, London).

<table>
<thead>
<tr>
<th>eicosanoid</th>
<th>% cross reactivity.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TXB$_2$</td>
<td>100</td>
</tr>
<tr>
<td>6-oxo-PGF$_{1\alpha}$</td>
<td>0.001</td>
</tr>
<tr>
<td>PGF$_{2\alpha}$</td>
<td>0.002</td>
</tr>
<tr>
<td>PGE$_2$</td>
<td>0.005</td>
</tr>
<tr>
<td>PGD$_2$</td>
<td>0.003</td>
</tr>
<tr>
<td>PGE$_1$</td>
<td>0.01</td>
</tr>
<tr>
<td>PGB$_2$</td>
<td>0.003</td>
</tr>
<tr>
<td>PGA$_2$</td>
<td>0.005</td>
</tr>
<tr>
<td>6-oxo-PGE$_{1\alpha}$</td>
<td>0.005</td>
</tr>
<tr>
<td>linolenic acid</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>arachidonic acid</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Cross reactivity = \[
\frac{\text{mass of PG required to displace 50\% [}^3\text{H-PG]}}{\text{mass of PG}_X \text{ required to displace 50\% [}^3\text{H-PG]}}
\]

PG = PG against which antiserum has been raised (e.g. TXB$_2$) and
PG$_X$ = other PG being tested for cross reactivity (e.g. PGE$_1$).
Figure 2.2. Representative standard curve from TXB$_2$ radioimmunoassay. A standard range of known concentrations of TXB$_2$ was prepared by serial dilution of a 1 $\mu$g/ml stock solution, to give decremental doses from 10 ng/ml to 0.16 ng/ml. These standard concentrations were incubated in duplicate as described in text; a standard curve being prepared for each RIA. The concentration of TXB$_2$ in unknown samples were calculated from the standard curve using Microsoft Excel.
\[ y = 40.010 + -37.749 \times \text{LOG}(x) \quad R^2 = 0.974 \]
2.4. Measurement of intracellular calcium.

2.4.1. The theory behind the use of fluorescent probes.

The main problem associated with measuring the [Ca\(^{2+}\)]\(_i\) is that its value is very low (10\(^{-7}\) M) with respect to the concentration outside the cell (10\(^{-3}\) M). As a consequence, any interference from other ions, such as magnesium, can greatly distort any measurement made. Another problem stemming from its low value is that any technique that requires inserting a micropipette into the cell can disrupt the integrity of the cell membrane sufficiently enough to admit enough Ca\(^{2+}\) from outside the cell to swamp the cytosol with Ca\(^{2+}\). Not only will this distort the measurement, but it will often destroy the cell.

One way to overcome these problems is to make use of fluorescent probes which are able to bind Ca\(^{2+}\) ions, and these have become the most popular method of measuring [Ca\(^{2+}\)]\(_i\). Fura-2 is one of the most widely used probes used to monitor [Ca\(^{2+}\)]\(_i\), but like many of these probes, it is not without pitfalls. At neutral pH, Fura-2 is negatively charged and so cannot permeate through cell membranes. However, the free acid can be esterified, thereby making the probe lipophilic, and hence cell permeant (Tsien, 1981). When cells are exposed to the esterified form of Fura-2, Fura-2 acetoxymethylester, (Fura-2 AM), the probe passively diffuses across the cell membrane into the cytosol. Most cells have naturally occurring esterases that hydrolyse Fura-2 AM to generate Fura-2 \textit{in situ}; this being trapped in the cytosol by virtue of its negative charge. There is a risk, however, that the indicator may be loaded into other cell compartments such as mitochondria and the endoplasmic reticulum, as well as into the cytosol, most probably through endocytosis of the dye (Malgaroli \textit{et al}, 1987). Homogenous distribution of the dye is a prerequisite for appropriate [Ca\(^{2+}\)]\(_i\) measurement. An additional problem is that de-esterification may be incomplete so that
a fluorescence intermediate, which is not ion sensitive, may be produced (Cannell and Thomas, 1994). All these confound analysis of the true value of [Ca^{2+}]_{i}.

When the level of [Ca^{2+}]_{i} increases, whether as a result of receptor activation or mechanical stimulation, the amplitude of fluorescence of Fura-2 increases, since the probe binds Ca^{2+} and fluoresces accordingly. In addition, there is a shift in the spectrum of Fura-2, which allows for the use of a ratio technique for determining [Ca^{2+}]_{i} from the fluorescence. When Fura-2 binds Ca^{2+}, an increase in fluorescence at the 340 nm wavelength occurs. This increase can be used ratiometrically against the fluorescence observed at 360 nm. Since 360 nm is the isobestic point of Fura-2, it is independent of the [Ca^{2+}]_{i} and therefore ideal to use as a reference measurement. However, recording spectral shifts at 340 nm requires expensive quartz optics, and often the concomitant decrease in fluorescence seen at 390 nm is used, since this can be achieved with cheaper glass optics (Cobbold and Rink, 1987). The 360/390 nm ratio does not depend on such factors such as the intensity of the excitation light or the concentration of the probe within the cell, since these affect the fluorescence of the two wavelengths in the same way, and cancel out when the ratio is taken.

2.4.2. Method used to measure changes in intracellular calcium.

Cells grown on 32 mm^2 glass coverslips were loaded in the dark with 0.5 μM Fura-2 AM for 30 minutes at room temperature in a shaking water bath, and subsequently washed to remove unloaded dye. All incubations and drug additions were carried out in a physiological buffer containing NaCl (142.5 mM), KCl (5.0 mM); MgCl_2 (1.0 mM) CaCl_2 (1.0 mM), glucose (10 mM) and HEPES (5.0 mM), buffered to pH 7.4. In those experiments using nominally calcium free buffer, the CaCl_2 was omitted and 200 μM EGTA added.
The cover slips were mounted on the stage of an inverted microscope (Nikon Diaphot, Japan) and the cells viewed at 400 x using a Nikon 40 x 1.3NA phase-contrast oil immersion fluorescence objective. A photograph of the microscope stage is shown in Figure 2.3. A field of approximately 20 cells were positioned in the light path of a photomultiplier (Thom EMI 9924B) and the path adjusted by an iris diaphragm. The field of cells were illuminated alternately with 360 and 390 nm narrow band filtered (Ealing Electro-optics filters) ultraviolet light from a 150 W Xenon source fed via a fluid filled light guide (Micro Instruments Oxford) through the epifluorescence port of the microscope. Filters were changed every 250 ms by a solenoid operated filter changer. The ultraviolet light was reflected onto the cells by a 430 nm dichroic mirror and emitted light passed from the cell through a 470 nm barrier filter, then a 500 nm broad-band filter, before reaching the photomultiplier. The signals from the photomultiplier were fed into a current-to-voltage converter and then through a low pass filter before being digitised by a CED 1401 Laboratory Interface (Cambridge Electronics Design), and fed into an IBM compatible personal computer, which also controlled the filter movement. The \([\text{Ca}^{2+}]_i\) was calculated on-line from the ratio (R) of the emission at 360 nm excitation to that at 390 nm from the equation:

\[
[\text{Ca}^{2+}]_i = \frac{K_d(F_0/F_1)(R - R_{\text{min}})}{(R_{\text{max}} - R)},
\]

where \(R_{\text{max}}\) was the ratio value at saturating calcium, \(R_{\text{min}}\) the ratio at limitingly low calcium, \((F_0/F_1)\) the ratio of fluorescence at 390 nm in low calcium to that in high calcium and \(K_d\) is the dissociation constant for Fura-2 (Gryniewicz et al, 1985). Before each experiment, background fluorescence calibration from unloaded cells were recorded and subtracted from the emission data accordingly. Cells were washed with buffer ± drug additions at 0.2-0.3 ml/min via a continuous gravity-fed perfusion system. The perfusion out-flow tip was positioned within 200 µm of the cell surface as
observed under the microscope, and complete solution changes in the region of the cell were achieved within 2 seconds using an aspirator positioned close to the field of cells under investigation (Di Benedetto et al, 1991; Gray, 1988).

2.4.3. Adaptation of the method for assessing changes in intracellular calcium.

Serious problems were encountered with the computer software needed to operate the equipment and analyse the data obtained. Consequently, a computer-independent system was designed and built by C.J. Courtice, Dept. of Pharmacology, The School of Pharmacy. Essentially, the computer was replaced by an electronic circuit which calculated the ratio of the two currents detected by the photomultiplier at each recorded wavelength of light, the results being displayed graphically on a three channel chart recorder (Gould TA550). The signal output represented the current ratio which was proportional to the amount of fluorescence detected by the photomultiplier. This ratio gives a convenient measurement of changes in fluorescence and, therefore \([\text{Ca}^{2+}]_i\), but it does not allow the conversion of these data into molar concentrations.

A calibration curve for this system is shown in Figure 2.4.

All Figures within this thesis from this adapted method were taken from actual chart recorder traces.

2.5. Materials.

Unless otherwise stated all reagents used in this thesis were obtained from Sigma Chemical Co., UK or BDH.
Figure 2.3. Photograph of microscope stage of the apparatus used to measure intracellular Ca\(^{2+}\). Glass cover slips, on which astrocyte cultures had been grown, were placed onto the stage of a Nikon microscope. The drug delivery perfusion system (left hand side of photograph) was positioned manually so that its tip was extremely close to those cells directly over the aperture of the microscope. The aspirator (on the right hand side) was positioned close to the cells to remove bathing media.
Figure 2.4. Current ratio calibration curve of known voltages against % full scale deflection of recorder. Incremental increases in voltage were applied directly to the photomultiplier amplifier, and the corresponding step-wise deflection on the chart recorder was monitored for each change. The purpose of this was to assess if voltage applied to the photomultiplier, and therefore the ratio of the signal at 360 and 390 nm, was directly proportional to the deflection on the chart recorder. In this way, % full-scale deflection on the chart recorder was shown to be proportional to the current ratio at 360/390 nm.
$y = -6.1516e-3 + 4.0187e-2x$

$R^2 = 1.000$
Chapter 3
Characterisation of purine and pyrimidine-stimulated TXA$_2$
release and changes in intracellular Ca$^{2+}$
3.1. Introduction.

Astrocytes are, potentially, an important source of eicosanoids in the CNS; however, the precise mechanism and conditions under which eicosanoid synthesis and release is evoked from these cells continues to remain elusive. Activation of a number of PI-linked receptors which are present on astrocytes results in the generation of IP3 and an increase in [Ca2+]i (Fatatis et al., 1994; Inagaki et al., 1991; Kastritsis et al., 1992; Marin et al., 1991; McCarthy and Salm, 1991; Murphy and Welk, 1990; Pearce and Murphy, 1988; Pearce et al., 1989). In most cell types, activation of PI-linked receptors leads to stimulation of eicosanoids; examples of these cell types include central and peripheral neurons, cerebellar cortical slices, cerebral vasculature, pheochromocytoma PC12 cells, aorta, thyroid FRTL-5 cells, renal tubule and the bladder (Chiu and Richardson, 1985; Choi et al., 1992; Jeremy et al., 1985, 1986; Kanterman et al., 1990a, b; Reichman et al., 1987; Smith, 1989; Tornquist et al., 1994). It is generally believed that an increase in [Ca2+]i activates phospholipase(s), which in turn cleaves AA from membrane phospholipids. Agonists which stimulate IP3 production, and thereby increase the [Ca2+]i, might therefore be predicted to evoke eicosanoid release. Evidence supporting this hypothesis comes from the observation that agents such as thapsigargin (which depletes intracellular Ca2+ stores by specifically inhibiting Ca2+ ATPase) and ionophores, both elevate the free [Ca2+]i, and stimulate eicosanoid production and release from astrocyte cultures (Bruner and Murphy, 1990b, 1993b; Keller et al., 1987; Murphy et al., 1985). Nevertheless, in cultured astrocytes the vast majority of PI-linked receptors fail to evoke the release of eicosanoids. Agents such as carbachol, noradrenaline, histamine, 5-hydroxytryptamine (5-HT), glutamate and somatostatin, are all incapable of eliciting eicosanoid release from astrocytes (Marin et al., 1991; Pearce and Murphy, 1988; Pearce et al., 1987).
ATP stimulates the formation of inositol phosphates (IPs) and evokes an increase in 
$[\text{Ca}^{2+}]_i$ (Bruner and Murphy, 1993b; Kastritsis et al, 1992; Neary et al, 1988, 1991; 
Pearce et al, 1989). Moreover, it is one of the few physiologically relevant signals 
capable of stimulating eicosanoid release from CNS astrocytes (Bruner and Murphy, 
1990b, 1993b; Gebicke-Haerter et al, 1988; Pearce et al, 1989), and this is thought to 
be via activation of P2Y-purinoceptors (Bruner and Murphy, 1990b).

The situation is further complicated, however, by the discovery of receptors on a 
variety of cell types which recognise both ATP and/or the pyrimidine nucleotide uridine 5'-triphosphate (UTP), these being defined as P2U receptors (O'Connor et al, 1991; 
O'Connor, 1992; Seifert and Schultz, 1989). Moreover, these receptors are not 
activated by the stable ATP analogues, 2-MeSATP or $\alpha,\beta$-methylene-ATP, indicating 
that they are neither P2Y or P2X. UTP has been demonstrated to have receptor-
mediated effects similar to those seen with ATP, including increases in $[\text{Ca}^{2+}]_i$, 
stimulation of PI turnover and activation of protein kinase C (Brown et al, 1991; 
Carew et al, 1994; Iredale and Hill, 1993; Keppens et al, 1992; Lin et al, 1993; Lin, 1994; 
Lustig et al, 1992; Motte et al, 1993; Munshi et al, 1993). Using an antibody against 
P2U receptors, Bruner and Murphy (1993a) have established that cultured astrocytes 
express these receptors, activation of which elicits PI breakdown, an increase in 
$[\text{Ca}^{2+}]_i$ and stimulates eicosanoid release. These data indicate that eicosanoid release 
from astrocytes might be controlled by at least two P2 purinoceptor subtypes. 
However, since UTP is a pyrimidine nucleotide its receptor cannot satisfactorily be 
accommodated within the P2 purinoceptor classification and has, therefore, been more 
appropriately termed a "nucleotide receptor". Using agonist potency orders, the major 
classes of nucleotide receptors have been characterised according to the scheme below 
The existence of a nucleotide receptor has been proposed to account for circumstances whereby purine and pyrimidine nucleotides elicit the same functional response. The characteristics for receptor activation which appear to distinguish the nucleotide receptor from others are high sensitivity to both UTP and ATP and low sensitivity to agonists such as 2-MeSATP. In addition to agonist potency order data, the concept of nucleotide receptors is supported by evidence that in some tissues, UTP and ATP interact with the same receptor. For example, Pfeilschifter (1990) has demonstrated that responses to both agonists were inhibited equally by pertussis toxin, modulated similarly by acute and chronic phorbol ester treatment, non-additive at maximal concentrations, similarly inhibited by reactive blue 2 and showed cross-desensitisation, observations which are all consistent with the proposal that ATP and UTP are acting through a common receptor. Similarly, Brown et al (1991) have shown that in human airway epithelial cells, ATP- and UTP-stimulated release of IPs was not additive, showed specific cross-desensitisation, and were affected by pertussis toxin to a similar extent.

A problem that has always restricted research involving P2 purinoceptor classification is the lack of selective, competitive antagonists. In their absence, receptor classification may be attempted although with less confidence by the use of selective nucleotide agonists and agonist potency orders. This is unsatisfactory since the apparent agonist potency depends not only upon agonist binding to the receptor, but also upon the entire signal transduction machinery (Fredholm et al, 1994). ATP is non-selective,
biologically unstable and degraded by sequential phosphorylation to ADP, AMP and finally, adenosine, degradation products which themselves may be biologically active at P2 or P1 purinoceptors (O'Connor, 1992). Perhaps more reliable in terms of receptor classification are agents such as 2-MeSATP, which show resistance to ectonucleotide degradation and selectivity for a particular P2 purinoceptor subtype. However, this approach has limitations in terms of expanding the current receptor classification to encompass non-ATP nucleotides, since stable and selective biologically active analogues of most nucleotide agonists are generally not available.

The trypanoside suramin appears to have some antagonist activity at P2 purinoceptors (Bailey and Hourani, 1994; Bultmann and Starke, 1994; von Kugelgen et al, 1990; Voogd et al, 1993), but does not appear to distinguish between the P2X and P2Y subtypes (Hoyle et al, 1990). However, suramin appears to possess a range of biological activities, such as inhibiting certain growth factors and enzymes, particularly 5'-nucleotidase, which might further limit its usefulness as a specific receptor antagonist (Betsholtz et al, 1986; Crack et al, 1994; Hourani and Chown, 1989; Voogd et al, 1993). In addition to suramin, reactive blue 2 has potentially been identified as a selective P2 purinoceptor antagonist on the basis of its ability to inhibit the contractile action of 2-MeSATP, but not α,β-methylene-ATP, in rabbit mesenteric artery (Burnstock and Warland, 1987).

3.1.1. Functional significance of nucleotide receptors.

According to O'Connor (1992), "for endogenous nucleotides to have physiological significance would require evidence of their release into the extracellular space in a biologically appropriate manner to achieve local concentrations sufficient to activate their receptors and elicit a functional response". It is now well established that ATP
fulfils these criteria in numerous cell types. The biological significance of extracellular UTP is less well defined but along with other uracil nucleotides, it is found in all cells, with platelets, kidney and brain tissue being especially rich sources (Keppler et al, 1970; Pfeilschifter, 1990; Seifert and Schultz, 1989). By analogy with adenine nucleotides, uracil nucleotides may be released from cells under a variety of pathological conditions such as trauma, hypoxia and inflammation (Gordon, 1986).

It appears that UTP has a role in inflammatory cell function and vascular reactivity following reports that it activates human macrophages, neutrophils and fibroblasts, shows both tissue-dependent vasorelaxant and vasoconstrictor properties, and stimulates eicosanoid release (Alonso-Torre and Trautmann, 1993; Bruner and Murphy, 1993a; Cockcroft and Stutchfield, 1989; Fine et al, 1989; Juul et al, 1992; Lustig et al, 1992; Motte et al, 1993; Needham et al, 1987; Seifert et al, 1989a; Vials and Burnstock, 1993; Walker et al, 1991). These observations have led to the suggestion that contraction of intracranial arteries by UTP plays an important role in the pathogenesis of vasospasm following cerebral injury (Shirasawa et al, 1983).

Purine and pyrimidine nucleotides appear to be involved in the regulation of epithelial ion transport. Mason et al (1991) have identified a receptor sensitive to both UTP and ATP, activation of which modulates chloride ion secretion in human airway epithelial cells from both normal and cystic fibrosis subjects. Cystic fibrosis is a genetic condition characterised by abnormal exocrine cell function, principally in the lungs, where it results in thickening of airways secretions, impaired mucociliary clearance and increased susceptibility to bacterial infection. These authors have found that UTP and ATP stimulated chloride ion secretion, which is defective in patients with cystic fibrosis, via activation of a cell surface receptor linked to increases in \([\text{Ca}^{2+}]_i\) in human nasal epithelium. Both nucleotide agonists were equally effective in this respect, whereas 2-MeSATP was ineffective, indicating the involvement of a P2U nucleotide
receptor. More recently, Gobran et al (1994) have used northern blot analysis to confirm the existence of the P2U receptor gene in airway epithelium from patients with cystic fibrosis.

3.1.2. P2 purinoceptor second-messenger systems.

The body of evidence now suggests that the family of P2 purinoceptors constitute a diverse group with respect to their second messenger systems. Most evidence concerning the P2X purinoceptor family is that it represents an intrinsic ion channel permeable to Na⁺, K⁺ and Ca²⁺ (Bean, 1992). P2Y purinoceptors, however, constitute G-protein linked receptors, which are predominantly coupled to stimulation of PLC activity and hence IP₃ formation and increases in [Ca²⁺]ᵢ (Harden et al, 1995; O'Connor et al, 1991). All G protein receptors have a predicted seven transmembrane-spanning structure and share conservation of sequence in their transmembrane-spanning domains. Exchange of GTP for GDP is promoted by agonist-occupied receptors, which leads to activation as a result of dissociation of the inactive heterotrimeric protein into a GTP-ligated α-subunit and a tightly associated dimer of β- and γ-subunits (Harden et al, 1995). In most cases, the GTP-ligated α-subunit then interacts and activates an effector enzyme or channel; this is commonly PLC in the case of P2 purinoceptors.

However, additional transduction mechanisms including modulation of cAMP generation and arachidonic acid mobilisation have also been demonstrated (Boyer et al, 1993; Bruner and Murphy, 1993a; Yamada et al, 1992). Pianet et al (1989) have reported that extracellular ATP and ADP produced a concentration-dependent decrease in cAMP levels in rat C6 glioma cells. Sensitivity of this response to inactivation by
pertussis toxin suggested that the effect of a P2 purinoceptor was probably mediated through G\textsubscript{i} and the inhibition of adenylyl cyclase.

Since the most predominantly described second-messenger response to P2Y purinoceptor activation is promotion of inositol lipid hydrolysis, it is important to establish whether a PLC-activating receptor could also couple through G\textsubscript{i} to inhibit adenylyl cyclase. This would not be expected based on the fidelity of both the coupling to the G\textsubscript{q} family of G proteins and PLC exhibited by nonpurinergic receptors (Harden et al., 1995). This observation therefore suggested the existence of an additional member of the P2Y purinoceptor family. To this end, Boyer et al. (1993) have reported that although P2 purinoceptor agonists markedly inhibited cAMP accumulation in C6 glioma cells, they had no effect on inositol lipid hydrolysis or on Ca\textsuperscript{2+} mobilisation. The authors interpretation of these results is that a P2Y purinoceptor is expressed in C6 glioma cells that couples G\textsubscript{i} and adenylyl cyclase but does not couple to the G\textsubscript{q} family of G proteins and PLC. These observations are in direct contrast to results obtained by the same laboratory with turkey erythrocytes where P2Y purinoceptor agonists stimulate PLC and the accumulation of IPs, but doesn't couple to adenylyl cyclase (Boyer et al., 1989). Furthermore, Lin and Chuang (1994) have reported that whilst nucleotide receptors in C6 glioma cells are linked to PLC by a pertussis toxin-insensitive G protein, the P2Y receptor is linked to adenylyl cyclase by a G protein which is pertussis toxin-sensitive. Thus, there is evidence of heterogeneity among P2Y purinoceptors, however, the functional significance of these has yet to be determined.

The overall signalling response promoted by P2U purinoceptors is that of a PLC-linked receptor (Harden et al., 1995). In human neutrophils, for example, both ATP and UTP activated PLC via a pertussis-toxin-sensitive G protein (Cockcroft and Stutchfield, 1989). Similar results have been reported in HL60 cells, in which UTP is a more potent agonist than ATP (Stutchfield and Cockcroft, 1990). Very little biochemical
information is available to indicate the existence of multiple P2U purinoceptors. On the basis of agonist potency orders, Xing et al (1992) have reported the existence of two subtypes of P2U receptor on HL60 cells: one which stimulates AA release and displaying a high affinity for agonist, and the other inhibitory and interacting with low affinity. Moreover, a novel receptor has been identified on rat glioma cells that could be a submember of the P2U purinoceptor family (Lazarowski and Harden, 1994). Activation of this receptor resulted in marked activation of PLC, but in contrast to the classical P2U purinoceptor, this receptor is activated by uridine nucleotides but not by adenine nucleotides. Uridine 5'-diphosphate (UDP) is the most potent agonist at this so-called uridine nucleotide receptor, with UTP being approximately 50-fold less potent than UDP. The physiological significance and tissue distribution of this novel receptor is unknown. The concept of UTP-specific pyrimidinoceptors at which ATP is inactive has been proposed previously (Seifert and Schultz, 1989).

3.1.3. Molecular biology of P2Y and P2U purinoceptors.

A chicken and turkey P2Y purinoceptor (Filtz et al, 1994; Webb et al, 1993) and a mouse and a human P2U purinoceptor (Erb et al, 1993; Lustig et al, 1993; Parr et al, 1994) represent the only G protein-linked P2 purinoceptors whose cDNA and amino acid sequences have been cloned. P2U and P2Y purinoceptors have relatively low amino acid homology (36% identity), with the greatest degree of homology occurring in the putative transmembrane-spanning regions. These sequences confirm that these receptors are members of the superfamily of G protein-coupled receptors, which contain seven putative α-helical transmembrane segments, with the amino terminal located on the extracellular side and their carboxy terminal located on the intracellular side of the membrane (Harden et al, 1995). Interestingly, both P2U and P2Y purinoceptors are surprisingly similar to G protein-coupled receptors for several peptide
ligands, notably somatostatin, vasoactive intestinal peptide and neuropeptide Y, whereas adenosine receptors have considerably less homology to P2 purinoreceptors than do the peptide receptors (Harden et al., 1995). The significance of this similarity between P2 purinoreceptors and peptide receptors remains unclear, but the peptides bradykinin and substance P are the only other ligands apart from ATP which have been shown to stimulate eicosanoid release from astrocytes. On the basis of evidence obtained from molecular biology techniques, a new classification has been proposed to take account of molecular subtypes of P2 purinoreceptors (Fredholm et al., 1994).

We set out to examine TXA$_2$ release from astrocytes in terms of the range of nucleotide agonists that might elicit TXA$_2$ release from these cells, and investigate whether there are common features in the pathways through which P2Y and P2U purinoreceptors regulate this release, or whether they operate independently. We were particularly interested to determine if there was a correlation between the activation of these receptors, the change in [Ca$^{2+}$]$_i$, PI turnover and the release of TXA$_2$ from astrocytes in culture. In particular, we attempted to characterise the subtypes of nucleotide receptor involved eicosanoid release from astrocytes, and to determine whether an increase in [Ca$^{2+}$]$_i$ and the presence of serum in the culture medium is an absolute requirement for this response.

3.2. Methods and Materials

Methods were as described in Chapter 2. All drug solutions were freshly prepared in distilled water except forskolin, ionomycin and 2-(p-amylcinnamoyl) amino-4-chlorobenzoic acid (ONO-RS-082), which were dissolved in DMSO. The final concentration of DMSO in contact with the cells did not exceed 0.1% v/v. Control experiments where cells were exposed to DMSO alone at 0.1% v/v showed that the
vehicle had no effect on \([\text{Ca}^{2+}]_i\) or \(\text{TXA}_2\) release. In those experiments in which suramin was used, the stock solution was protected from light.

All drugs were purchased from Sigma, except ONO-RS-082 which was obtained from Cascade Biochem Ltd, UK, and 2-MeSATP which was purchased from Research Biochemicals Inc., USA. Suramin was the kind gift of Bayer UK.

### 3.3. Results

#### 3.3.1. The effect of purine and pyrimidine nucleotides on \(\text{TXA}_2\) release.

We set out to examine to what extent, if any, the range of purine and pyrimidine nucleotides were capable of eliciting \(\text{TXA}_2\) release from astrocytes, and the concentration dependence of those tri- and diphosphate nucleotides which did evoke responses are shown in Figure 3.1. Both ATP and ADP (both purine nucleotides) were found to be similar in terms of efficacy and potency (\(EC_{50}\) values being 10 µM and 18 µM respectively). The selective P2Y receptor agonist 2-MeSATP was found to be considerably more potent than ATP (\(EC_{50} = 0.6\) µM) but slightly less effective, being capable of stimulating a maximum response which was only 80% of that seen with ATP.

The pyrimidine nucleotides UTP and UDP were found to have similar potencies to ATP (\(EC_{50} = 19\) µM and 16 µM respectively), but were much more effective than ATP in stimulating \(\text{TXA}_2\) from these cells. Indeed, UTP was capable of stimulating almost twice as much \(\text{TXA}_2\) release than ATP. The monophosphates of adenosine and uridine were also tested, and at concentrations up to 250 µM were without effect (89 ± 16% and 82 ± 5% of control respectively, \(n = 3\)). Similarly, uridine failed to stimulate
Figure 3.1. Concentration dependence of purine (panel A) and pyrimidine (panel B) nucleotide-stimulated TXA₂ release from astrocytes. Cultures were incubated for 15 min with various concentrations of nucleotide and the release of TXA₂ measured and expressed as a percentage of the maximal response to ATP, this was taken as the effect (657 ± 69% stimulation over basal) elicited by 100 μM ATP. Each point is the mean of at least four determinations (SEM <15%). The nucleotides used were ATP (●), ADP (○) 2-MeSATP (□) GTP (▲), UTP (▲), UDP (▼), CTP (▼) and TTP (■).

EC₅₀ values were calculated by visual inspection.
TXA$_2$ (94 ± 13% of control, n = 4). Although adenosine also failed to evoke TXA$_2$ release, it was found to reduce basal release to 60 ± 8% of control (n = 4). This effect is in accordance with a previous report (Pearce et al, 1989).

The effects of the purine nucleotide guanosine 5'-triphosphate (GTP) and the pyrimidine nucleotides deoxythymidine 5'-triphosphate (TTP), cytidine 5'-triphosphate (CTP) and xanthosine 5'-triphosphate (XTP) were also examined. All of these nucleotides, with the exception of XTP, stimulated TXA$_2$ release from astrocytes with potencies similar to ATP (EC$_{50}$ values being 16 μM, 13 μM and 20 μM for GTP, TTP and CTP respectively). Generally, these nucleotides were only half as effective as that produced by ATP. At a concentration of 250 μM, the diphosphate species of guanosine, thymidine and cytosine caused only modest (approximately 20 - 30% of that elicited by an equivalent dose of ATP) increases in TXA$_2$ release (data not shown).

3.3.2. The effect of removing microglia on nucleotide-stimulated TXA$_2$ release.

The effect of removing microglia from astrocyte cultures on nucleotide-stimulated TXA$_2$ release was examined from cultures prepared in growth medium supplemented with 5 mM LME. Table 3.1 shows that in the absence of microglia, all nucleotides examined were capable of stimulating TXA$_2$ release, but to a slightly reduced extent. Moreover, the effects of UTP and UDP were not substantially greater than that evoked by ATP.
Table 3.1. Effect of nucleotides on TXA₂ release from microglia-free cultures.

<table>
<thead>
<tr>
<th>Additions</th>
<th>TXA₂ release (% stimulation over basal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>388 ± 7</td>
</tr>
<tr>
<td>ADP</td>
<td>384 ± 8</td>
</tr>
<tr>
<td>2-MeSATP</td>
<td>252 ± 43</td>
</tr>
<tr>
<td>UTP</td>
<td>407 ± 4</td>
</tr>
<tr>
<td>UDP</td>
<td>384 ± 8</td>
</tr>
<tr>
<td>GTP</td>
<td>322 ± 18</td>
</tr>
<tr>
<td>CTP</td>
<td>175 ± 18</td>
</tr>
<tr>
<td>TTP</td>
<td>241 ± 8</td>
</tr>
</tbody>
</table>

Cultures were maintained in growth medium containing 5 mM LME to prevent the proliferation of microglia. Incubations were for 15 min and each nucleotide was used at a concentration of 250 µM except 2-MeSATP which was at 5 µM. Results are expressed as a percent stimulation over basal (1.6 ± 0.15 ng/ml) and are means ± SEM from at least three determinations.
Figure 3.2. Changes in $[Ca^{2+}]_i$ with increasing concentrations of ATP. The bars above the trace indicate the period (30 sec) over which the cells were exposed to the nucleotide. This experiment was performed on a single field of cells and is representative of similar experiments carried out on at least 10 fields from different batches of cultures.
3.3.3. Characterisation of purine- and pyrimidine-induced changes in [Ca^{2+}]_i.

Astrocyte cultures were challenged with ATP over the dose range 0.1-10 |iM, and the change in [Ca^{2+}]_i assessed. Cells responded to ATP with a dose related increase in [Ca^{2+}]_i as illustrated in Figure 3.2. This figure illustrates that changes evoked by ATP were characteristically biphasic, and were comprised of a secondary component which was sustained throughout the drug exposure. This latter phase of the response oscillates around a mean [Ca^{2+}]_i, and is derived from the influx of Ca^{2+} from the extracellular environment. Figure 3.2 also shows that a subsequent challenge with the same concentration of ATP resulted in a change in [Ca^{2+}]_i which was slightly attenuated than that obtained during the initial challenge. Doses of ATP >10|μM were not able to evoke further increases in [Ca^{2+}]_i.

Naive cells were challenged with a range of various purine and pyrimidine nucleotides at a concentration of 10 |μM, and the change in [Ca^{2+}]_i assessed. These results are illustrated in Figure 3.3. ATP, ADP, 2-MeSATP and UTP were all similar in that they induced Ca^{2+} transients which were all characteristically biphasic, comprising an initial spike-like transient increase and a secondary plateau. Dose response curves for these nucleotides are shown in Figure 3.4, and demonstrates that there is a rank order of potency in their ability to increase [Ca^{2+}]_i. The specific P2Y receptor agonist, 2-MeSATP is the most potent nucleotide agonist (EC50 = 3 nM), ATP and ADP have similar potencies (EC50= 0.7 and 0.15 |μM respectively) and UTP was the least potent (EC50= 3 |μM).

In comparison, when cells were challenged with GTP, TTP, CTP or UDP, changes in [Ca^{2+}]_i were observed which were generally smaller than those seen with ATP, ADP, 2-MeSATP or UTP, and which lacked the secondary plateau; however, the changes in
Figure 3.3. Representative changes in \([\text{Ca}^{2+}]_i\) evoked by various nucleotides in astrocytes. Traces are compiled from a number of experiments on different preparations showing changes in \([\text{Ca}^{2+}]_i\) when cells were challenged for 30 sec with various nucleotides at a concentration of 10 \(\mu\text{M}\).
1.5

0.5

ATP  ADP  2-MeSATP  UTP  CTP  TTP  GTP  UDP

$\left[ \text{Ca}^{2+} \right]_i$ (µM)
Figure 3.4. Concentration dependence of nucleotide-stimulated changes in $[\text{Ca}^{2+}]_i$.

Cell fields from a number of different cultures were challenged for 30 sec with various concentrations of nucleotide and the increase in $[\text{Ca}^{2+}]_i$ recorded. The peak response elicited was then expressed as a percentage of that evoked by a maximally effective dose (10 μM) of ATP. Each point is the mean (SEM <15%) of at least four determinations. The nucleotides used were ATP (●), ADP (○), 2-MeSATP (□) and UTP (△).
induced by GTP, although usually spike-like, were also occasionally found to be biphasic. In contrast, the nucleotides XTP and inosine 5'-triphosphate (ITP) did not affect the $[Ca^{2+}]_i$.

The $Ca^{2+}$ transients induced by GTP, TTP, CTP or UDP were characteristically transitory and, unlike ATP, long-term exposure of these nucleotides to a cell field did not result in a sustained change in $[Ca^{2+}]_i$. Moreover, the $Ca^{2+}$ transients evoked by these nucleotides were found to desensitise rapidly and repeated agonist challenge had no further effect on $[Ca^{2+}]_i$. However, there were some atypical instances where this desensitisation was somewhat delayed, as is illustrated in Figure 3.5. Thus, there is a marked, but not complete, reduction in GTP- and TTP-evoked changes in $[Ca^{2+}]_i$ when cells undergo repeated challenge with these agonists. Repeated exposure of cells to CTP did, however, not result in any change in $[Ca^{2+}]_i$ at any time.

The desensitisation of GTP-, TTP-, CTP- or UDP-evoked $Ca^{2+}$ transients was seen with all effective concentrations examined, which limited our ability to characterise them in detail. Table 3.2 is therefore compiled from data obtained from a number of experiments from different batches of cultures, and shows both the proportion of cells responding to each of the nucleotides and their relative efficacies. This table shows that all cell fields examined responded to ATP, ADP and 2-MeSATP, and shows that the peak changes in $[Ca^{2+}]_i$ were essentially of the same order. GTP, the other purine nucleotide tested, was only half as efficacious as ATP (46 ± 8%), and elicited a response in 75% of cell fields.

The pyrimidine nucleotides, however, elicited changes in $[Ca^{2+}]_i$ which were generally much smaller than those evoked by ATP, the exception being UTP which evoked changes in $[Ca^{2+}]_i$ that was 73 ± 2% of that induced by ATP. Furthermore, changes in $[Ca^{2+}]_i$ evoked by the pyrimidine nucleotides were not detected as frequently as those
Table 3.2. Proportion of cell fields responding to nucleotide additions with an increase in [Ca^{2+}]_{i}.

<table>
<thead>
<tr>
<th>Additions</th>
<th>No. of positive responses/ no. of cell fields examined</th>
<th>Peak increase in [Ca^{2+}]_{i} (%) ATP response</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>76/76 (100%)</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>ADP</td>
<td>14/14 (100%)</td>
<td>97 ± 9</td>
</tr>
<tr>
<td>2-MeSATP</td>
<td>13/13 (100%)</td>
<td>90 ± 6</td>
</tr>
<tr>
<td>UTP</td>
<td>25/63 (40%)</td>
<td>73 ± 2</td>
</tr>
<tr>
<td>UDP</td>
<td>4/17 (24%)</td>
<td>25 ± 6</td>
</tr>
<tr>
<td>GTP</td>
<td>6/8 (75%)</td>
<td>46 ± 8</td>
</tr>
<tr>
<td>CTP</td>
<td>9/19 (47%)</td>
<td>42 ± 5</td>
</tr>
<tr>
<td>TTP</td>
<td>7/19 (37%)</td>
<td>54 ± 8</td>
</tr>
</tbody>
</table>

A number of cell fields were challenged with a nucleotide at a concentration of 10 |μM for 0.5 min and the change in [Ca^{2+}]_{i} recorded. The number of cell fields responding with an increase in intracellular Ca^{2+} and the number of experiments performed are indicated. The values in parenthesis represent the proportion of positive responses recorded. The peak increase in [Ca^{2+}]_{i} for each nucleotide is expressed as a percentage of that evoked by ATP. These data are from experiments carried out on cell fields from a number of culture preparations.
Figure 3.5. Atypical effect of repeated challenge with GTP, CTP and TTP on [Ca^{2+}]_{i}. Each nucleotide was at a concentration of 10 μM, with each exposure period being approximately 3 min apart. The bars above the trace indicate the period (30 sec) over which the cells were exposed to the nucleotide. These data are compiled from experiments performed on different cell fields.
evoked by ATP. UDP was the least effective nucleotide both in terms of the number of positive responses recorded and the peak increase in \([\text{Ca}^{2+}]_i\).

Macrophage/microglia are often seen in astrocyte cultures as a contaminating cell. We wanted to confirm that the responses described above were indeed due to astrocytes, and not an effect of the macrophages. To this end, cell fields which were particularly dense in terms of macrophages were visually selected, the aperture of iris diaphragm over the photomultiplier was reduced so that the cells completely filled the optical path, and the change in \([\text{Ca}^{2+}]_i\) due to ATP, ADP and UTP assessed. The results in Figure 3.6 confirm that although challenge with these nucleotides did result in a change in basal \([\text{Ca}^{2+}]_i\) in the macrophages, the changes are relatively small in comparison to those observed in astrocytes, and can be largely discounted in terms of the overall change in \([\text{Ca}^{2+}]_i\).

3.3.4. The effect of extracellular \(\text{Ca}^{2+}\) on nucleotide-evoked \(\text{Ca}^{2+}\) mobilisation and \(\text{TXA}_2\) release.

In an attempt to identify the source(s) of \(\text{Ca}^{2+}\) mobilised by the different nucleotides, cells were washed in buffer devoid of extracellular \(\text{Ca}^{2+}\), and supplemented with 0.2 mM EGTA. The EGTA acts to chelate free \(\text{Ca}^{2+}\) that may still be present in the buffer. Of those nucleotides examined, this regime resulted in a reduction in the extent of the change in \([\text{Ca}^{2+}]_i\), which was further characterised by the absence of the secondary plateau phase of the response (where normally present). These results are summarised in Table 3.3. Under these conditions, the responses elicited by ATP, ADP and 2-MeSATP were reduced by 60-70% whereas those of UTP and CTP were reduced by only 35-40%.
Figure 3.6. Effect of ADP, ATP and UTP on the [Ca\textsuperscript{2+}]\textsubscript{i} in macrophages. Cell fields were chosen which were particularly dense in macrophages, the aperture of the iris diaphragm of the photomultiplier was reduced so that only the changes in fluorescence from these cells was recorded, and the change in [Ca\textsuperscript{2+}]\textsubscript{i} assessed. The bars above the trace indicate the period (30 sec) over which the cells were exposed to the drugs.
Table 3.3. Effect of extracellular Ca\(^{2+}\) on nucleotide-stimulated intracellular Ca\(^{2+}\) mobilisation.

<table>
<thead>
<tr>
<th>Additions</th>
<th>([\text{Ca}^{2+}]_i) (% control response)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>27 ± 5</td>
</tr>
<tr>
<td>ADP</td>
<td>36 ± 8</td>
</tr>
<tr>
<td>2-MeSATP</td>
<td>26 ± 1</td>
</tr>
<tr>
<td>UTP</td>
<td>67 ± 14</td>
</tr>
<tr>
<td>CTP</td>
<td>60 ± 12</td>
</tr>
</tbody>
</table>

Cell fields were challenged for 0.5 min with various nucleotides at a concentration of 10 \(\mu\text{M}\) in Ca\(^{2+}\)-free medium containing 0.2 mM EGTA and the changes in \([\text{Ca}^{2+}]_i\) recorded. The peak responses elicited under these conditions are expressed as a percentage of those obtained in sister cultures incubated in normal Ca\(^{2+}\) containing medium. Results are means ± SEM from at least three determinations.
The intracellular Ca\(^{2+}\) chelator 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester (TMB-8) has been reported to specifically antagonise the release of Ca\(^{2+}\) from intracellular stores, and is often used to distinguish between dependence on extracellular and intracellular Ca\(^{2+}\) (Palmer et al, 1992; Yamada et al, 1988). We were interested, therefore, to see what effect TMB-8 had on ATP-induced changes in [Ca\(^{2+}\)]\(_i\). Accordingly, cells were challenged with ATP, then washed in buffer containing 1 mM TMB-8 for 5 min, and subsequently challenged again with ATP. Under this regime, as can be seen in Figure 3.7, the initial phase of increased [Ca\(^{2+}\)]\(_i\) normally evoked by ATP was substantially diminished, whereas the subsequent plateau phase appears unaffected by TMB-8. This figure also illustrates that any effect of TMB-8 on intracellular Ca\(^{2+}\) is transitory, since the biphasic change in [Ca\(^{2+}\)]\(_i\) usually induced by ATP can be restored upon subsequent challenge with ATP.

The dependence on extracellular Ca\(^{2+}\), and the contribution of Ca\(^{2+}\) release from intracellular stores, on nucleotide-stimulated TXA\(_2\) release was assessed by incubating cultures in either Ca\(^{2+}\)-free buffer (with 0.2 mM EGTA) or Ca\(^{2+}\)-free buffer containing 1 mM TMB-8. Results from these experiments are shown in Table 3.4, and indicate that nucleotide-stimulated TXA\(_2\) release is largely dependent on the presence of extracellular Ca\(^{2+}\), with only 20-30% of the response obtained in Ca\(^{2+}\)-containing buffer remaining when extracellular Ca\(^{2+}\) is removed. The Ca\(^{2+}\) ionophore ionomycin was included as a positive control as one would predict that its effects are entirely dependent on the presence of extracellular Ca\(^{2+}\). Accordingly, ionomycin-stimulated TXA\(_2\) release was completely abolished when extracellular Ca\(^{2+}\) was removed.

To determine whether the remaining nucleotide-stimulated TXA\(_2\) release was due to release of Ca\(^{2+}\) from internal stores, cells were incubated in Ca\(^{2+}\)-free medium containing TMB-8. Table 3.4 shows that under these conditions, TXA\(_2\) release was no greater than basal.
Figure 3.7. Representative trace showing the effect of TMB-8 on ATP-evoked changes in current ratio of Fura 2-AM. A control response to ATP (10 μM) was established, the cells were then bathed with buffer containing TMB-8 (1 mM) for approximately 5 min, and the cells challenged once again with ATP. The bars below the trace indicate the period over which the cells were exposed to the ATP (30 sec) or TMB-8 (5 min). The change in current ratio is representative of the change in [Ca²⁺]ᵢ.
The graph shows the current ratio over time with designated periods labeled ATP and TMB-8. The x-axis represents time in minutes, ranging from 0 to 12, and the y-axis represents the current ratio ranging from 0 to 3.0. The graph indicates fluctuations in the current ratio with specific time intervals marked by ATP and TMB-8.
Table 3.4. Effect of extracellular Ca\(^{2+}\) and TMB-8 on nucleotide-evoked TXA\(_2\) release from astrocytes.

<table>
<thead>
<tr>
<th>Additions</th>
<th>+Ca(^{2+})</th>
<th>-Ca(^{2+})</th>
<th>-Ca(^{2+}) + TMB-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>414 ± 4</td>
<td>193 ± 25</td>
<td>91 ± 3</td>
</tr>
<tr>
<td>ADP</td>
<td>412 ± 27</td>
<td>154 ± 7</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>2-MeSATP</td>
<td>300 ± 13</td>
<td>137 ± 7</td>
<td>107 ± 3</td>
</tr>
<tr>
<td>UTP</td>
<td>523 ± 9</td>
<td>184 ± 9</td>
<td>109 ± 7</td>
</tr>
<tr>
<td>UDP</td>
<td>480 ± 41</td>
<td>212 ± 39</td>
<td>96 ± 7</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>395 ± 6</td>
<td>117 ± 14</td>
<td>ND</td>
</tr>
</tbody>
</table>

Cultures were incubated with the above for 15 min in either Ca\(^{2+}\) containing medium, Ca\(^{2+}\)-free medium to which 0.2 mM EGTA had been added, or Ca\(^{2+}\)-free medium containing 1 mM TMB-8. The effect of each nucleotide is expressed as a percentage of basal TXA\(_2\) release under each of the conditions listed above, these were 1.01 ± 0.13 ng/ml, 0.47 ± 0.07 ng/ml and 0.39 ± 0.02 ng/ml respectively. Each agonist was used at a concentration of 250 μM except 2-MeSATP and ionomycin which were used at 5 μM. Results are means ± SEM from at least three determinations. ND indicates not determined.
3.3.5. The effect of suramin and reactive blue 2 on nucleotide-stimulated TXA\textsubscript{2} release.

Suramin is widely used as a putative P2 receptor antagonist. We were interested to see what effect it had on adenine and uridine nucleotide-stimulated TXA\textsubscript{2} release, and whether these results could tell us anything of the receptor subtype(s) involved in this response. The results obtained with the five main nucleotides are shown in Figure 3.8, and indicate that suramin inhibited TXA\textsubscript{2} release induced by all nucleotides examined. The effect of 2-MeSATP was particularly sensitive to suramin, the results giving an IC\textsubscript{50} of 4 \textmu M. Suramin was slightly less potent in its ability to inhibit the effect of ATP and UTP (IC\textsubscript{50} = 25 \textmu M and 18 \textmu M respectively), whilst the effects of ADP and UDP appeared even more resistant to antagonism by suramin, the IC\textsubscript{50} values being 45 \textmu M and 90 \textmu M respectively. The effects of GTP, TTP and CTP were also antagonised by suramin, the IC\textsubscript{50} values being 15 \textmu M, 5 \textmu M and 14 \textmu M respectively (Data not shown).

We also examined the effects of another P2 receptor antagonist, reactive blue 2 (or cibacron blue 2 as it is sometimes known), which has been reported to have some selectivity towards P2Y receptors (Dalziel and Westfall, 1994). Based upon our results with suramin, a concentration of 25 \textmu M reactive blue was chosen, and tested against 250 \textmu M concentrations of ATP, ADP, UTP, UDP and 2-MeSATP. The results obtained are shown in Figure 3.9, and show that nucleotide-stimulated TXA\textsubscript{2} release for all the nucleotides examined is reversed by reactive blue. However, the effects of the nucleotide diphosphates, ADP and UDP, appear particularly susceptible to the action of the antagonist, their effects being reduced by 91 \pm 1\% and 83 \pm 17\% respectively (n = 3 in each case).
Figure 3.8. Effect of suramin on nucleotide-stimulated TXA$_2$ release. Cultures were incubated for 15 min with nucleotides at 250 μM except 2-MeSATP which was at 5 μM in the presence or absence of suramin at concentrations of 2.5 μM (open bars), 25 μM (striped bars) and 250 μM (solid bars). The release of TXA$_2$ was calculated as a percentage of that evoked by each nucleotide in the absence of suramin. Results are means ± SEM from at least three determinations.
Figure 3.9. Effect of reactive blue 2 on nucleotide-stimulated TXA$_2$ release. Cultures were incubated with nucleotides at 250 µM except 2-MeSATP which was at 5 µM in the presence or absence of reactive blue at a concentration of 25 µM. The release of TXA$_2$ was calculated as a percentage of each nucleotide in the absence of reactive blue 2. Results are means ± SEM from at least three determinations.
3.3.6. The effect of combined nucleotide additions.

The effect of combined additions of nucleotides on TXA₂ release and [Ca²⁺]ᵢ were examined. There was no evidence of additivity in terms of TXA₂ release when cells were challenged with ATP plus ADP or UTP plus UDP, as is shown in Table 3.5. Furthermore, no additivity was found between ATP and UTP, the observed response being equivalent to that of UTP, the more effective agonist. The ability of ATP to elicit TXA₂ release was attenuated by two-thirds during the concomitant addition of 2-MeSATP to the cultures. However, this effect was not observed when cells were exposed to ADP, UTP or UDP in the presence of 2-MeSATP.

Combined nucleotide additions had no effect on [Ca²⁺]ᵢ, which is illustrated in Figure 3.10. Challenge with ATP plus ADP, 2-MeSATP or UTP did not result in additional increases in [Ca²⁺]ᵢ beyond that elicited by ATP alone, nor did 2-MeSATP attenuate the response evoked by ATP.

3.3.7. The effect of forskolin and ONO-RS-082.

The effect of forskolin on nucleotide-stimulated TXA₂ release is shown in Figure 3.11. Forskolin directly stimulates adenylyl cyclase and has been used extensively to increase cyclic AMP and to elicit cyclic AMP-dependent physiological responses (Laurenza et al, 1989). Concentrations of forskolin below 10 μM failed to alter the ability of any of the nucleotides tested to promote TXA₂ release from the cultured cells. At 10 μM, however, forskolin produced a reduction of approximately 25% in the responses to ATP, ADP and UDP. The effect of 2-MeSATP remained essentially unchanged, and
Table 3.5. Effect of combined additions on TXA$_2$ release from astrocytes.

<table>
<thead>
<tr>
<th>Additions</th>
<th>TXA$_2$ release (% stimulation over basal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>678 ± 45</td>
</tr>
<tr>
<td>ADP</td>
<td>667 ± 94</td>
</tr>
<tr>
<td>ATP + ADP</td>
<td>658 ± 67</td>
</tr>
<tr>
<td>UTP</td>
<td>930 ± 65</td>
</tr>
<tr>
<td>UDP</td>
<td>890 ± 123</td>
</tr>
<tr>
<td>UTP + UDP</td>
<td>839 ± 54</td>
</tr>
<tr>
<td>ATP + UTP</td>
<td>912 ± 68</td>
</tr>
<tr>
<td>2-MeSATP</td>
<td>391 ± 77</td>
</tr>
<tr>
<td>ATP + 2-MeSATP</td>
<td>233 ± 42</td>
</tr>
<tr>
<td>UTP + 2-MeSATP</td>
<td>1112 ± 311</td>
</tr>
<tr>
<td>ADP + 2-MeSATP</td>
<td>839 ± 8</td>
</tr>
<tr>
<td>UDP + 2-MeSATP</td>
<td>850 ± 81</td>
</tr>
</tbody>
</table>

Cultures were incubated with the above for 15 min and the release of TXA$_2$ expressed as a percentage stimulation over basal (0.33 ± 0.3 ng/ml, n=7). All nucleotides were used at a concentration of 250 μM except 2-MeSATP which was at 5 μM. Results are means ± SEM from at least five determinations.
Figure 3.10. Representative traces showing the effect of combined nucleotide additions on the current ratio of Fura 2-AM. Cells were challenged with ATP, ADP, 2-MeSATP or UTP at a concentration of 10 μM, then with various combinations of nucleotides at the same concentration, and the change in current ratio recorded. This is representative of the change in [Ca^{2+}]_i. The bars below the traces indicate the period (30 sec) over which the cells were exposed to the nucleotides. The change in current ratio is representative of the change in [Ca^{2+}]_i.
ATP + ADP

ATP + 2-MeSATP

ATP + UTP
Figure 3.11. Effect of forskolin on nucleotide-stimulated TXA$_2$ release. Cultures were incubated for 15 min with nucleotides at 250 μM except 2-MeSATP which was at 5 μM in the presence or absence of forskolin at concentrations of 0.1 μM (open bars), 1 μM (striped bars) and 10 μM (solid bars). The release of TXA$_2$ was calculated as a percentage of that evoked by each nucleotide in the absence of forskolin. Results are means ± SEM from at least three determinations.
**Figure 3.12.** Effect of ONO-RS-082 on nucleotide-stimulated TXA$_2$ release. Cultures were incubated for 15 min with nucleotides at 250 μM except 2-MeSATP which was at 5 μM in the presence or absence of ONO-RS-082 at concentrations of 0.35 μM (open bars), 3.5 μM (striped bars) and 35 μM (solid bars). The release of TXA$_2$ was calculated as a percentage of that evoked by each nucleotide in the absence of ONO-RS-082. Results are means ± SEM from at least three determinations.
that due to UTP was reduced to about 50%. The effect of forskolin on ATP-evoked changes in $[Ca^{2+}]_i$ were also assessed, and these experiments showed that 10 $\mu$M forskolin had no effect either on resting or ATP-induced changes in astrocyte $[Ca^{2+}]_i$ (data not shown).

ONO-RS-082 has been reported to inhibit PLA$_2$ in human platelets (Banga et al, 1986), which possess the cytosolic form of PLA$_2$ only (Dennis, 1994), and consequently it was used to examine any differences in the extent various nucleotides were selectively coupled to PLA$_2$ in astrocytes. The results are shown in Figure 3.12. ONO-RS-082 attenuated nucleotide-stimulated TXA$_2$ release in a concentration-dependent manner, with no major differences in potency of the drug against particular nucleotides. The IC$_{50}$ values of ATP, ADP and 2-MeS ATP were 8 $\mu$M, 3 $\mu$M and 11 $\mu$M respectively. The IC$_{50}$ values for UTP and UDP were similar at 3 $\mu$M and 5 $\mu$M. At a concentration of 35 $\mu$M, ONO-RS-082 had little effect on basal TXA$_2$ release ($87 \pm 8\%$ of control, $n = 3$).

3.3.8. The effect of serum depletion on nucleotide-stimulated TXA$_2$ release.

To investigate the serum-dependence of nucleotide-stimulated TXA$_2$ release, cells were maintained in serum free growth medium for 4 days prior to their use. Figure 3.13 shows that this regime resulted in a reduction in TXA$_2$ release for nucleotide-stimulated and basal release. Generally, TXA$_2$ release stimulated by both purine and pyrimidine nucleotides was reduced, however, purine nucleotides were inhibited to a greater extent than the pyrimidine nucleotides (ATP = 86.5 $\pm$ 2%, ADP = 68.6 $\pm$ 5%, 2-MeS ATP = 92.1 $\pm$ 1%; UTP = 59.2 $\pm$ 3% and UDP = 50.0 $\pm$ 4% inhibition, $n = 3$).
Figure 3.13. Effect of serum depletion on nucleotide-stimulated TXA$_2$ release from astrocytes. Cultures were maintained in culture medium for 21 days in vitro. This culture medium was then changed to one that was devoid of foetal calf serum, and the cultures maintained for a further 4 days in vitro. Cultures were then incubated for 15 min with nucleotides at 250 µM except 2-MeSATP which was at 5 µM. The release of TXA$_2$ was calculated as a percentage of that evoked by each nucleotide in serum-containing culture medium. Results are means ± SEM from at least three determinations.
3.3.9. The effect of purine and pyrimidine stimulation on $[^3\text{H}]$ inositol phosphate accumulation.

Dose-response relationships for purine- and pyrimidine-stimulated $[^3\text{H}]$IP accumulations were examined and the results are shown in Figure 3.14. ATP and ADP evoked the largest concentrations of $[^3\text{H}]$IPs, the maximum responses elicited were essentially the same as were their EC$_{50}$ values (ATP, 7 $\mu$M; ADP, 10 $\mu$M). However, 2-MeSATP was only 60% as effective as ATP in promoting PI breakdown, although it was considerably more potent. UTP and the purine, ITP also stimulated $[^3\text{H}]$IP formation in these cells. In both cases the maximum response elicited was approximately 50% of that induced by ATP. UTP was found to be slightly more potent (EC$_{50} = 2 \mu$M) than ATP whereas the EC$_{50}$ for ITP was in excess of 100 $\mu$M.

Accumulations of $[^3\text{H}]$IPs in response to maximally effective agonist concentrations used either alone or in combination are shown in Figure 3.15. Once again, ATP and ADP were found to be the most effective agonists, with UTP and 2-MeSATP being only half as effective. A combined addition of ATP and ADP produced an accumulation of $[^3\text{H}]$IPs which was essentially the same as that evoked by either agonist alone. Incubations with UTP in combination with either ATP or ADP gave contrasting results in that $[^3\text{H}]$IP accumulation following the addition of ADP and UTP together was 92% of that predicted for a fully additive response whereas that for ATP and UTP together was just 76% of the predicted value. Combined additions of 2-MeSATP plus both ATP and UTP were also tested. In the presence of 2-MeSATP, ATP-stimulated $[^3\text{H}]$IP accumulation was reduced to a level below that elicited by either agonist alone. In contrast, when UTP and 2-MeSATP were added together their effects were almost completely additive (80% of the predicted response).
Figure 3.14. Dose-response relationships for nucleotide-stimulated [³H] inositol phosphate accumulation in astrocytes. The response by each agonist concentration used is expressed as a percentage of the response to a maximally effective dose (250 μM) of ATP, and are means (SEM <12%) of 3-5 determinations. The following agonists were used: ATP (●), ADP (○), 2-MeSATP (■), UTP (□) and ITP (▲).
% MAX. ATP RESPONSE

LOG (M) AGONIST
Figure 3.15. The effect of combined nucleotide additions on $[^{3}\text{H}]$ inositol phosphate accumulation in astrocytes. Cultures were incubated with ATP, ADP, UTP (all at 250 μM) and 2-MeSATP (10 μM) either alone or in combination. Results are means ± SEM from 3-5 determinations and are expressed as a percent stimulation over basal (260 ± 38 dpm, n=5) $[^{3}\text{H}]$ inositol phosphate accumulation.
% STIMULATION

ATP
ADP
UTP
2-MeSATP
ATP + ADP
ATP + UTP
ADP + UTP
ATP + 2-MeSATP
UTP + 2-MeSATP
2-MeSATP could also be acting as a partial agonist at the P2Y receptor.
3.4. Discussion.

The results presented in the chapter have suggested that astrocytes in culture possess both P2Y and P2U purinoceptors, activation of which leads to PI turnover, an increase in $[Ca^{2+}]_i$ and TXA$_2$ release. These findings are in accord with previous reports from studies on astrocytes (Bruner and Murphy, 1990, 1993a; Kastritsis et al, 1992). Furthermore, novel evidence has been presented to show that these, or separate classes of nucleotide receptors which are present on these cells respond similarly to a range of other purine and pyrimidine nucleotides. However, in the absence of specific competitive antagonists for nucleotide/purinoceptors, it is difficult to comment with any certainty on the subclass(es) of receptors involved in the responses described within this chapter.

In terms of nucleotide-stimulated TXA$_2$ release, the P2Y selective agonist 2-MeSATP was the most potent agonist examined, with ATP, ADP, UTP and UDP being at least an order of magnitude less potent. This is in accord with the agonist potency order for the adenine nucleotides in terms of eicosanoid release from astrocytes as reported by Bruner and Murphy (1990b, 1993a, b). The finding that in addition to the adenine tri- and diphosphate nucleotides, both 2-MeSATP and UTP are capable of eliciting TXA$_2$ release suggests that eicosanoid release from astrocytes may be coupled to more than one type of P2 purinoceptor. However, 2-MeSATP is consistently less effective than either ATP or UTP in stimulating TXA$_2$ release from these cells, which might suggest that the P2U subtype is predominant in terms of eicosanoid release from astrocytes. Moreover, not only is 2-MeSATP effective in its own right, but when it is used in combination with ATP (but not UTP or any other nucleotide examined), it reduced the effect of ATP, providing more evidence that ATP may be liganded to both P2Y and P2U purinoceptors. Thus, as has been the suggestion in other tissues (O'Connor et al,
1991; Yu and Ferrier, 1993), astrocytes in culture may possess either P2Y, P2U or a mixed population of both subtypes.

ATP, ADP, 2-MeSATP and UTP all stimulated dose-dependent accumulations in 
$[^{3}\text{H}]$IPs, with 2-MeSATP being the most potent, but least effective nucleotide. Similarly, although slightly more potent than ATP, UTP was about 50% less effective than either ATP or ADP in stimulating $[^{3}\text{H}]$IP accumulation. The results with 2-MeSATP contrast with those reported by Kastritsis et al (1992) who found this agonist to be equally as effective ATP in stimulating $[^{3}\text{H}]$IP accumulation in astrocytes. This disparity might be explained by the ATP dose ranges used in that the maximum concentration of ATP used by these authors was 10 $\mu$M which, in this chapter, was equivalent to the EC$_{50}$ value. Studies on a number of other cell types have shown that ATP concentrations in the high micromolar range are required to elicit maximal PI breakdown (Huang and Sun, 1988; Munshi et al, 1993; Murrin and Boarder, 1992; Pavenstadt et al, 1992; Sato et al, 1992).

A combined addition of ATP and ADP produced an accumulation of $[^{3}\text{H}]$IPs which was essentially the same as that evoked by either agonist alone. This suggests that ATP and ADP act on the same class of purinoceptor, which is in agreement with the report by Pearce et al (1989). Incubations with UTP in combination with either ATP or ADP gave contrasting results in that $[^{3}\text{H}]$IP accumulation for ADP and UTP was 92% of that predicted for a fully additive response, whereas that for ATP and UTP was just 76% of the predicted value. This suggests that ADP and UTP act on distinct classes of purinoceptor. However, the fact that UTP did not fully antagonise the effect of ATP, as has been shown in PC12 cells (Murrin and Boarder, 1992), but produced partial additivity once again suggests a mixed population of receptors but with ATP being recognised by both. In this respect, it is interesting to note the existence of a novel 'PC12' purinergic receptor has recently been proposed which appears functionally
distinct from other P2 purinoceptors (Kim and Rabin, 1994). Moreover, the effects of 2-MeSATP and UTP on astrocyte \(^{3}\text{H}\)IP accumulation were almost completely additive. This result is in agreement with Purkiss et al (1994), who reported a similar result in aortic endothelial cells. These two nucleotide agonists were not additive in terms of TXA_2 release from astrocytes, which is not surprising in that it might be expected that different receptors are coupled to discrete PI pools, whereas it seems unlikely that they are linked similarly to cyclooxygenase.

The putative P2 antagonist suramin reduced the ability of all purine and pyrimidine nucleotides examined to stimulate TXA_2 release. However, ADP and UDP appeared more resistant to this antagonism than ATP, UTP and 2-MeSATP. Similarly, the effect of all nucleotides examined were antagonised by reactive blue 2, another putative P2 antagonist. This is in accord with previous reports which have shown that both ATP and UTP are antagonised by both of these compounds (Bailey and Hourani, 1994; Pfeilschifter, 1990; von Kugelgen et al, 1990). Hourani and Chown (1989) have reported that suramin can act as an endonucleotidase inhibitor, and have suggested that it may preferentially interfere with the degradation of adenine, rather than uridine nucleotides. However, the data presented in this chapter suggests that adenine and uridine nucleotides were affected equally by suramin, and indeed 2-MeSATP was antagonised by the greatest extent by suramin. Interestingly, ADP and UDP appeared most susceptible to antagonism by reactive blue. This raises some interesting possibilities regarding P2 purinoceptor identity. In this chapter, nucleotide triphosphate agonists were antagonised principally by suramin, whereas the diphosphate species were antagonised more effectively by reactive blue 2. It may be that reactive blue 2 is a stronger antagonist at a receptor subclass at which nucleotide diphosphate agonists specifically interact, such as the nucleotide receptor on C6 glioma cells which appears to be preferentially activated by UDP that has been reported by Lazarowski and Harden (1994). Furthermore, differences between ADP and ATP in terms of the Ca^{2+}
transients they induce in rat hepatocytes has been reported (Dixon et al, 1990; Green et al, 1994), and these authors argue that these nucleotides do not act via a single purinoceptor. Platelets express a subclass of purinoceptor (P2T) which is not only responsive solely to ADP, but is antagonised by ATP (Greco et al, 1992; Sage et al, 1990). Moreover, evidence now exists which suggests that ADP-activated P2T receptors are more widely distributed than platelets, and have been reported to occur in cultured hematopoietic cell lines (Murgo et al, 1994). However, antagonism between ATP and ADP was not observed in the results presented in this chapter.

It has been shown previously that agents which increase cyclic AMP levels are capable of markedly attenuating ATP-stimulated eicosanoid release from astrocytes (Gebicke-Haerter et al, 1991). We were interested, therefore, to determine whether there was any differential sensitivity to adenylyl cyclase activation with regard to the ability of the various nucleotides to promote TXA₂ release. At a concentration of forskolin (10 μM) which ought to maximally activate adenylyl cyclase (Seamon et al, 1981), the responses to ATP, ADP and UDP where reduced by approximately 25%, whereas that of 2-MeSATP was largely unaffected. The effect of UTP was reduced by 50% under the same conditions, suggesting that P2U receptors may be more sensitive to raised intracellular cyclic AMP levels than the P2Y subtype. However, if ATP is equally as potent as UTP at the P2U subtype, then this explanation doesn’t really explain the discrepancy. Forskolin has been shown to inhibit a number of membrane transport proteins and channel proteins through a mechanism that does not involve the production of cyclic AMP (Laurenza et al, 1989), and it may be possible that forskolin is interfering with the binding of UTP, but not ATP, to its receptor, or even inhibiting UTP at a specific pyrimidinoceptor at which ATP does not bind. The results presented in this chapter conflict with those reported by Gebicke-Haerter et al, (1991) in that nucleotide-evoked TXA₂ release was largely unaffected by forskolin. However, it should be noted that these authors examined a different species of eicosanoid to TXA₂,
and it could be that cyclic AMP selectively modifies the synthesis of some eicosanoids but not others. Nevertheless, these results indicate that an inhibition of adenylyl cyclase is not a requirement for P2-purinoceptor-evoked eicosanoid release from astrocytes.

The precursor for eicosanoid synthesis is believed to be arachidonic acid which can be liberated from membrane phospholipids by phospholipases. It appears that in astrocytes PLA$_2$, but not PLC or PLD, is involved in this process (Keller et al., 1987; Bruner and Murphy, 1990a; Pearce et al., 1987). There are a number of isozymes of PLA$_2$ which can be broadly divided into those which are located in the cytoplasm or are secreted from the cell (Dennis, 1994; Glaser et al., 1993). The brain contains significant PLA$_2$ activity (Witter and Kanfer, 1985), and immunocytochemical studies on intact tissue and cultured astrocytes have shown that both forms of the enzyme are associated with these cells (Oka and Harita, 1991; Stephenson et al., 1994). Given the previous report that P2Y purinoceptors on astrocytes are directly coupled to PLA$_2$ (Bruner and Murphy, 1993b), we set out to determine whether nucleotides selectively activated one or the other form of PLA$_2$. ONO-RS-082 was found to inhibit TXA$_2$ release in a dose-dependent manner for all nucleotide agonists examined, with 35 µM ONO-RS-082 virtually abolishing TXA$_2$ release from the cells. This suggests that stimulation of either receptor subtype (P2Y and/or P2U) in astrocytes results in the activation of the cytosolic form of PLA$_2$. This finding might have implications for the physiology of reactive astrocytes, which are seen after any form of brain insult. Increases in PLA$_2$ activity and free fatty acid levels have been observed in several forms of brain injury. Evidence exists for PLA$_2$ activation following brain damage induced by free radicals and cerebral ischaemia (Chan et al., 1982; Kempski et al., 1987; Rordorf et al., 1991). Astrocytes containing cytosolic PLA$_2$ may, therefore, be ideally situated to react to inflammatory insult, and to this end are known to produce cytokines and growth factors.
Serum depletion of astrocyte cultures prior to their use resulted in a reduction in nucleotide-stimulated TXA$_2$ release. This regime also reduced basal TXA$_2$ release. ATP and 2-MeSATP were affected to the greatest extent, with the release evoked by UTP, UDP and ADP being affected less. Since serum contains a variety of hormones and growth factors necessary for cell survival in vitro, it could be argued that these results are simply an expression of cell death. However, experiments conducted in this laboratory using a measurement of lactate dehydrogenase have confirmed that cell death is minimal against those cells constantly exposed to serum (Amin and Pearce, unpublished observations). An alternative proposal is that serum depletion results in a down-regulation of the inducible form of COX (COX-II), and TXA$_2$ evoked by nucleotides under these conditions is derived from the constitutive form of COX (COX-1). COX-II activity is known to be dramatically increased by serum in 3T3 fibroblasts (DeWitt et al, 1991). If this hypothesis is correct, then it appears that COX-II is the predominant isozyme under normal culture conditions. Moreover, maintenance of cerebellar astrocytes in serum-free media results in a reduction of COX-II immunoreactivity, whereas that of COX-1 was unaffected (Marriott et al, 1995). Furthermore, this regime may result in microglial death, and it necessarily follows that these contribute significantly to TXA$_2$ release from astrocyte cultures. Specifically, under conditions of injury, the microglia may release factors such as cytokines which up-regulate the expression of COX-II activity in astrocytes. To this end, interleukin-1 has been shown to induce COX-II mRNA in vitro (Wu et al, 1991).

Microglial cells play important roles in mediating immune responses in the central nervous system (Thomas, 1992). Pathological conditions lead to a activation of these cells which are then thought to influence the regenerative process (Kazuyuki and Kohsaka, 1993; Streit et al, 1988). In CNS tissue culture, cells become initially activated by the isolation procedure, and are capable of both proliferation and
phagocytosis (Gebicke-Haerter et al., 1989; Rieske et al., 1989). Although microglia constitute only a small proportion (5-10%) of the total cell number, they are capable of making substantial contributions to the extracellular concentrations of neuroactive mediators such as nitric oxide (Simmons and Murphy, 1992). Interestingly, nitric oxide has been shown to interact with both forms of cyclooxygenase to cause an increase in the enzymatic activity (Salvemini et al., 1993). Moreover, P2Y purinoceptor agonists have been shown to activate rat microglia (Norenberg et al., 1994). When microglial cells were removed from astrocyte cultures by the addition of LME to the medium, the ability of all nucleotides examined to evoke TXA2 release was attenuated. In those cultures which were not treated with LME, both UTP and UDP were found to be more effective than ATP in promoting TXA2 release, however, in the presence of LME the potentiation of UTP and UDP compared to that of ATP was abolished. This reasons why uridine nucleotides should be more effective than those of adenine remain unclear, however, one possible explanation is that they are are acted upon by different ecto-nucleotidase enzymes. If the ecto-nucleotidase that acted upon uridine nucleotides had a slower rate constant than that of adenine ecto-nucleotidases, then the stepwise hydrolysis of the triphosphate to diphosphate and so on would be slower for UTP than ATP. A consequence of this is that the active tri- and diphosphate nucleotide species of uridine exist for a longer time, and may be able to stimulate more TXA2 release as a result of this. These results are contrary to the study by Bruner and Murphy (1993a), who report that both the efficacy and potency of UTP in terms of astrocyte TXA2 release was ten-fold less than the values reported in this chapter. However, the cultures used by Bruner and Murphy (1993a) were passaged and serum deprived prior to experimentation. It is possible that these treatments affected the expression/distribution of microglial P2Y and P2U purinoceptors and their signal transduction pathways. These results suggest that microglia possess purinoceptors coupled to the release of eicosanoids, and represent the first indication that both purine and pyrimidine nucleotides are capable of eliciting this response in this cell type.
We then set out to examine any correlation between nucleotide-stimulated TXA\textsubscript{2} release and changes in \([\text{Ca}^{2+}]_i\). ATP, ADP and 2-MeSATP were equally as effective in increasing \([\text{Ca}^{2+}]_i\), whereas 2-MeSATP was less effective than either ATP or ADP in stimulating TXA\textsubscript{2} release from these cells. The changes in \([\text{Ca}^{2+}]_i\) induced by UTP and particularly UDP were less than those observed with ATP, ADP or 2-MeSATP, but the uridine tri- and diphosphates were the most effective nucleotides in terms of TXA\textsubscript{2} release. It is worthy of comment that few (24%) cell fields that were challenged with UDP responded with a change in \([\text{Ca}^{2+}]_i\). The relatively small changes in \([\text{Ca}^{2+}]_i\) which were recorded are from a field of between 10 and 20 cells, and this result may simply reflect a low level of P2U purinoceptor expression within a group of astrocytes in culture, hence the overall recorded change in \([\text{Ca}^{2+}]_i\) is low. Clearly, the magnitude of the change in \([\text{Ca}^{2+}]_i\) is not a determining factor in the ability of various nucleotides to evoke TXA\textsubscript{2} release from astrocytes. Furthermore, a recent study in astrocytes has suggested that different ligands release \text{Ca}^{2+} from common stores, and that each ligand is capable of evoking maximal release (Shao and McCarthy, 1995). This may explain why the changes in \([\text{Ca}^{2+}]_i\) evoked by the various nucleotides, as presented in this chapter, were not additive.

It has been demonstrated previously that eicosanoid production in astrocytes is dependent on both the influx of \text{Ca}^{2+} across the plasma membrane from the extracellular environment and the release of \text{Ca}^{2+} from internal stores (Bruner and Murphy, 1990b; Pearce et al, 1989). Accordingly, when both sources of \text{Ca}^{2+} were essentially removed (by addition of TMB-8 to and the removal of \text{Ca}^{2+} from the bathing medium) from cells investigated in this chapter, nucleotide-evoked TXA\textsubscript{2} release was completely abolished. We were interested, therefore, to determine the relative contributions made by either of these mechanisms by the various nucleotides, and relate these to differences in TXA\textsubscript{2} release. The results presented here suggest that
the majority of TXA$_2$ release is dependent upon influx of Ca$^{2+}$ into the cell from the outside. Thus, in the absence of extracellular Ca$^{2+}$, nucleotide-evoked TXA$_2$ release was reduced to approximately 20-30% of that normally observed when Ca$^{2+}$ is present in the bathing medium. These results correspond well in terms of changes in [Ca$^{2+}$]$_i$ evoked by ATP, ADP and 2-MeSATP in the absence of extracellular Ca$^{2+}$. However, the changes in [Ca$^{2+}$]$_i$ evoked by UTP (and CTP), were reduced to around 60% in the absence of extracellular Ca$^{2+}$. This suggests that those purinoceptors which are activated by UTP are more dependent upon release of Ca$^{2+}$ from internal stores in terms of TXA$_2$ release.

The results presented in this chapter raise some important questions concerning the relationships between agonists and their potencies in stimulating the productions of IPs, increasing the [Ca$^{2+}$]$_i$ and stimulating TXA$_2$ release from astrocytes. For example, the relative potency order for 2-MeSATP is [Ca$^{2+}$]$_i$ > IP > TXA$_2$. That of UTP, however, is [Ca$^{2+}$]$_i$ = IP > TXA$_2$. The reasons for this are unknown, and may suggest differences in the coupling of P2Y and P2U purinoceptors to their effector systems. It may be possible, for example, that UTP has a higher requirement for occupancy at its receptor than 2-MeSATP, and activation of its signal transduction pathway may reflect this.

Evidence exists which suggests that nucleotides which are not based on adenine or uracil are also able to elicit a range of responses in central and peripheral cells and tissues (Bailey and Hourani, 1994; Lazarowski and Harden, 1994; Lin, 1994; Lin et al, 1993; Seifert and Schultz, 1989, Seifert et al, 1989b; Stutchfield and Cockcroft, 1990; Theobald, 1992; Vials and Burnstock, 1993). Results presented in this chapter are the first report, however, that cultured astrocytes respond to nucleotides such as GTP, TTP and CTP with an increase in both [Ca$^{2+}$]$_i$ and TXA$_2$ release. It is still not known whether these nucleotides interact with classical P2Y/P2U purinoceptors, and if their
receptors represent a distinct class from those that recognise ATP and/or UTP. Interestingly, the proportion of cells responding to the purine nucleotide GTP with an increase in \([\text{Ca}^{2+}]_i\) was similar to that responding to ATP, ADP and 2-MeSATP, whilst those responding to the pyrimidine nucleotides TTP and CTP were not dissimilar to the numbers responding to UTP. Furthermore, \(\text{Ca}^{2+}\) transients evoked by GTP were occasionally biphasic, which is a hallmark of P2Y purinoceptor activation. The P2 purinoceptor antagonist suramin inhibited TXA\(_2\) release evoked by these nucleotides. There were, however, some major differences in the responses elicited by GTP, TTP and CTP compared to those seen with ATP and UTP. For example, changes in \([\text{Ca}^{2+}]_i\) evoked by the former nucleotides were monophasic and desensitised very rapidly, and it was virtually impossible to evoke a further \(\text{Ca}^{2+}\) transient, this phenomenon was not observed with ATP or UTP. Accordingly, it is impossible to attribute these nucleotides to a receptor classification, but with advances in receptor cloning techniques, it may soon be possible to be more definite. Moreover, it still remains to be determined under what conditions nucleotides such as GTP, TTP and CTP are released from cells, and the significance of this. It is exciting to speculate, however, that they are released from astrocytes into the extracellular environment upon trauma or injury to the brain, and may play a role in the reaction of the CNS to these insults.

Since purine (and probably pyrimidine) nucleosides and nucleotides are important metabolically, cells possess efficient systems for their degradation and/or uptake into cells. Such removal mechanisms are present on virtually all cells; this is in contrast to the situation for many other neurotransmitters, where sites of loss are abundant in the nerves that use them, but virtually absent elsewhere (Fredholm et al, 1994). Adenosine is rapidly taken up and/or metabolised by transporters and enzymes, and it has been estimated that the half-life of adenosine injected into the blood stream is in the order on a second (Moser et al, 1989).
Nucleotides are also rapidly degraded, and their products taken up by cells. Some of the nucleotide analogues and their breakdown products interfere not only with receptors, but also with the removal systems (Fredholm et al, 1994). Moreover, the extent and rate to which the different purine and pyrimidine nucleotides are metabolised by ectonucleotidase enzymes remain unknown. It has been estimated that the lifetime of ATP at the purinoceptor is 50 msec, after which it is broken down to adenosine by ecto-ATPase (Cunnane and Manchanda, 1988). This is significant because astrocytes maintain close contact with other cell types of the CNS, and express both P1 and P2 purinoceptors, they are, therefore, likely targets for neuronally released purines. The fact that ATP is released in quanta (i.e. as small but massive packets) into narrow synapses is what enables ATP to escape destruction by ecto-ATPase before binding to intrajunctional purinoceptors, whether neuronal or astrocytic (Stjarne et al, 1994). Clearly these responses will depend upon the intricate balance between entry (by diffusion) and removal (by degradation and diffusion) of each nucleotide species with the microenvironment of their receptors (Gleeson et al, 1989). Cultured glial cells have been shown to possess 5'-nucleotidases (Kreutzberg et al, 1978; Lai and Wong, 1991), which suggests that these cells play a role in regulating the concentration of ATP, ADP, AMP and adenosine in the vicinity of the synaptic cleft. Although adenosine has several effects in the nervous system, it does not stimulate TXA2 release from astrocytes (Pearce et al, 1989). Our results are in agreement with this, however, we found that adenosine inhibited basal TXA2 release from these cells. It is possible that in vivo this situation exists to finely control TXA2 release. Whether distinct 5'-nucleotidases exist for the range of purine and pyrimidine nucleotides, and the consequences of this, has yet to be elucidated. Overall, these considerations are important because evidence for nucleotide receptor subtypes which is based on relative agonist potency in complex biological systems must be regarded as tentative, rather than definitive.
In conclusion, throughout this chapter I have examined and attempted to characterise the potential of various purine and pyrimidine nucleotides to evoke TXA$_2$ release from cultured astrocytes. This has involved analysis of the importance of Ca$^{2+}$ in this process, and the possible contribution of microglial cells has been discussed. Whether these results reflect the reactive state of astrocytes following CNS trauma and injury, and the possibility that cultured astrocytes are themselves reactive is still open to discussion. High micromolar concentrations of nucleotides may be released if the cell is damaged in some way (Forrester and Williams, 1977; Gordon, 1986). Once released into the extracellular environment, the various nucleotides interact with receptors, however, it is not known if these receptors are expressed constitutively on astrocytes or are induced following some form of insult to the brain. Whatever the circumstances of their release, nucleotides remain one of the few physiologically relevant signals to evoke eicosanoid release from astrocytes. In the next chapter I shall investigate why other agonists, such as NA, are not able to evoke eicosanoid release from astrocytes.
Chapter 4

Interactions between $\alpha_1$-adrenoceptors and P2 purinoceptors in the regulation of $\text{Ca}^{2+}$ mobilisation and $\text{TXA}_2$ release
4.1. Introduction.

In the previous chapter, I have discussed the characterisation of nucleotide-stimulated TXA$_2$ release from astrocytes, and assessed the role of P2 purinoceptors in this process. Astrocytes are also known to possess adrenoceptors (Hosli and Hosli, 1982; McCarthy and De Vellis, 1978; Ruck et al, 1991; Salm and McCarthy, 1989), and stimulation of the $\alpha_1$ subtype by NA results in the production of IPs (Pearce et al, 1985, 1986; Wilson and Minneman, 1991; Wilson et al, 1990). Furthermore, NA has been shown to increase [Ca$^{2+}$]$_i$ in astrocytes (Dave et al, 1991; Fatatis et al, 1994; Inagaki et al, 1991; McCarthy and Salm, 1991; Salm and McCarthy, 1990; Shao and McCarthy, 1993). Despite the fact that stimulation of both P2 purinoceptors and $\alpha_1$-adrenoceptors results in an increase in [Ca$^{2+}$]$_i$ and turnover of inositol phospholipids, activation of the latter class of receptor fails to evoke eicosanoid release from astrocytes.

Precisely why P2, but not $\alpha_1$, receptor activation stimulates eicosanoid production and release from astrocytes has yet to be elucidated. One hypothesis that has been proposed is that P2Y receptors are coupled to G proteins in a unique way. Receptors which stimulate eicosanoid release from cells are all coupled to G proteins; moreover, receptors at which eicosanoids themselves act are also G protein linked (Smith, 1989). Gebicke-Haerter et al (1991) have shown that when astrocytes are incubated with pertussis toxin, ATP-evoked mobilisation of AA is abolished. Bruner and Murphy (1993b) have reported that ATP-evoked TXA$_2$ release and PI hydrolysis are inhibited by pertussis toxin in a concentration-dependent fashion. However, the concentration of toxin necessary to inhibit PI hydrolysis was 50 times greater than that required to inhibit eicosanoid release. It has been suggested that in addition to having a G protein
coupling to PLC, P2Y receptors on astrocytes are also directly coupled to PLA₂ via another G protein (Bruner et al, 1993). The proposal is that although other agonists activate PLC, and hence increase IP₃ and [Ca²⁺]ᵢ, they fail to evoke eicosanoid release because they do not stimulate G protein coupling to PLA₂. Although an interesting concept, it has yet to be proved unequivocally.

4.1.1. Modulation of ATP-mediated eicosanoid release by NA.

Despite its inability to evoke eicosanoid release from astrocytes, NA is capable of inhibiting ATP-induced eicosanoid release from these cells (Pearce et al, 1989). The co-existence of receptors and modulation of their second messengers is not uncommon in astrocytes (Hannson, 1989). The actions of ATP and NA with respect to IP accumulation are additive, indicating the two receptors access distinct PI pools; however, their combined ability to increase Ca²⁺ efflux is not additive (Pearce et al, 1989), implying the two receptors mobilise Ca²⁺ from a common store.

An interaction between ATP and NA is not unique to astrocytes; indeed, ATP has been shown to inhibit effects mediated by NA in other tissues (Allgaier et al, 1994; Torodov et al, 1994; von Kugelgen et al, 1994). In rat brain cortex, for example, ATP and 2-MeSATP decreased electrically stimulated release of [³H]-NA, whereas α,β-methylene-ATP (a P2X purinoceptor agonist) caused no change (von Kugelgen et al, 1994). This latter class of purinoceptor is believed to be a ligand-gated cation channel, and is thought to mediate fast synaptic transmission of ATP at synapses (Evans et al, 1992). Another P2X agonist, β,γ-methylene-ATP, appears to act as a partial agonist with respect to the stimulation of IPs in astrocytes (Kastritis et al, 1992), but does not stimulate eicosanoid release from these cells (Bruner and Murphy, 1990b).
In addition, it has been shown that 5-HT similarly inhibits ATP-induced eicosanoid release from astrocytes (Murphy and Welk, 1990). The inhibition of ATP by 5-HT could be reversed by methysergide (a 5-HT antagonist), suggesting the direct involvement of 5-HT receptors. In this study, the accumulation of IPs due to ATP were elevated in the presence of 5-HT in an apparently additive manner. This additive effect could also be reversed by methysergide. The mechanism by which 5-HT inhibits ATP-induced eicosanoid release does not, therefore, appear to involve the purinergic receptor or its coupling to inositol phospholipid pools. Bruner et al (1993) have suggested that 5-HT receptors may be negatively coupled to PLA₂, or that some other cell-signalling event is altered when astrocytes are stimulated with 5-HT.

Although adenosine (P1) receptors are present on astrocytes (Hosli and Hosli, 1988), their activation does not stimulate the formation of IPs nor evoke eicosanoid release (El-Etr et al, 1989; Pearce et al, 1989). Despite its inactivity, 2-chloroadenosine has been shown to increase formation of IPs which were induced by α₁-adrenoceptor agonists in mouse striatal astrocytes (El-Etr et al, 1989). Furthermore, α₁-adrenergic receptor-mediated increases in [Ca²⁺]ᵢ were sustained when striatal astrocytes are stimulated in the presence of adenosine agonists (Delumeau et al, 1991). This suggests that α₁-adrenoceptor-mediated increases in [Ca²⁺]ᵢ alone are not sufficient to stimulate eicosanoid release from astrocytes.

Marin et al (1991) have shown the peptide, somatostatin, enhances the ability of methoxamine, an α₁-adrenoceptor agonist, to stimulate the formation of IPs in striatal mouse astrocytes. Pre-treatment of the cells with pertussis toxin totally suppressed the ability of somatostatin to potentiate IP formation, whereas, the α₁-adrenergic-mediated response remained unchanged. Mepacrine, an inhibitor of PLA₂, slightly inhibited the accumulation of IPs induced by methoxamine alone, but completely suppressed the potentiating effect of somatostatin. Furthermore, neither somatostatin nor α₁-
adrenergic agonists alone stimulate AA release from these cells, but co-application significantly increases release. These authors propose that somatostatin stimulates a G protein coupling to PLA₂, and that α₁-adrenoceptor activation leads to an increase in \([\text{Ca}^{2+}]_i\) and activation of protein kinase C. Stimulation of each pathway alone may not be sufficient to fully activate PLA₂ but, together, might provide the appropriate stimuli required for maximum enzyme activation.

In an attempt to identify potential differences in the source and characteristics of the changes in \([\text{Ca}^{2+}]_i\) induced by ATP and NA, and thereby explain differences with respect to eicosanoid release, experiments were carried out which examined agonist-induced \(\text{Ca}^{2+}\) transients in astrocytes in detail. Specifically, whether these two agonists compete for the same source of intracellular \(\text{Ca}^{2+}\), if when chronically applied to cells, differences in the characteristics of \(\text{Ca}^{2+}\) transients evoked by ATP and NA could be detected, and to investigate and characterise any effect of NA on TXA₂ release induced by different nucleotide agonists. Overall, the hypothesis being that some qualitative or quantitative difference in the way ATP and NA increase the \([\text{Ca}^{2+}]_i\) might exist, which may then explain why the latter does not stimulate eicosanoid release from astrocytes.

4.2. Methods and Materials.

All methods were as described in Chapter 2. Stock solutions of drugs were freshly prepared in distilled water (except prazosin which was dissolved in acidified methanol) and stored in the dark at 4°C. All drugs were obtained from Sigma, except 2-MeSATP, which was purchased from Research Biochemicals Inc., Natick, USA.
4.3. Results.

4.3.1. Effect of acute exposure of ATP and NA on [Ca\(^{2+}\)]\(_i\).

Astrocyte cultures were challenged with ATP and then NA for 30 s over the concentration range 0.1-10 \(\mu\)M, and the change in [Ca\(^{2+}\)]\(_i\) assessed. Experiments were performed on cells derived from a number of different cultures and of the 76 cell fields examined, the majority (>95%) were found to respond to ATP with an increase in [Ca\(^{2+}\)]\(_i\). This is in accordance with previously published observations (McCarthy and Salm, 1991). When 39 cell fields were similarly challenged with NA, 31 (79%) were found to respond with an increase in [Ca\(^{2+}\)]\(_i\). The concentration dependence of ATP-evoked changes in [Ca\(^{2+}\)]\(_i\) has been presented in the previous chapter (Figure 3.2). The change in [Ca\(^{2+}\)]\(_i\) due to NA are also concentration-dependent, and these results are shown in Figure 4.1.

Typical responses to 10 \(\mu\)M ATP and NA in the presence and absence of extracellular Ca\(^{2+}\) are shown in Figure 4.2. When Ca\(^{2+}\) was present in the buffer, both agonist-induced Ca\(^{2+}\) transients were strikingly similar in that they were biphasic comprising a rapid increase in [Ca\(^{2+}\)]\(_i\) that decayed within 10 s to a plateau of Ca\(^{2+}\) which was sustained for the duration of the agonist challenge. This plateau phase represents approximately 30-40% of the peak change in [Ca\(^{2+}\)]\(_i\). Small oscillations in [Ca\(^{2+}\)]\(_i\) were noted during this phase but there were no apparent differences in their frequency. Once either agonist was removed, the [Ca\(^{2+}\)]\(_i\) returned immediately to pre-stimulation levels.

It is widely accepted that the characteristic biphasic Ca\(^{2+}\) transient induced by many agonists linked to IP\(_3\) sensitive Ca\(^{2+}\) pools involves release of Ca\(^{2+}\) from intracellular stores, and an influx of Ca\(^{2+}\) into the cell from the extracellular environment (Berridge, 1993a). To investigate whether there were any differences in the source of Ca\(^{2+}\)
Figure 4.1. Representative changes in $[Ca^{2+}]_i$ with increasing concentrations of NA. The bars above the trace indicate the period (30 sec) over which the cells were exposed to the drug. This experiment was performed on a single field of cells and is representative of similar experiments carried out on at least 8 fields from different batches of cultures.
[Ca^{2+}]_i (μM)

0 0.5 1.0 1.5

0.1 1.0 10.0

time (seconds)

500

131
Figure 4.2. ATP and NA-stimulated changes in [Ca^{2+}]_i in astrocytes bathed in Ca^{2+}-containing and Ca^{2+}-free buffers. Cultures were challenged for 30 sec (indicated by the solid bar) with either ATP (10 μM) or NA (10 μM) in Ca^{2+}-containing buffer to establish a control response. The buffer was then changed to one containing zero CaCl_2 plus 0.2 mM EGTA (indicated by the open bar) and superfusion of the cells continued for 10 min before challenging them again with either ATP or NA. These traces are representative of a number of experiments carried out on cultures from different preparations.
mobilised by ATP or NA, the bathing media was switched to one devoid of Ca^{2+} and containing 0.2 mM EGTA, and the cells washed in this buffer for 10 min. Cells from which control responses had previously been obtained with ATP or NA in Ca^{2+} containing buffer, were then challenged with either agonist in Ca^{2+}-free buffer, and typical results are shown in the latter portion of Figure 4.2. ATP-induced changes in [Ca^{2+}]_i were still observed when Ca^{2+} was removed from the buffer, however, the extent of change in [Ca^{2+}]_i was much reduced and the characteristic biphasic response obtained when extracellular Ca^{2+} was present in the buffer was no longer seen. NA evoked increases in [Ca^{2+}]_i obtained in Ca^{2+}-free buffer were also markedly attenuated, and the characteristics of the Ca^{2+} transient similarly altered in appearance. Overall, there did not appear to be any discernible difference in responses obtained with either ATP or NA with respect to changes in [Ca^{2+}]_i.

4.3.2. Effect of chronic exposure of ATP and NA on [Ca^{2+}]_i.

During experiments where TXA_2 release evoked by various stimuli were determined, cells were exposed to agonists for 15 min. We were interested, therefore, to investigate whether differences in the Ca^{2+} transients obtained with ATP or NA differed over a similar time course. Thus, astrocytes in culture were challenged with ATP and NA for 10 min, and the resulting intracellular Ca^{2+} transients assessed. Representative responses are illustrated in Figure 4.3a and b. The agonist-induced changes in [Ca^{2+}]_i obtained with 10 μM ATP and NA are similar in profile. Both responses were once again biphasic, with the secondary component of the response being maintained throughout the drug exposure period. No repetitive spike-like Ca^{2+} transients were observed, nor were there any obvious differences in the mean level of oscillation or characteristics of the plateau phase induced by either agonist, this component
Figure 4.3. Representative traces showing the effect of chronic challenge of 10 μM ATP (A) and 10 μM NA (B) on [Ca^{2+}]_{i}. Cells were challenged with an agonist over a period of approximately 12 min, and the change in [Ca^{2+}]_{i} recorded. This experiment was performed on a single field of cells and is representative of similar experiments carried out on at least 7 fields from different batches of cultures.
This experiment was designed to test whether ATP and NA mobilised Ca\(^{2+}\) from the same or different pools of Ca\(^{2+}\).
representing about 30% of the initial change in \([Ca^{2+}]_i\). Again, the \([Ca^{2+}]_i\) returns to pre-stimulation levels when the cells are returned to drug free buffer.

4.3.3. The effect of additive doses of ATP and NA on \([Ca^{2+}]_i\).

We were interested to see whether the change in \([Ca^{2+}]_i\) induced by ATP and NA was additive or not. The hypothesis under investigation is that competition for available intracellular \(Ca^{2+}\) may explain why NA inhibits the ability of ATP to evoke eicosanoid release. Alternatively, these two agonists mobilise \(Ca^{2+}\) from discrete pools of intracellular \(Ca^{2+}\), hence the change in \([Ca^{2+}]_i\) would be additive. Accordingly, cells were challenged with a combination of concentrations of ATP and NA. Figure 4.4 is representative of results obtained from these experiments, and shows intracellular \(Ca^{2+}\) transients evoked by 10 \(\mu\)M ATP ± incremental doses of NA. A control response to 10 \(\mu\)M ATP and NA were initially obtained. Combined challenges of ATP and incremental doses of NA did not evoke \(Ca^{2+}\) transients which were additive.

4.3.4. The effect of NA on nucleotide-evoked TXA2 release from astrocytes.

It has been reported previously that although activation of \(\alpha_1\)-adrenoceptors in astrocytes results in PI hydrolysis and increase in \([Ca^{2+}]_i\), it does not stimulate the release of eicosanoids (Pearce and Murphy, 1988; Pearce et al, 1989). Moreover, it has also been shown that when NA and ATP are incubated in combination, the release of TXA2 (evoked by ATP) is reduced (Pearce et al, 1989). We were interested in characterising this response in more detail, and determining whether NA was likewise capable of inhibiting TXA2 release evoked by other purine and pyrimidine nucleotides.
Figure 4.4. Representative traces showing the effect of NA on ATP-evoked changes in $[Ca^{2+}]_i$. Cells were challenged with ATP and NA (10 μM) to establish control responses. They were then challenged with the same concentration of ATP in the presence of incremental doses of NA, and the change in $[Ca^{2+}]_i$ recorded. This experiment was performed on a single field of cells and is representative of similar experiments carried out on at least 6 fields from different batches of cultures. (a = 10 μM ATP, b = 10 μM NA, c = 10 μM ATP + 0.1 μM NA, d = 10 μM ATP + 1.0 μM NA, e = 10 μM ATP + 10 μM NA, f = 10 μM ATP).
These results are illustrated in Figure 4.5, and show the inhibition of nucleotide-evoked TXA₂ release by incremental concentrations of NA. Despite not stimulating TXA₂ release alone (116 ± 21%, n=6, 100 μM), NA reduced TXA₂ release induced by all the nucleotides examined (ATP, ADP, 2-MeSATP, UTP, and UDP). This effect was evident at concentrations of NA as low as 0.1 μM (the lowest concentration used), and as the concentration of NA was increased, then the extent of the inhibition of TXA₂ release increased concomitantly. In terms of the effectiveness of the inhibition by NA of nucleotide-stimulated TXA₂ release, the effect of 2-MeSATP was inhibited to the greatest extent (IC₅₀ of NA <0.1 μM). IC₅₀ values for the NA-mediated inhibition of TXA₂ release for the other nucleotides were ATP (0.5 μM), ADP (0.4 μM), UTP (0.6 μM) and UDP (2.0 μM).

Figure 4.6 illustrates that although NA does not stimulate TXA₂ release from astrocytes, it increases the [Ca²⁺]ᵢ in those cells in which nucleotides such as ATP, ADP and UTP are also able to evoke Ca²⁺ transients. Note that the change in [Ca²⁺]ᵢ evoked by adenosine is characteristically different from those induced by ATP, ADP, UTP or NA.

4.3.5. Identification of the adrenoceptor subtype involved in the inhibition of nucleotide-evoked TXA₂ release.

NA acts at adrenoceptors, but the subtype involved in the inhibition of TXA₂ release reported in the previous section has not been identified. Consequently, we set out to determine which subtype of adrenoceptor is involved in the response, and the results obtained with ATP and UTP are shown in Figure 4.7. Control responses were obtained with ATP in the absence and then presence of a previously determined inhibitory dose of NA, the latter treatment resulting in an 80% reduction of ATP-
Figure 4.5. Effect of NA on nucleotide-stimulated TXA$_2$ release from astrocytes. Cultures were incubated with nucleotides at a concentration of 250 μM, except 2-MeSATP which was at 5 μM, in the presence of various concentrations of NA which was added to the cultures 10 min prior to nucleotide challenge. Nucleotide-stimulated release of TXA$_2$ was calculated as a percentage over basal release. The effect of nucleotides in the presence of NA were then expressed as a percentage of the response evoked by the nucleotide alone. Results are means ± SEM from at least four determinations. NA alone did not stimulate TXA$_2$ release. The nucleotides used were ATP (●), ADP (○) 2-MeSATP (■), UTP (▲) and UDP (△).
Figure 4.6. Representative changes in $[\text{Ca}^{2+}]_i$ recorded from the same field of cells evoked by NA and various nucleotides. All agonists were used at a concentration of 10 μM. The bars above the trace indicate the period (30 sec) over which the cells were exposed to the nucleotide. This experiment was performed on a single field of cells and is representative of similar experiments carried out on at least 43 fields from different batches of cultures.
Figure 4.7. Effect of adrenoceptor antagonists on ATP and UTP-stimulated TXA$_2$ release. Cultures were stimulated for 15 min with ATP or UTP (250 μM) ± NA (100 μM) in the presence of adrenoceptor antagonists (1 μM). Antagonists were added to cultures 10 min prior to the addition of the nucleotide. The effects of the nucleotide alone were calculated as a percentage over basal and were taken as the control responses. The effects of nucleotides in the presence of NA + antagonists were then expressed as a percentage of these values. The results are means ± SEM from a least three determinations.

Prazosin can also act as an antagonist at α$_{2B}$ adrenoceptors.
evoked TXA2 release. The effect of 1 μM prazosin and propanolol on NA-mediated inhibition of TXA2 were then examined. This regime was similarly repeated with UTP. Antagonism of α1-adrenoceptors by prazosin affected NA-mediated inhibition of both ATP and UTP, with approximately 60% of control release evoked by ATP being restored. Propanolol, a non-selective β-adrenoceptor antagonist, did not affect the NA-mediated TXA2 inhibition seen with either ATP or UTP.

4.4. Discussion

The results presented in this thesis show that when astrocytes are challenged with either ATP or NA, a concentration-dependent increase in the [Ca^{2+}]_i is observed. However, only ATP is able to stimulate eicosanoid release from these cells. Of those cells challenged with NA, 79% responded with an increase in [Ca^{2+}]_i, which indicates that not all astrocytes in culture possess α1-adrenoceptors and is in agreement with other studies in these cells (Salm and McCarthy, 1990; McCarthy and Salm, 1991). It may be possible that the reason NA does not evoke eicosanoid release from astrocytes is because cells that express α1-adrenoceptors do not synthesise these agents. This proposal seems unlikely since these results also demonstrate that >95% of astrocytes in culture express P2 purinoceptors, and hence are capable of synthesising eicosanoids. Similar results with acetylcholine (ACh) have been reported in a glioma cell line, which by nature are a homogenous population of cells (DeGeorge et al, 1986a). Moreover, Figure 4.6 illustrates that P2 purinoceptor and α1-adrenoceptors can be co-localised within the same field of cells.

Both agonist-induced Ca^{2+} transients were similar in that there was a short delay from exposure of cells to either drug and an increase in [Ca^{2+}]_i, which was characteristically biphasic. It has been suggested that this short delay of onset of response is due to the
time taken by IP$_3$ to bind to its receptors (Berridge and Irvine, 1989). In the absence of extracellular Ca$^{2+}$, an increase in [Ca$^{2+}$]$_i$ was still observed when cells were exposed to either agonist; however, both responses were much attenuated and monophasic. This suggests that despite activating different receptors, ATP and NA induce both the entry of Ca$^{2+}$ into the cell from the extracellular environment (presumably a channel-mediated entry), and release from intracellular stores. Since the absence of extracellular Ca$^{2+}$ does not result in complete abolition of Ca$^{2+}$ transients, it appears that a component of the response is derived from internal stores of Ca$^{2+}$. The exact nature and location of these stores in astrocytes remains to be elucidated, but it is generally accepted that receptor activation involves release of Ca$^{2+}$ following binding of IP$_3$ to receptors localised in intracellular Ca$^{2+}$ stores, probably in the endoplasmic reticulum (Berridge, 1993a; Shuttleworth, 1994; Tsien and Tsien, 1990). The mechanism by which store depletion can control plasma membrane Ca$^{2+}$ permeability remains largely unknown. However, an organelle-derived Ca$^{2+}$-influx factor has been found in 1321N1 astrocytoma cells (Randriamampita and Tsien, 1993).

Neary et al (1988) have reported ATP-stimulated Ca$^{2+}$ influx into cultured rat astrocytes and postulated that a ‘purinergic receptor-operated Ca$^{2+}$ channel’ exists in astroglia. However, contrary to this finding, Magoski and Walz (1992) have reported the existence of a highly sensitive ATP-activated Na$^+$ and K$^+$ cation channel in astrocytes which does not gate Ca$^{2+}$. Nevertheless, it is interesting to speculate that activation of an ‘ATP’ Ca$^{2+}$ channel could perhaps be necessary for stimulating eicosanoid release from astrocytes, and may explain why other agonists are ineffective in this respect. However, this hypothesis does not explain how Substance P and bradykinin could stimulate eicosanoid release from astrocytes (Marriott et al 1991; Marriott and Wilkin, 1993).
It has been postulated that since eicosanoid release is Ca\(^{2+}\) dependent (Bruner and Murphy, 1990b; 1993b; Keller et al, 1987; Murphy et al, 1985), NA may be unable to increase the [Ca\(^{2+}\)]\(_i\) to a level sufficient to activate PLA\(_2\) and hence evoke TXA\(_2\) release (Pearce et al, 1989). Here, we report that increases in [Ca\(^{2+}\)]\(_i\) evoked by either ATP or NA are of the same order of magnitude, so Ca\(^{2+}\) mobilisation \textit{per se} is not sufficient to stimulate eicosanoid release from astrocytes. Moreover, a previous report has shown that when astrocytes are treated with pertussis toxin, ATP-stimulated TXA\(_2\) release is abolished, even though the change in [Ca\(^{2+}\)]\(_i\) evoked by ATP remains unchanged (Bruner and Murphy, 1993b). Pertussis toxin, however, does not affect Ca\(^{2+}\) transients which are evoked by NA in astrocytes (Fatatis et al, 1994).

The nature of many Ca\(^{2+}\) transients reported in various cells have been shown to be agonist specific, and are known to differ both qualitatively and quantitatively (Berridge, 1990; Gray, 1988; Jacob, 1991; Sanchez-Bueno and Cobbold, 1993; van den Pol et al, 1992). When astrocytes are challenged with glutamate, for example, Ca\(^{2+}\) transients are generated which oscillate, but which are spike-like in appearance (Charles et al, 1991). Similar findings have been reported in hepatocytes, when cells have been chronically exposed to vasopressin (Sanchez-Bueno and Cobbold, 1993). Moreover, differences in the characteristics of Ca\(^{2+}\) transients induced by chronic exposure to various agonists have previously been reported in astrocytes (Inagaki et al, 1991). Such oscillatory activity has been put forth as a means by which cells encode information based on oscillation frequency (Rapp, 1987). We were interested, therefore, to see whether longer term exposure to ATP and NA might identify differences in terms of the characteristics, oscillations and extent of the change in [Ca\(^{2+}\)]\(_i\) induced by either agonist over the time course used to investigate TXA\(_2\) release. Differences of this nature could perhaps explain why ATP is able to stimulate eicosanoid release from astrocytes. Chronic exposure of cells to ATP or NA resulted in a biphasic increase in [Ca\(^{2+}\)]\(_i\); the secondary phase of the response being sustained.
throughout prolonged exposure to either agonist. We could detect no qualitative or quantitative difference with either agonist under these conditions; indeed, the Ca$^{2+}$ transients obtained with ATP or NA are virtually superimposable. Unlike glutamate, neither agonist induced spike-like Ca$^{2+}$ transients, and conclude that chronic treatment with ATP or NA does not identify agonist specific Ca$^{2+}$ transients.

It is important to appreciate that all reports of agonist specific Ca$^{2+}$ transients have been carried out on single cells using very low concentrations of drug. Astrocytes in vivo do not exist as isolated single, functional units, and evidence is growing to suggest that they act as a syncitium. Cells in culture used throughout this thesis were grown to confluence, and this may be why agonist specific Ca$^{2+}$ transients were not observed in this study. Moreover, intercellular Ca$^{2+}$ waves have been described in glial cell cultures (Charles et al, 1991, 1993; Cornell-Bell and Finkbeiner, 1991; Finkbeiner, 1992), and the suggestion is that these provide a means of cell-cell communication, and/or may serve to co-ordinate the responses of many cells within a tissue. The extent and functional consequence of agonist specificity in terms of intercellular Ca$^{2+}$ waves has yet to be determined. Eicosanoid release is dependent on the presence of extracellular Ca$^{2+}$ (Bruner and Murphy, 1993b), and it is interesting to speculate that intercellular Ca$^{2+}$ waves induced by ATP are important in terms of co-ordinating eicosanoid release from astrocytes. Unfortunately, the equipment used throughout this study to measure [Ca$^{2+}$]$_i$ was unable to perform spatio-temporal analysis of these Ca$^{2+}$ waves, so these questions remain unanswered.

There was no evidence of additivity between ATP and NA in terms of the intracellular Ca$^{2+}$ they evoke, suggesting both agonists access a common source of intracellular Ca$^{2+}$. Recent evidence suggests that, in astrocytes, different ligands release Ca$^{2+}$ from a common store (Shao and McCarthy, 1995). Interestingly, in Figure 4.4 where the effect of NA on ATP-evoked changes in [Ca$^{2+}$]$_i$ is illustrated, the final control
challenge with ATP induced less change in \([\text{Ca}^{2+}]_i\) than the initial exposure. This raises the possibility that the stores of \(\text{Ca}^{2+}\) within the cells have been depleted, perhaps as a result of \(\alpha_1\)-adrenoceptor activation, or that P2 purinoceptor desensitisation may have occurred. This lack of additivity is at odds with studies performed on thyroid FRTL-5 cells, where there is evidence of partial additivity of ATP and NA on changes in \([\text{Ca}^{2+}]_i\) (Bizzarri and Corda, 1994). However, these cells differ from astrocytes in that NA-induced changes in \([\text{Ca}^{2+}]_i\) are not concentration-dependent, but are all-or-none (Bizzarri and Corda, 1994). It is interesting to note in this regard that NA is capable of stimulating eicosanoid release from these cells (Tornquist et al, 1994). It is not known whether the all-or-none nature of the \(\text{Ca}^{2+}\) transient evoked by NA in FRTL-5 cells has any consequence for eicosanoid release from these cells.

NA was found to inhibit TXA\(_2\) release evoked by the various nucleotides examined in a concentration-dependent manner. This is similar to the inhibition of ATP-induced eicosanoid release by 5-HT (Murphy and Welk, 1990). The release of TXA\(_2\) stimulated by 2-MeSATP was reduced by NA to the greatest extent. The inhibition of TXA\(_2\) release due to ATP, ADP and UTP were all similar, and UDP was affected least. Whether this is indicative of differences in receptor classification or function between the nucleotides has yet to be elucidated.

An additional reason as to why NA inhibited ATP stimulated TXA\(_2\) release might be competition between \(\alpha_1\)-adrenoceptors and P2 purinoceptors for G proteins. G proteins are not thought to be permanently coupled to receptors, but rather are fluid within the plasma membrane. In hepatocytes, for example, different receptors share an ability to stimulate IP\(_3\) formation by activating the same G protein pool (Dasso and Taylor, 1992, 1994). Moreover, it has been shown that activation of V1-vasopressin receptors prevented \(\alpha_1\)-adrenoceptors from associating with G proteins (Dasso and Taylor, 1992). Whether \(\alpha_1\)-adrenoceptors might compete with P2 purinoceptors for G
proteins in a similar fashion has yet to be determined, but an interaction of this kind might explain why NA is capable of inhibiting the effects of ATP.

It is not known whether the inhibitory effect of NA on the ability of ATP (or other nucleotides) to synthesise eicosanoids actually occurs \textit{in vivo}. It is interesting to speculate, however, that this form of functional co-existence of receptors on cells may offer a physiological opportunity to regulate cell function \textit{in vivo}; one consequence of which could be the regulation of eicosanoid release from astrocytes. An environment in which eicosanoid release from cells could not be finely controlled at a cellular level might have profound effects on the cerebral vasculature and synapses.

The results presented in this chapter indicate that the ability of NA to inhibit nucleotide-induced TXA$_2$ release is mediated via stimulation of $\alpha$1-adrenoceptors. When cells were incubated with NA in the presence of propanolol, inhibition was unaffected, suggesting that $\beta$-adrenoceptors are not involved in the process of NA-mediated inhibition of eicosanoid release. Adrenergic modulation of a number of responses induced by various agonists appears to involve the $\alpha$1-adrenoceptor subtype. To this end, Fatatis \textit{et al} (1994) have shown that synergism between vasoactive intestinal peptide and NA in terms of changes in [Ca$^{2+}$]$_i$ in astrocytes is completely reversed by prazosin. However, in the results presented in this chapter, prazosin only partially reversed the inhibiting effect of NA on ATP-evoked TXA$_2$ release. Whether this indicates that molecular subtypes of the $\alpha$1-adrenoceptor are also involved in the inhibition has yet to be determined.

The precise reasons why activation of P2 purinoceptors, but not $\alpha$1-adrenoceptors, stimulates eicosanoid release from astrocytes are still unknown. On the basis of results obtained with pertussis toxin, Bruner \textit{et al} (1993) have proposed the existence of a direct coupling of G protein to PLA$_2$, which may be unique to P2 purinoceptors. There
is indeed evidence to suggest that a single receptor subtype can be linked to more than one second messenger system (Felder et al, 1990; Jones et al, 1991). What still remains unclear is whether the purinergic receptor coupled to PLC is the same as that coupled to PLA₂. It is interesting to note that in their protocol, Bruner and Murphy (1993b) change their usual culture media to one which is serum free 16-18 hours prior to experimentation. Since this regime can affect the expression of the COX-II (Marriott et al, 1995) and hence eicosanoid synthesis, their results should be treated with caution.

In all experiments discussed in this chapter, cells were continuously maintained in culture media which was supplemented with serum.

Stjärne et al (1994) have suggested that there may be spatio-temporal differences between ATP and NA in respect of their release and behaviour in the synaptic cleft of the neuromuscular junction. It is of interest therefore that in astrocytes, NA-evoked Ca²⁺ transients have been shown to be initiated focally in one region of the cell, whereafter a wave of Ca²⁺ is seen to propagate throughout the cytoplasm (Yagodin et al, 1994). Furthermore, spatio-temporal analysis of IP₃-mediated Ca²⁺ transients recorded in Xenopus oocytes has revealed that Ca²⁺ waves can occur either as spirals or puffs (Parker and Ivorra, 1990, 1993). The kinetics of wave initiation and propagation of ATP in astrocytes has yet to be examined; however, it is interesting to speculate that spatio-temporal variations between ATP and NA-induced Ca²⁺ transients could explain differences in their ability to stimulate eicosanoid release, due to differential localisation of cyclooxygenase within the cell for example.

Determining the functional significance of astrocyte-derived eicosanoids is hampered by the fact that it is still unclear under what conditions these cells are stimulated to release them. The release of eicosanoids has been investigated in primary cultures derived from human astrocytic glioma biopsies which were GFAP positive (Murphy et al, 1990) and from glioma C62B cells (DeGeorge et al, 1986a and b, 1987). Unusually,
Murphy et al (1990) found that NA (in addition to ATP) evoked TXA$_2$ release from cultures derived from high grade astrocytomas. This was not the case in lower grade astrocyte-derived tumours. The muscarinic agonist carbachol was not able to evoke release from neoplastic cells, which is what is normally observed when non-neoplastic astrocyte cultures are challenged with this agonist. In the studies by DeGeorge et al (1986a and b, 1987), however, ACh and carbachol were able to stimulate AA accumulation within glioma C62B cells, but other agonists proved ineffective. The unexpected effectiveness of NA and ACh/carbachol in C62B cells might suggest there is a transformation in receptor coupling such that associated changes in [Ca$^{2+}$]$_i$ following receptor activation are different, or perhaps there is a change in the way PLA$_2$ couples to the receptor or G proteins. Alternatively, there may be a change in the expression of certain classes of receptor. The significance of eicosanoid release from these tumours in terms of their vascular supply or associated pathology remain to be determined; however, if the precise transformation affecting receptor coupling could be identified, then this could yield information concerning receptor-mediated stimulation of eicosanoid production in normal astrocytes.

In conclusion, the results in this chapter have demonstrated that ATP and NA are capable of evoking Ca$^{2+}$ transients within cells. The two agonists increase the [Ca$^{2+}$]$_i$ in an essentially similar way, and appear to have access to the same source of intracellular Ca$^{2+}$. Neither agonist evoked repetitive spike-like Ca$^{2+}$ transients during longer term exposure to the cells, and no difference could be detected in the amplitude and frequency of these transients. NA, acting at $\alpha$1-adrenoceptors, is capable of inhibiting nucleotide-evoked TXA$_2$ release from astrocytes in a dose-dependent manner. The precise reasons why agonists such as NA fail to evoke eicosanoid release from astrocytes has still to be elucidated.
Chapter 5
Ryanodine-induced intracellular mobilisation of Ca$^{2+}$ in astrocytes
5.1. Introduction.

In the preceding chapters I have examined and characterised the ability of various nucleotides to evoke eicosanoid release from astrocytes. This process has been shown to be dependent upon the presence of Ca\(^{2+}\) (Murphy et al, 1988), and it has generally been assumed to involve IP\(_3\)-sensitive Ca\(^{2+}\) release channels. To this end, Berridge (1993a) has illustrated the role of IP\(_3\)-sensitive stores in regulating a wide variety of physiological events. These are not, however, the only Ca\(^{2+}\) release channel known to be present in cells, and many possess Ca\(^{2+}\) channels that are distinct from (and insensitive to) IP\(_3\). This latter class of Ca\(^{2+}\) channel is characterised by the ability to bind the plant alkaloid ryanodine, and because of this sensitivity, receptors linked to IP\(_3\)-insensitive Ca\(^{2+}\) release channels are referred to as ryanodine receptors (RYRs). Both classes of receptor share considerable structural and functional homologies (Tsien and Tsien, 1990). They are thought to be responsible for the release of Ca\(^{2+}\) from intracellular stores, and hence for the increase in cytosolic Ca\(^{2+}\) that is observed following transduction of many extracellular stimuli, including neurotransmitters.

We were interested to assess the contribution of ryanodine-sensitive Ca\(^{2+}\) release channels to the Ca\(^{2+}\) transients observed when astrocytes in culture were challenged with various nucleotides, and therefore set out to determine whether these cells respond with an increase in [Ca\(^{2+}\)]; when similarly exposed to ryanodine. Moreover, since RYRs had not been reported to be present in astrocytes, it was essential that a thorough characterisation of any response was conducted, and the class of RYR examined. Since different Ca\(^{2+}\) pools have been shown to be involved in TXA\(_2\) formation in human platelets (Brune and Ullrich, 1991), we were curious to investigate whether ryanodine was capable of influencing the release of TXA\(_2\) from astrocytes, and to what
extent this occurred. In this chapter, I shall review what is known about ryanodine, the receptors at which it acts, and their function. I shall then proceed to present evidence that astrocytes in culture possess RYRs, and discuss their effect on eicosanoid release.

5.1.1. Chemistry of ryanodine.

Ryanodine was first characterised after isolation and crystallisation from the ground wood and root of *Ryania speciosa vahl* by Rogers *et al* (1948). Ryanodine is a neutral alkaloid that is soluble (in its purest form) in water and organic solvents, such as methanol. Advances in techniques of separation and analysis have made it possible to purify various ryanodine congeners from *Ryania* extract, and to determine their chemical properties (Ogawa, 1994). It is now known that the extract contains at least three biologically active components: ryanodine, 9,21-didehydroryanodine and 18-hydroxyryanodine (Humerickhouse *et al*, 1993), but their individual biological activities are not known.

5.1.2. Structure of ryanodine receptors.

Ryanodine receptors were originally described in the sarcoplasmic reticulum of skeletal and cardiac muscle, and although they share considerable homology, they are the product of different genes and have been designated RYR1 and RYR2 respectively. The two receptors are known to be 66% identical (McPherson and Campbell, 1993a). A gene coding for a third type of RYR has now been isolated, cloned and designated RYR3 (Sorrentino and Volpe, 1993). The RYR3 subtype is reported to share an overall homology of 70% with RYR1 and RYR2. Along the areas that are well conserved between all three RYRs, the RYR3 presents a few regions where sequence
homology with RYR1 and RYR2 is poor, in particular, the region of the domain D2, which is practically absent in RYR3 (Sorrentino and Volpe, 1993). The significance of this is unknown.

IP$_3$ and RYRs are remarkably similar in their general organisation, and it is thought this may reflect a common evolutionary origin (Berridge, 1993b). All members of the two families are tetramers composed of large subunits (300 and 550 kDa, respectively). A significant degree of homology is recognised in the domain located toward the COOH-terminus that is thought to span the membrane and participate in the assembly of the channel (Pozzan et al, 1994). This free C-terminal tail may play a part in channel opening because monoclonal antibodies that bind to this region can either inhibit or enhance Ca$^{2+}$ release (Fadool and Ache, 1992; Miyazaki et al, 1992). The rest of the molecule, where no homology exists, protrudes into the cytosol. Overall, the two types of receptor assembly resemble geometric cauliflower's, approximately of the same width (20 nm) but of considerably different heights: 10 and 18 nm for the IP$_3$ and RYRs, respectively (Berridge, 1993a). It has been suggested that RYRs possess an unusual ion-conducting structure comprised of a central (membrane-spanning) channel that branches into four radial channels in the cytoplasmic region of the receptor assembly (Meissner, 1994a). The characteristics of both IP$_3$ and RYRs are compared in Table 5.1.

Two isoforms of RYR, α and β, have been purified from bullfrog skeletal muscle (Murayama and Ogawa, 1992). Oyamada et al (1994) report that about 70% of the amino acid sequence identity is present between the α and β isoforms of the bullfrog. The primary structure of the α-isoform is highly (80%) homologous to the ryanodine-binding protein cloned from rabbit skeletal muscle (RYR1), and is expressed mainly in skeletal muscle. The β-isoform, however, is more than 85% identical with that derived from rabbit brain (RYR3), and is widely expressed. The ion channel properties of the
Table 5.1. Structural and functional properties of intracellular Ca\(^{2+}\)-release channels (adapted from Ehrlich et al, 1994).

<table>
<thead>
<tr>
<th>Properties</th>
<th>IP(_3) receptor</th>
<th>Ryanodine receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structure</td>
<td>Tetramer</td>
<td>Tetramer</td>
</tr>
<tr>
<td>Subunit Molecular Mass</td>
<td>310 kDa</td>
<td>560 kDa</td>
</tr>
<tr>
<td>Genes</td>
<td>3-4</td>
<td>3</td>
</tr>
<tr>
<td>Channel Conductance</td>
<td>8-26 pS</td>
<td>100 pS</td>
</tr>
<tr>
<td>Physiological activators</td>
<td>IP(_3)</td>
<td>Ca(^{2+}) (&gt;1μM)</td>
</tr>
<tr>
<td></td>
<td>Ca(^{2+}) (&lt;300nM)</td>
<td>cADP ribose*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mechanical coupling</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(skeletal muscle)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>adenine nucleotides</td>
</tr>
<tr>
<td>Pharmacological activators</td>
<td>none discovered</td>
<td>caffeine**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>heparin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ryanodine (&lt;10μM)</td>
</tr>
<tr>
<td>Pharmacological inhibitors</td>
<td>heparin</td>
<td>ruthenium red</td>
</tr>
<tr>
<td></td>
<td>caffeine</td>
<td>ryanodine (&gt;10μM)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mg(^{2+})</td>
</tr>
</tbody>
</table>

* In RYR2 only; ** In RYR1 and RyR2 only.
two isoforms have been investigated in chicken skeletal muscle (Percival et al, 1994), and the α-RYR was found to have two gating modes, which differ in the extent they are activated by Ca\(^{2+}\). β-RYR channel openings were found to be longer than the α-RYR isoform in either gating mode, are activated by Ca\(^{2+}\) to a greater extent, and inactivated by millimolar Ca\(^{2+}\) in the absence, but not presence, of adenine nucleotides. Both isoforms are activated by caffeine, are inhibited by ruthenium red and Mg\(^{2+}\), and bind calmodulin (Percival et al, 1994). Exactly how the two individual isoforms contribute physiologically to the control of cellular Ca\(^{2+}\), particularly if they are functional within the brain, has yet to elucidated.

5.1.3. Distribution of ryanodine receptor subtypes.

It is now well established that all RYR1 and RYR2 subtypes are present in the CNS, with RYR2 being the predominant form (McPherson et al, 1991; Nakanishi et al, 1992; Kuwajima et al, 1992). Gene expression studies have shown that the RYR3 subtype has a much wider tissue distribution than either RYR1 or RYR2, and is also found in the CNS (Sorrentino and Volpe, 1993). The localisation of all three RYR isoforms has shown them to be present throughout wide regions of the brain, including the parietal, frontal and temporal lobes of the cerebrum, thalamus/hypothalamus, cerebellum and brain stem (Furuichi et al, 1994; Ledbetter et al, 1994). Interestingly, both these workers found that the RYR3 subtype was almost absent from cerebellum; however, it has been reported to be abundant in the corpus striatum, caudate putamen, thalamus and hippocampus (Hakamata et al, 1992). It is of interest that none of these investigations have reported the existence of RYRs in glial cells. Table 5.2 illustrates the tissue distribution of the various RYR mRNAs.
The relationship between RYRs and IP$_3$ receptors in the brain is complex with the localisation of one or other in some neurons and both in other neurons (Sharp, et al, 1993). Contrasting distributions have been reported both for large brain areas as well as at the cellular level (Verma, et al, 1992). These authors report that hippocampal cells and cerebellar Purkinje cells possess both types of receptor. Overall, the level of expression of the various RYRs, including RYR2 and RYR3, appear to be much lower than those of the IP$_3$ receptor; however, since the unit conductance for RYR channels is about tenfold higher than that of IP$_3$ receptor channels, quantitative differences may be less evident at a functional level (Fleischer and Inui, 1989).

5.1.4. Functional aspects of ryanodine receptors.

Studies with purified RYR1 and RYR2 incorporated in lipid bilayers have shown that they are, upon activation, permeable to Ca$^{2+}$ with a single-channel conductance of around 100 pS. Ca$^{2+}$ flux through both of these RYRs is stimulated by caffeine, Ca$^{2+}$, calmodulin and adenine nucleotides, and is inhibited by Mg$^{2+}$ and ruthenium red (Fleischer and Inui, 1989). Hakamata et al (1992) have shown that the RYR3 subtype also has calmodulin-binding and phosphorylation sites. However, at nanomolar concentrations, calmodulin has been reported to inhibit ryanodine binding to purified brain receptor (McPherson and Campbell, 1993b). Meissner (1994a) has suggested that modulation by various endogenous effector molecules allows a complex pattern of regulation of the various RYR subtypes.

Single channel measurements have shown that ryanodine may exert its actions in vertebrate muscle by inducing, at nanomolar to micromolar concentrations, the formation of an open subconductance state, whereas at concentrations in the high micromolar range, ryanodine completely closes the release channel (Buck et al, 1992;
Table 5.2. Distribution of expression of the three ryanodine receptor genes (adapted from Sorrentino and Volpe, 1993).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>RYR1* (Skeletal)</th>
<th>RYR2* (Cardiac)</th>
<th>RYR3** (Brain)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skeletal muscle</td>
<td>++++</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Liver</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Kidney</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Lung</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Stomach</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Spleen</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Ileum</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Jejunum</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Heart</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Brain</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
</tbody>
</table>

* In rabbit; ** In mink.

Ca\(^{2+}\) release experiments in cultured mink lung epithelial (MvILu) cells treated with transforming growth factor β (TGF-β) are the only cells definitely known to possess functional RYR3s (Giannini et al, 1992). This regime resulted in the induction of RYR3 that is responsive to ryanodine, but not to caffeine. Lack of response to caffeine is unexpected since caffeine is known to activate RYRs, probably by prolonging the opening time of the channel, and has, therefore, been considered characteristic of RYR function (Meissner, 1994b; Rousseau et al, 1988).

The expression of the RYR3 gene in MvILu cells is TGF-β-dependent since virtually no RYR3 mRNA is detected in untreated cells (Giannini et al, 1992). Induction by TGF-β is, at least in part, controlled at the transcriptional level, and does not require new protein synthesis, which indicates that expression of the RYR3 gene is involved in the primary TGF-β response (Sorrentino and Volpe, 1993). Furthermore, RYR3 does not correlate to the growing state of the cells or differentiation, but seems to be confined to the cellular actions of TGF-β.

Both IP\(_3\) and RYRs are similar in that they may be regulated by luminal and cytoplasmic Ca\(^{2+}\) (Cheek et al, 1994). This is important as it indicates that both receptors could operate as Ca\(^{2+}\)-induced Ca\(^{2+}\)-release (CICR) channels, although IP\(_3\) receptors require the simultaneous presence of IP\(_3\) as an endogenous modulator (Berridge, 1993a). The recently identified nucleotide, cyclic adenosine diphosphate ribose (cADPR) can mobilise Ca\(^{2+}\) from a variety of cells (Lee et al, 1994). cADPR is the most potent Ca\(^{2+}\)-mobilising agent described to date, and the biochemical machinery for its synthesis and degradation has been identified in all cells so far examined (Galione, 1992). It mobilises Ca\(^{2+}\) via an IP\(_3\)-independent mechanism, and
it is now thought that cADPR is an endogenous regulator of at least once class of RYR (Galione, 1993); however, it has been shown to increase the open probability of RYR2, but not necessarily RYR1 or RYR3 (Meszaros et al, 1993). The cADPR molecule functions similarly to IP$_3$; it increases the probability of channel opening of the RYR by increasing its Ca$^{2+}$ sensitivity (Putney, 1993).

The question that remains largely unanswered concerns the relationships between the two Ca$^{2+}$ pools and, in particular, whether the two pools correspond to, at least functionally, independent Ca$^{2+}$ stores, whether they fully coincide, or are in direct luminal continuity, and thus in rapid equilibrium with each other (Pozzan et al, 1994). RYRs have been shown to be concentrated in endoplasmic reticulum (ER) areas of the subplasmalemma cytoplasm, with marked heterogeneity among adjacent cisternae (Parys et al, 1992). In contrast, these authors found the ER cisternae of the deep cytoplasm mostly devoid of RYRs. Zachetti et al (1991) have found that, in PC12 cells, RYR and IP$_3$ receptors are linked to the same Ca$^{2+}$ store. However, Pozzan et al (1994) hypothesises that overlapping of the two pools occurs in most if not all cells, albeit to a variable extent. Further, this situation could be due not only to a partial colocalisation of RYR and IP$_3$ receptors in individual areas of the ER, but also to the proximity of RYR and IP$_3$ receptor specialised membrane areas. It appears that colocalisation of IP$_3$ and RYRs may have physiological relevance in vivo. In the dendrites of Purkinje neurons, for example, IP$_3$ and RYRs are clustered together near the attachment of the spine stalk (Meldolesi et al, 1992). Diffusion of second messengers to this area could trigger larger and more persistent responses. This deserves special attention, since in various neurons the spines (and their impinging synapses) are the cell areas where processes such as cellular memory are known to take place (Meldolesi et al, 1992).
5.1.5. Diseases related to ryanodine receptor dysfunction.

A mutation of the RYR1 gene is responsible for the inherited skeletal muscle disorder malignant hyperthermia (MH) (Ledbetter et al., 1994). This syndrome is most readily characterised by skeletal muscle contracture and accelerated muscle metabolism in response to halogenated anaesthetics. MH can induce cardiac tachycardia, an abrupt rise in body temperature, and extremely high fever, which can lead to tissue damage or death (Ogawa, 1994).

Central core disease (CCD) of muscle is a rare myopathy usually characterised by hypotonia and muscle weakness in infancy (Ogawa, 1994). Genetic analysis of several families with CCD has indicated that the disorder is inherited as an autosomal dominant trait and is closely associated with a predisposition to MH. It is now established that CCD occurs as a result of a genetic mutation of the RYR1 gene (Haan et al., 1990).

Airey et al. (1993) have shown that a failure to make the α isoform of the RYR1 is associated with the Crooked Neck Dwarf mutation in chickens. At the present time, however, no disease has been attributed to defects in the RYR2 or RYR3 subtype.

5.2. Methods and Materials.

All methods were as described in Chapter 2. All drug solutions were made up in distilled water except caffeine and ryanodine. A stock solution of caffeine was prepared in boiling distilled water, and ryanodine was dissolved in methanol. In all cases drug dilutions were made in appropriate buffer (at room temperature) before addition to the cultured cells. The ryanodine stock was diluted such that cultures were
never exposed to a methanol concentration in excess of 0.5% v/v. Control experiments where cells were exposed to methanol alone at 0.5% v/v showed that ryanodine vehicle had no effect on \([\text{Ca}^{2+}]_i\).

Ryanodine was purchased from Sigma. For some experiments, ryanodine was also obtained from Research Biochemicals Inc., Natick, U.S.A. All other drugs and chemicals were obtained from either Sigma or BDH.

5.3. Results.

5.3.1. Effect of ryanodine on \([\text{Ca}^{2+}]_i\).

Astrocyte cultures were challenged with ryanodine over a concentration range of 0.1-50 \(\mu\)M and the change in \([\text{Ca}^{2+}]_i\) assessed. This concentration range was chosen on the basis of previous work showing that RYRs are activated at low concentrations of ryanodine but inhibited at concentrations in excess of 10 \(\mu\)M (Meissner, 1986). Experiments were performed on cells derived from a number of different cultures and of the 20 cell fields examined, 60% were found to respond to ryanodine with an increase in \([\text{Ca}^{2+}]_i\). A typical example of the response obtained is shown in Figure 5.1. The response to 10 \(\mu\)M ATP on the same field of cells is shown to illustrate both the qualitative and quantitative differences in the effects elicited by both stimuli. The response to ATP is characterised by a rapid (approximately 7 s to peak height) increase in \([\text{Ca}^{2+}]_i\) which decays within 10 s to a plateau of oscillating \([\text{Ca}^{2+}]_i\) which is approximately 30% of the peak change in \([\text{Ca}^{2+}]_i\) recorded. This level is maintained throughout the drug exposure period. Once the drug is removed and the cells bathed in drug free buffer, the level of \([\text{Ca}^{2+}]_i\) returns immediately to pre-stimulation levels. In contrast, the response observed with ryanodine was much smaller in terms of peak
Figure 5.1. Representative trace showing changes in $[\text{Ca}^{2+}]_i$ in astrocytes challenged consecutively with ryanodine (Rya., 1 μM) and ATP (10 μM). The bars above the trace indicate the period (30 sec) over which the cells were exposed to the drugs. This experiment was performed on a single field of cells and is representative of similar experiments carried out on at least 20 fields from different batches of cultures.
change in \([Ca^{2+}]_i\), did not show the secondary plateau phase seen with ATP, and the time to peak \([Ca^{2+}]_i\) was slower than that of ATP, taking 11 s to reach peak height.

5.3.2. The effect of multiple drug additions of ryanodine on \([Ca^{2+}]_i\).

When cells were challenged with two consecutive additions of ryanodine 3 min apart, the second drug application failed to elicit a measurable change in \([Ca^{2+}]_i\). However, when cells are similarly challenged with repeated doses of ATP, the responses were found to be almost identical, as illustrated in Figure 5.2. Thus, responses to ryanodine were found to desensitise rapidly, and recovery from this generally took between 30 and 45 min. This limited the ability to examine dose-response relationships on the same field of cells, and the dose response curve shown in Figure 5.3 is therefore compiled from experiments performed on a number of cell fields from different batches of cultures. Ryanodine was found to be ineffective at concentrations below 0.5 \(\mu\)M; maximal responses were recorded at 1 \(\mu\)M giving an EC\(_{50}\) of approximately 600 nM.

5.3.3. The effect of ryanodine on \([Ca^{2+}]_i\) in calcium-free buffer.

It is thought that ryanodine mobilises \(Ca^{2+}\) from intracellular pools (Cheek et al, 1994; Sorrentino and Volpe, 1993). To establish that this was the case in astrocytes, we exposed three cell fields to ryanodine in nominally \(Ca^{2+}\)-free buffer containing 0.2 mM EGTA. Ryanodine-induced changes in \([Ca^{2+}]_i\) were still observed in \(Ca^{2+}\)-free buffer but the magnitude of the response was considerably greater (179 ± 36%) than those found in the same cell fields in \(Ca^{2+}\)-containing buffer. This is illustrated in Figure 5.4. This is in contrast to ATP-stimulated changes in \([Ca^{2+}]_i\) which were reduced, particularly the initial phase of the response, in \(Ca^{2+}\)-free buffer. This suggests that
Figure 5.2. Changes in [Ca$^{2+}$]$_i$ in cells exposed to multiple additions of ATP and ryanodine. Cells were challenged twice with ATP (10 µM) for 30 sec, then twice with ryanodine (Rya., 1 µM), each exposure period being approximately 3 min apart. These responses are typical of those found in two other experiments. The bars above the trace indicate the drug additions.
Figure 5.3. Dose-response relationship for ryanodine-stimulated changes in $[\text{Ca}^{2+}]_i$. A number of cell fields were challenged over 30 sec periods with various concentrations of ryanodine. The changes in $[\text{Ca}^{2+}]_i$ were calculated by subtracting the basal $[\text{Ca}^{2+}]_i$ (usually between 50 and 150 nM) from the peak response elicited by the drug. Results are means ± SEM from three determinations.
change in $[\text{Ca}^{2+}]_i$ (µM)
P2-purinoceptor activation promotes both Ca\(^{2+}\) release from internal stores and Ca\(^{2+}\) influx from the extracellular medium.

It was also observed that desensitisation of ryanodine-induced responses which were normally observed in Ca\(^{2+}\)-containing buffer, can be delayed quite significantly when extracellular Ca\(^{2+}\) is removed. This is illustrated in Figure 5.5. where the upper trace shows desensitisation in Ca\(^{2+}\)-containing buffer, whereby no change in \([\text{Ca}^{2+}]_i\) is seen with subsequent challenge with ryanodine. However, as the lower trace shows, repetitive additions of ryanodine in Ca\(^{2+}\)-free buffer continue to increase \([\text{Ca}^{2+}]_i\) with negligible desensitisation.

5.3.4. Effect of caffeine on ryanodine-induced calcium transients.

Responses evoked by ryanodine have been characterised by their sensitivity to caffeine (Fleischer and Inui, 1989). When six cell fields were challenged with 10 mM caffeine there was no change in \([\text{Ca}^{2+}]_i\). However, it was noted that prior addition of caffeine attenuated the effect of a subsequent challenge by ATP (Figure 5.6A). By comparing this figure with the characteristic ATP response shown in Figure 5.1, it can be seen that the initial ATP-stimulated spike of intracellular Ca\(^{2+}\) mobilisation was markedly reduced in cells pre-treated with caffeine; this effect was observed in four out of six experiments. Ryanodine had no effect on \([\text{Ca}^{2+}]_i\) in these experiments. In the two experiments where the response to ATP was unaffected by caffeine, ryanodine did have an effect on \([\text{Ca}^{2+}]_i\) (Figure 5.6B). To determine whether the reduction in ATP-evoked Ca\(^{2+}\) mobilisation was the result of a prior exposure to caffeine and not ryanodine and caffeine (as shown in Figure 5.6A), experiments were carried out where cells, which had not been exposed to ryanodine, were challenged with ATP in the presence of caffeine. In five experiments performed under these conditions, the ability of ATP to
Figure 5.4. Ryanodine- and ATP-stimulated changes in $[Ca^{2+}]_i$ in astrocytes bathed in $Ca^{2+}$-containing and $Ca^{2+}$-free buffers. Cultures were challenged for 30 sec (indicated by the solid bar) with either ryanodine (Rya., 500 nM, upper trace) or ATP (10 µM, lower trace) in $Ca^{2+}$-containing buffer to establish a control response. The buffer was then changed to one containing zero $CaCl_2$ plus 0.2 mM EGTA (indicated by the open bar) and superfusion of the cells continued for 10 min before challenging them again with either ryanodine or ATP. These results are typical of those found in two other experiments.
Figure 5.5. The effect of multiple additions of ryanodine on changes in current ratio of Fura 2-AM in astrocytes bathed in Ca\(^{2+}\)-containing and Ca\(^{2+}\)-free buffers. Cultures were challenged with 1 μM ryanodine (solid bar) for 30 sec in Ca\(^{2+}\)-containing buffer. This was repeated two minutes later (upper trace), where no change in current ratio could be seen, after which the bathing medium was switched to one containing zero CaCl\(_2\) plus 0.2 mM EGTA. The cells were washed for a further 30 min in this buffer. They were then challenged with the same concentration of ryanodine (lower trace) for 30 sec, and bathed in drug free buffer for two mins. The cells were challenged with subsequent doses of ryanodine in the same way, and the change in current ratio assessed. The change in current ratio is representative of the change in [Ca\(^{2+}\)]\(_i\).
Figure 5.6A. Representative trace showing the effect of caffeine on ATP-evoked changes in $[\text{Ca}^{2+}].$. Cells were challenged consecutively for 0.5 min with ryanodine (Rya., 1 µM), caffeine (Caff., 10 mM), and ATP (10 µM) as indicated by the bars above the trace. This result is typical of those found in four of the six experiments performed.
Figure 5.6B. Cells were challenged as described in Figure 5.6A. Note that in this field of cells ryanodine induced an increase in $[\text{Ca}^{2+}]_i$ but that caffeine alone was ineffective and had no effect on the response elicited by a subsequent addition of ATP. This result was found in two of the six experiments performed.
Figure 5.7. A representative trace showing ATP-stimulated changes in $[\text{Ca}^{2+}]_i$ in the presence and absence of ryanodine. Cells were initially challenged with 10 $\mu$M ATP to establish a control response then exposed to 50 $\mu$M ryanodine (Rya.) for 5 min before a further addition of 10 $\mu$M ATP, the bars above the trace indicate the drug additions. This experiment was performed on two other cell fields with essentially similar results.
Table 5.3. The effect of ryanodine on TXA$_2$ release from astrocytes.

<table>
<thead>
<tr>
<th>Additions</th>
<th>TXA$_2$ release (% basal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP (250 μM)</td>
<td>1,074 ± 28</td>
</tr>
<tr>
<td>Ryanodine (1 μM)</td>
<td>77 ± 6</td>
</tr>
<tr>
<td>Ryanodine (5 μM)</td>
<td>100 ± 6</td>
</tr>
<tr>
<td>Ryanodine (10 μM)</td>
<td>94 ± 1</td>
</tr>
</tbody>
</table>

Cultures were incubated for 15 min with one of the above and the amount of TXA$_2$ released into the bathing medium measured by radioimmunoassay. Results are means ± SEM from three determinations assayed in duplicate and are expressed as a percentage of basal TXA$_2$ release (0.35 ± 0.3 ng/ml, n = 6).
Table 5.4. The effect of ryanodine and caffeine on ATP-stimulated TXA$_2$ release from astrocytes.

<table>
<thead>
<tr>
<th>Additions</th>
<th>TXA$_2$ release (% basal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP (50 µM)</td>
<td>260 ± 41</td>
</tr>
<tr>
<td>Ryanodine (5 µM)</td>
<td>101 ± 16</td>
</tr>
<tr>
<td>Ryanodine (50 µM)</td>
<td>114 ± 13</td>
</tr>
<tr>
<td>Caffeine (10 mM)</td>
<td>85 ± 5</td>
</tr>
<tr>
<td>ATP (50 µM) + Ryanodine (5 µM)</td>
<td>271 ± 35</td>
</tr>
<tr>
<td>ATP (50 µM) + Ryanodine (50 µM)</td>
<td>224 ± 36</td>
</tr>
<tr>
<td>ATP (50 µM) + Caffeine (10 mM)</td>
<td>78 ± 4</td>
</tr>
</tbody>
</table>

Cultures were incubated for 15 min with additions indicated above and the released TXA$_2$ measured by radioimmunoassay. Results are means ± SEM from 3-5 determinations assayed in duplicate and are expressed as a percentage of basal TXA$_2$ release (0.44 ± 0.12 ng/ml, n = 7).
increase $[\text{Ca}^{2+}]_i$ was reduced by $24 \pm 2\%$. Experiments were also carried out to determine whether prior exposure of ryanodine would affect the response to ATP. Three cell fields were incubated with ryanodine at a concentration (50 $\mu$M) expected to block Ca$^{2+}$ release from ryanodine-sensitive stores and then challenged with ATP. Figure 5.7 shows that this regime had no effect on the ATP-stimulated change in $[\text{Ca}^{2+}]_i$.

5.3.5. Effect of ryanodine, caffeine and ATP on TXA$_2$ release.

The ability of ryanodine to stimulate TXA$_2$ release from astrocyte cultures was examined over a concentration range which had been shown to be capable of increasing $[\text{Ca}^{2+}]_i$. However, ryanodine was ineffective in this respect, and no release of TXA$_2$ was seen (Table 5.3). The effect of a maximally effective dose of ATP (250 $\mu$M) is also shown since this was included as a positive control. Table 5.4 shows the effect of combined additions of ryanodine and caffeine on ATP-stimulated TXA$_2$ release. At concentrations of 5 and 50 $\mu$M, ryanodine was without effect on the ATP response. These concentrations of ryanodine have been reported to be excitatory and inhibitory respectively (Meissner, 1986). Although 10 mM caffeine had no effect when used alone, it completely abolished the increase in TXA$_2$ release elicited by ATP.

5.4. Discussion.

The results presented here show that a population of cultured cortical astrocytes respond to ryanodine with an increase in $[\text{Ca}^{2+}]_i$, probably as a result of Ca$^{2+}$ release from internal stores, since Ca$^{2+}$ transients were observed in cells bathed in Ca$^{2+}$-free buffer. However, this finding is in disagreement with previous studies on cultured glia.
(Charles et al, 1993) and with immunohistochemical localisation of RYRs in intact tissue (Kuwajima et al, 1992). Charles et al (1993) found that cultured glia failed to show changes in $[Ca^{2+}]_i$ when challenged with ryanodine. This discrepancy could be explained in that their cultures contained a mixed population of whole brain glia (only 60% of which were astrocytes), which contrasts with the cultures used throughout this thesis which were derived from the cerebral cortex and contained approximately 90% protoplasmic astrocytes. Given that ryanodine did not evoke $Ca^{2+}$ transients in all cultures examined, it is possible that there is a degree of regional and cellular heterogeneity among cultured glia, this having been suggested previously (McCarthy and Salm, 1991; Wilkin et al, 1990). Moreover, it has been shown that astrocytes in culture lose their responsiveness to certain neuroligands with time (Shao and McCarthy, 1993), and there may be a possibility this applies to RYRs in culture too, whereby expression is observed only during a certain stage in the development of the cells.

The accepted hallmark of ryanodine-activated $Ca^{2+}$ pools is their sensitivity to caffeine, which seems to prolong the opening time of the RYR-linked $Ca^{2+}$ release channel by locking it in a sub-conductance state, thereby slowing the kinetics of channel opening and closing (Ehrlich et al, 1994; Rousseau et al, 1988). This appears to be the case for RYR1 and RYR2; however, Giannini et al (1992) have shown that RYR3 is insensitive to caffeine in Mv1Lu cells. In the astrocyte RYR examined in this chapter, no caffeine-induced changes in $[Ca^{2+}]_i$ were observed in any of the cell fields assessed, which tends to suggest that cortical astrocytes possess the RYR3 subtype. Moreover, since Kuwajima et al (1992) limited their localisation of RYRs in intact brain tissue to RYR1 and RYR2, this may explain why they failed to detect RYRs in glia. Despite this apparent inactivity, the results presented here show caffeine markedly attenuated the ATP-stimulated increase in $[Ca^{2+}]_i$ in the majority of cells tested. Caffeine appeared to inhibit the initial phase of the ATP-induced mobilisation of $Ca^{2+}$ which is believed to be
a mixture of Ca\(^{2+}\) release from internal stores and an associated influx of Ca\(^{2+}\) from the bathing media. Precisely how caffeine interferes with Ca\(^{2+}\) mobilisation in these cells remains to be established; however this effect has been noted in oocytes (Parker and Ivorra, 1991) and smooth muscle cells (Pacaud et al, 1994).

Although the characteristics of the astrocytic RYR described in this chapter display RYR3-like insensitivity to caffeine as reported by Giannini et al (1992), we hesitate to state unequivocally that astrocytes possess the RYR3 subtype. In the cells described by Giannini et al (1992), the RYR3 subtype were not constitutive, but were induced by treatment with TGF-\(\beta\). Further, ryanodine-evoked [Ca\(^{2+}\)]\(_i\) mobilisation could only be demonstrated following prior sensitisation with a Ca\(^{2+}\)-mobilising agonist. The cultures used in this study were not exposed to growth factors other than those likely to be present in foetal calf serum which is routinely added to the culture medium. Indeed, Dettbarn et al (1995) have described a caffeine-insensitive RYR in brain microsomes whose expression was not dependent on the presence of growth factors. With regard to the use-dependence of the RYR examined in this study, we found ryanodine was capable of evoking an increase in [Ca\(^{2+}\)]\(_i\) in cells that had received no prior treatment with a [Ca\(^{2+}\)]\(_i\) mobilising stimulus; indeed there was no qualitative or quantitative difference between the ryanodine-induced responses in naive cells and those previously exposed to ATP.

The use of TGF-\(\beta\) to stimulate RYR3 expression in Mv1Lu cells (Giannini et al, 1992) raises an interesting possibility. Three highly homologous forms of TGF-\(\beta\) are expressed in mammals, and are thought to be involved in cell proliferation and differentiation and in inflammatory processes, where they can be either proinflammatory or anti-inflammatory depending on the status of the target cell (Oppenheim and Neta, 1994). Injury to the CNS invariably results in astrocytes changing into the so-called 'reactive' state, and it appears that astrocytes in culture
express many of the characteristics usually associated with reactive cells in vivo (McMillan et al, 1994). One of the reasons for this appears to be the exposure of astrocytes to factors released from microglia. Amoeboid microglia are thought to be the major source of TGF-β and other cytokines within the CNS (McMillan et al, 1994), and are invariably found as a small (5-10% of total cell numbers) contaminating cell type in astrocyte cultures. It may be that RYR expression in cultured astrocytes is linked to the induction of the reactive state, possibly via their interaction with cytokines released from activated microglia.

It is generally accepted that an increase in [Ca^{2+}]_i is a stimulus for the synthesis and release of eicosanoids from astrocytes (Murphy et al, 1988). We were interested, therefore, to see whether ryanodine was capable of eliciting TXA_2 release from these cells. The results show that ryanodine neither stimulated TXA_2 release nor did it modify the effect of ATP on this response. In general, the peak increase in [Ca^{2+}]_i elicited by ryanodine was at least two-fold lower than that evoked by ATP, so it could be that ryanodine is unable to raise the [Ca^{2+}]_i to a level sufficient to initiate TXA_2 release. Caffeine by itself had no effect on TXA_2 release from these cells, however it completely abolished ATP-stimulated TXA_2 release. Clearly, caffeine is interfering with the sequence of events leading to eicosanoid synthesis in astrocytes, and this may be to the reduction in the initial peak component of [Ca^{2+}]_i transient induced by ATP.

Up until recently, it was thought caffeine had no effect on IP_3 receptors, but this view is now beginning to change. For example, Ehrlich et al (1994) have shown that 10 mM caffeine abolished IP_3 receptor channel activity in microsomes derived from cerebellum. Brown et al (1992) have shown that caffeine affects opening of the IP_3-operated Ca^{2+} release channels rather than IP_3 metabolism or its ability to bind to its receptor. Accordingly, caffeine may be acting as an antagonist at IP_3-linked P2-purinoceptors on astrocytes (rather than modulating the RYR channel), and thereby
reducing a large component of the ATP-induced IP₃-sensitive Ca²⁺ transient. It has recently been shown that the intracellular caffeine-sensitive Ca²⁺ pool is composed of functionally discrete stores that possess heterogeneous sensitivities to caffeine (Cheek et al., 1994). These stores are mobilised by caffeine in a concentration-dependent fashion, and when stimulated, individual stores release their Ca²⁺ in an 'all-or-none' manner. Such a release may underlie the ability of a cell’s Ca²⁺ response to be graded, and may also explain why astrocyte RYRs appeared unresponsive to caffeine alone. If, as it seems to be the case in adrenal chromaffin cells, low caffeine concentrations are unable to release the caffeine- and ryanodine-sensitive [Ca²⁺]ᵢ pool, then perhaps the astrocyte ryanodine-sensitive Ca²⁺ pool may be activated by higher concentrations of caffeine. However, it is important to remember that the use of caffeine to detect ryanodine-sensitive Ca²⁺-pools is far from ideal, not only because it interferes with the IP₃-sensitive pool and that the RYR3 subtype appears to be caffeine-insensitive, but also because it decreases the Ca²⁺ threshold for RYR activation (Pozzan et al., 1994).

We also noted that caffeine only interfered with ATP-stimulated Ca²⁺ mobilisation in cell fields which were unresponsive to ryanodine. The reasons for this are unclear, however, this finding raises two interesting possibilities. The first is that in astrocytes which express both RYRs and IP₃ receptors, the IP₃ receptors are in some way different from those present in cells which do not possess RYRs. There is increasing evidence for molecularly different subtypes of IP₃ receptors, some of which have different regulatory sites (Mikoshiba, 1993), and some have been found in the same cell (Ross et al., 1992). The consequences of this heterogeneity could be considerable; for instance, in their sensitivity to second messengers, which could vary considerably. Indeed, truncated IP₃ receptors obtained by site-directed mutagenesis have been found to be more responsive to IP₃ as compared with their wild counterparts (Pozzan et al., 1994). As yet, we have no idea of the type(s) of IP₃ receptor expressed in cultured
astrocytes except that they appear to have different affinity for IP₃ than do their neuronal counterparts (Khodakhah and Ogden, 1993).

The second possibility is based upon the proposal that an increase in [Ca²⁺]ᵢ is a key event in eicosanoid release from astrocytes. Given that caffeine completely abolished ATP-evoked TXA₂ release and reduced ATP-stimulated changes in [Ca²⁺]ᵢ in cells which were unresponsive to ryanodine, it could be that suggested that TXA₂ release is a function of the population of astrocytes which do not express RYRs.

The change in [Ca²⁺]ᵢ induced by ryanodine was markedly higher in buffer that was Ca²⁺ free, a condition which might be expected to decrease resting [Ca²⁺]ᵢ, particularly with the proposed involvement of RYRs in the process of CICR. It should be noted, however, that there was no evidence of CICR in ryanodine-induced changes in [Ca²⁺]ᵢ seen in our experiments on astrocytes. This process appears to be the preserve of the RYR2 subtype, which is the predominant form in the CNS (McPherson et al, 1991). This form of stimulation may play a role in amplifying Ca²⁺ signals within neurons.

We also noted that the rapid desensitisation of ryanodine-induced [Ca²⁺]ᵢ transients seen in buffer containing Ca²⁺ can be delayed quite significantly when cells are bathed in Ca²⁺-free buffer. It could be that the RYRs in astrocytes are subject to a degree of tonic inhibition under normal resting [Ca²⁺]ᵢ concentrations, this being removed during incubation in Ca²⁺-free buffer. Modulation of this nature could be achieved through either a Ca²⁺-sensitive regulatory site on the RYR itself, or a loosely associated Ca²⁺-binding protein which is removed by treatment with Ca²⁺-free medium. This has been observed with IP₃ receptors in peripheral tissues (Zhao and Muallem, 1990). A candidate for this Ca²⁺-binding protein could be calmodulin. McPherson and Campbell (1993b) have shown calmodulin, at nanomolar concentrations, dramatically inhibits the binding of ryanodine to purified brain receptor. Calmodulin is present in neurons at
concentrations greater than 1 μM (Miller, 1991), and it may be possible that it provides a negative feedback system to regulate $[\text{Ca}^{2+}]_i$. It has also been demonstrated that calmodulin inhibits Ca$^{2+}$ release from sarcoplasmic reticulum of skeletal and cardiac muscle (Meissner and Henderson, 1987). It is likely there is a direct interaction of calmodulin with the Ca$^{2+}$-release channel, or with a membrane component associated with the channel, since calmodulin-dependent protein kinase is not involved (Coronado et al, 1994). When Ca$^{2+}$ is released from internal stores, it could interact with calmodulin, leading to inhibition of the RYR with a subsequent decrease in Ca$^{2+}$ release, and this may have a role in the phenomenon of Ca$^{2+}$ oscillations (McPherson et al, 1993b). Hakamata et al (1992) have shown that the RYR3 subtype possesses a potential calmodulin binding site.

The β-isoform of muscle RYR1 has been shown to be RYR3-like (Oyamada et al, 1994). Interestingly, while ryanodine binding to α-RYR is negligible in Ca$^{2+}$-free media, ryanodine binding to β-RYR was about one fifth of maximum under the same conditions (Ogawa, 1994). In high (1 M) NaCl media, the β-isoform had very much increased Ca$^{2+}$ sensitivity. This could be related to redistribution of ions between RYR molecules and their surrounding media, or disinhibition of the RYR in the presence of high Ca$^{2+}$ concentrations (Ogawa, 1994). Whether this has relevance to the activity of the RYR in astrocytes has yet to be elucidated.

The change in $[\text{Ca}^{2+}]_i$ elicited by ryanodine is relatively small and desensitises rapidly in comparison to that evoked by ATP, which is presumed to be via stimulation of IP$_3$-linked Ca$^{2+}$ release channels. These findings might be indicative of differences in the abundance, capacity and/or regulation of the two Ca$^{2+}$ pools. It is known from binding studies that IP$_3$ receptor density in the cerebral cortex is approximately 15-fold greater than that of RYRs (Smith and Nahorski, 1993). However, this requires clarification in astrocytes. Immunohistochemical localisation of RYR within the CNS have shown
them to be present in neurons, with glial labelling being either very low or undetectable (Kuwajima et al., 1992; Nakanishi et al., 1992). Interestingly, similar findings were reported when studies were performed to map IP₃ receptors in intact tissues (Ross et al., 1989), yet it is quite clear that cultured glia possess an array of membrane receptors linked to inositol phospholipid metabolism and [Ca²⁺]ᵢ mobilisation (Pearce and Murphy, 1988).

We could find no evidence of interaction between IP₃ and RYRs in astrocytes. This cannot be explained simply on the basis of the different Ca²⁺ pools being located in different cells because all the cells that responded to ryanodine also responded to ATP, which points to a degree of colocalisation; this situation is known to exist in certain neurons (Nakanishi et al., 1992). However, we are yet to determine whether ryanodine alters the Ca²⁺ response to other ligands, particularly as Sanchez-Bueno and Cobbold (1993) have demonstrated that, in hepatocytes, ryanodine inhibits CICR induced by certain agonists but not others.

High doses of ryanodine are known to act in an inhibitory manner at RYRs (Meissner, 1986). It necessarily follows that the higher the dose used, the greater the extent of different ryanodine congeners that is likely to be encountered. This is particularly relevant given that commercially available ryanodine is known to consist of various congeners (Ogawa, 1994). During the experiments outlined in this chapter, ryanodine was purchased from two commercial sources, Sigma and Research Biochemicals Inc. It is interesting to note that with the latter compound, we were unable to evoke any change in [Ca²⁺]ᵢ using doses of ryanodine that we had previously shown to be effective in this respect. When I examined the appropriate data sheets supplied by the companies concerned, I noted ryanodine obtained from Sigma was >99% pure. However, the product purchased from Research Biochemicals Inc. was found to be a mixture of ryanodine and didehydroryanodine (60:40). Caution should, therefore, be
exercised when using purchasing ryanodine, and attempts should be made to obtain a product with the highest percent purity.

In summary, the work presented in this chapter has illustrated the existence of a ryanodine-sensitive Ca^{2+} pool in cultured cortical astrocytes. The contents of this pool cannot be mobilised by caffeine, which suggests that they possess the caffeine-insensitive RYR3 subtype. These results do not correspond entirely with RYR3-mediated Ca^{2+} release in other cells, and it could be that these cells possess a unique class of astrocytic RYR, or alternatively one of the splice variants of the RYR3 (Sorrentino and Volpe, 1993). Functionally, RYR-evoked changes in [Ca^{2+}]_{i} influence neither eicosanoid release nor the effect of Ca^{2+}-mobilising agents such as ATP.
Chapter 6
Concluding comments and future directions
The main aims of this thesis were:

• to characterise pharmacologically the purinoceptors coupled to eicosanoid release and to determine whether pyrimidines act in a similar fashion at either the same or distinct receptor sites.

• to examine the relationship between P2 purinoceptor-second messenger coupling, intracellular Ca\(^{2+}\) mobilisation and eicosanoid release from astrocytes.

• to explore the possibility that specific intracellular Ca\(^{2+}\) stores are responsible for regulating eicosanoid release from these cells.

CNS astrocytes in culture have been shown to possess cell surface receptors for ATP, termed P2 purinoceptors, activation of which leads to PI breakdown, increases in [Ca\(^{2+}\)]\(_i\) and the release of eicosanoids. The work presented in Chapter 3 has extended these observations to examine and characterise nucleotide-stimulated eicosanoid release from cultured astrocytes. These results showed that a range of purine and pyrimidine di- and triphosphate nucleotides were capable of inducing TXA\(_2\) release from these cells and that subtypes of P2 purinoceptors were involved in this phenomenon. Furthermore, a range of these agents were capable of increasing [Ca\(^{2+}\)]\(_i\) and stimulating PI turnover. The precise identity of those purinoceptors activated by the different nucleotides remains to be elucidated, and in the absence of specific receptor antagonists, classification must be dependent upon agonist potency studies; however, the response appeared to involve P2Y, P2U and possibly other as yet unidentified classes of nucleotide receptor.

There were curious discrepancies between the various nucleotides in terms of those signalling parameters examined and the functional significance of this has yet to be explained. For example, the change in [Ca\(^{2+}\)]\(_i\) evoked by UDP was relatively small, yet, along with UTP, was the most effective nucleotide examined in terms of TXA\(_2\)
release. Furthermore, the rapid desensitisation of the change in [Ca$^{2+}$]$_i$ evoked by CTP and TTP remains unexplained, yet these were also capable of stimulating TXA$_2$ release from astrocytes. Overall, the results presented in Chapter 3 have demonstrated that ATP is not unique among the purine and pyrimidine nucleotides in its ability to stimulate eicosanoid release from astrocytes. Moreover, all those nucleotides which stimulated TXA$_2$ release were found to evoke an increase in [Ca$^{2+}$]$_i$, albeit to varying extents and with differing characteristics. Ca$^{2+}$ is considered a requirement for eicosanoid release, however, these results suggest that the mechanisms controlling these processes are perhaps even more complex than was previously thought.

Results presented in Chapter 3 after cultures were incubated in serum-free medium showed that this regime resulted in a reduction in the total release of TXA$_2$ by the cells in culture. It has been suggested that serum-free conditions result in an absence of COX-II within the astrocytes. COX-II is inducible and this finding may have significance in the induction and maintenance of reactive gliosis. The targets for those eicosanoids which are released from these cells is unknown, but these results indicate their release may be part of a response of the CNS to a disease state or damage.

The results presented in Chapter 3 demonstrated that eicosanoid release was reduced when astrocyte cultures were grown in the absence of microglia, which indicates that microglial cells contribute to the total concentration of TXA$_2$ released from a culture of astrocytes. Microglial cell activation is also believed to be important in the induction of the reactive state, and in addition to eicosanoid release, may be responsible for the release of cytokines and growth factors. To this end, experiments carried out on cultures of pure microglial cells would be extremely interesting.

The reasons why the vast majority of PI-linked receptors in astrocytes fail to stimulate eicosanoid release is still unknown. It was reasoned that there may have been
differences in the characteristics and extent of the change in \([Ca^{2+}]_i\) evoked by ATP and NA, this increase in \(Ca^{2+}\) being a requirement for eicosanoid release. The results presented in Chapter 4 illustrate, however, that there were no discernible differences between ATP and NA in terms of the characteristics of the \(Ca^{2+}\) transients they evoked. The inhibition of ATP-stimulated \(TXA_2\) release by NA has been reported previously (Pearce et al., 1989). The results in Chapter 4 demonstrated that this inhibitory effect by NA was not unique to ATP and is a phenomenon that affected a range of other eicosanoid-stimulating nucleotides too. It is interesting to speculate that since nucleotides may be one of the triggers involved in the induction of reactive gliosis \(in vivo\), that part of this process involves the synthesis and release of eicosanoids. The majority of other PI-linked receptors may not be associated with the reactive state, and hence activation does not result in eicosanoid release.

It has been a general assumption that the requirement for \(Ca^{2+}\) in eicosanoid synthesis has involved the mobilisation of \(Ca^{2+}\) from \(IP_3\)-sensitive pools. We were interested to examine this hypothesis in detail, and investigate whether \(IP_3\)-insensitive \(Ca^{2+}\) pools were present in astrocytes, and to determine their contribution, if any, to eicosanoid release. The work presented in Chapter 5 is the first demonstration that an \(IP_3\)-insensitive ryanodine-activated \(Ca^{2+}\) pool is present in astrocytes. The contents of this pool of \(Ca^{2+}\) cannot be mobilised by caffeine, which suggests that they possess the caffeine-insensitive \(RYR3\) subtype, however, it also remains a possibility that these cells possess a unique class of astrocytic RYR. If astrocytes do possess the \(RYR3\) subtype, it remains to be determined whether they are present in cells constitutively, or are induced by factors unknown. RYR-evoked changes in \([Ca^{2+}]_i\) influence neither eicosanoid release nor the effect of eicosanoid stimulating agents such as ATP.

The role of RYR-sensitive \(Ca^{2+}\) pools in astrocytes remain unknown, and this reflects the current understanding of \(Ca^{2+}\) homeostasis in non-excitable cells in general. It is
not known, for example, why in some cells IP$_3$ and RYRs appear to be co-localised, while in others they are segregated into separate stores. It has been postulated that their presence may reflect a common evolutionary link. Whatever the functional significance of RYRs in astrocytes are, they do not appear not be involved in eicosanoid synthesis.

It is still not known whether astrocytes respond to nucleotides in vivo, and if so, under what conditions. Furthermore, it is not known whether nucleotides are actually released under normal conditions within the CNS, but it is interesting to note that they are released if cells are damaged in some way (Burnstock, 1993; Gordon, 1986). It is possible that it is this source of nucleotides which targets astrocytes in the CNS. Evidence in support of this notion has come from studies showing that a variety of nucleotides increase GFAP content and DNA synthesis (Neary et al, 1991; 1994) and that P2 purinoceptors in particular stimulate the activity of mitogen-activated protein kinase (Neary and Zhu, 1994), both of these events are associated with the proliferative response of astrocytes to damage. Nucleotides may be involved in the induction and maintenance of reactive gliosis in vivo. Reactive gliosis is frequently associated with conditions such as trauma, stroke and demyelinating disorders, and is believed to be involved in CNS repair and regeneration. Because ATP (and other nucleotides) are present in high micromolar concentrations in cells and are released following tissue injury (Forrester and Williams, 1977; Gordon, 1986), extracellular nucleotides may induce changes in astrocytic morphology and contribute to reactive gliosis. The significance of astrocyte-derived eicosanoids has yet to be determined, but they may also be involved in the reactive state. One possibility is that they function in an immunoregulatory role, or serve to modulate the flow of blood to a region of trauma. Our understanding of reactive gliosis and the factors which regulate it are of fundamental importance, since this condition may be associated with brain pathologies.
Future Directions

The hypothesis that astrocytes in culture reflect the reactive state is clearly a concept that requires more attention. Moreover, the potential role of ATP and the other nucleotides in the induction and maintenance of the various stages of gliosis ought to be examined. It would be particularly interesting, for example, to compare and contrast nucleotide-evoked changes in $[\text{Ca}^{2+}]_i$ and eicosanoid release from acutely isolated astrocytes with those recorded from cultured cells maintained in vitro. These experiments could be extended to include the effect of cerebral stab wounds (a model of reactive gliosis), and examine acutely isolated astrocytes from animals which have experienced actual neurological insults such as occlusion of the middle cerebral artery, and hence ischaemia.

Nucleotide-evoked changes in $[\text{Ca}^{2+}]_i$ in terms of analysis of wave propagation and waveform would identify any differences in initiation, foci and characteristics of the various Ca$^{2+}$ transients to be elucidated. Astrocytes are ideal for spatio-temporal analysis since their flat epitheloid morphology provides ideal optical conditions to follow spatial changes in fluorescence. Furthermore, confocal microscopy of brain slices would enable three-dimensional imaging of complete tissue samples, and enable astrocyte function to be examined in the presence of neurons. Not only is Ca$^{2+}$ an integral component of receptor-mediated signal transduction events, it is also ultimately involved in cell death. Since reactive gliosis can often result in the death of cells around the foci of an insult, a greater understanding of Ca$^{2+}$ transients and their induction by nucleotides is required. Moreover, since glia do not appear to be heterogeneous with respect to their response to injury in the CNS (Hoke and Silver, 1994), studies on regions of the CNS where regeneration occurs in comparison to those on areas where regeneration fails would be extremely useful.
Immunohistochemical studies using antibodies raised against the various types of RYR could also be carried out in an attempt to identify the subtype(s) of RYR present in astrocytes and under what conditions they are expressed. Similar experiments could be performed in an attempt to identify the level of expression of the COX isozymes under various parameters of serum depletion. Moreover, it would be interesting to elucidate to what extent these factors are dependent upon culture conditions, and to this end experiments carried out on acutely isolated astrocytes and intact brain slices would be useful.

In conclusion, this thesis has presented functional evidence of different purine and pyrimidine nucleotides in astrocytes. Evidence has been presented which suggests that various subtypes of purinoceptor are expressed in astrocytes, and that differences exist between the nucleotides in terms of change in $[\text{Ca}^{2+}]_i$, PI turnover and TXA$_2$ release. Finally, a cytoplasmic Ca$^{2+}$ pool activated by ryanodine has been reported in these cells, but which do not appear to be involved in eicosanoid release. The circumstances and relevance of these findings in terms of astrocyte function in vivo has been discussed.
References


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