The characterisation of cloned P2Y receptors transfected into 1321N1 astrocytoma cells

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by

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Contents

Abstract

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The P2Y receptors are a growing family of G protein-coupled receptors for both adenine and uracil nucleotides. Several of these receptors have recently been cloned and transfected into null cells for detailed examination. Work in this thesis was primarily involved with the characterisation of four of these receptors, the turkey and bovine $P2Y_1$, and the human $P2Y_2$ and $P2Y_4$ receptors, transfected into the human astrocytoma cell line, 1321N1. It also describes the preliminary characterisation of two vascular smooth muscle cell types, and the generation of $P2Y_1$-GST fusion proteins for the production of antibodies.

1321N1 cells were found to release nucleotides in response to sheer stress, but this was overcome by adaptation of the stimulation method. 1321N1 cells expressing the four receptors were assayed for PLC activity, and the rank order of agonist potency for each receptor was generally consistent with reports in the literature. Nucleotide interconversion at the surface of 1321N1 cells was also examined indirectly using the ecto-ATPase inhibitor ARL 67156 and hexokinase. Although there was no significant effect of nucleotide breakdown on the agonist concentration-responses, it was found that upstream conversion of nucleoside diphosphates to triphosphates occured, giving the false impression of activity of nucleoside diphosphates.

The antagonists suramin, PPADS and NF023 were examined. Suramin was a competitive antagonist at the $P2Y_1$ receptor, had weaker effects at the $P2Y_2$ receptor, and was inactive at the $P2Y_4$ receptor. PPADS was selective for the $P2Y_1$ receptor over the $P2Y_2$ and $P2Y_4$ receptors, and NF023 appeared to have non-competitive actions. ATP was found to antagonise the response to UTP at the $P2Y_4$ receptor. The time course of PLC activation appeared to rapidly desensitise with each of the receptors. Pertussis toxin reduced the responses to the $P2Y_2$ receptor, but not to the $P2Y_1$ and $P2Y_4$ receptors, and responses to each of the receptors was inhibited by activation of protein kinase C.

A preliminary study with human saphenous vein smooth muscle cells showed that responses to nucleotides were small and difficult to characterise. In contrast, nucleotides elicited a robust response at spontaneously hypertensive rat smooth muscle cells, consistent with action at a $P2Y_4$ receptor.

In conclusion, this thesis has provided a detailed examination of several cloned P2Y receptors and also demonstrated how information gained from such systems can aid the characterisation of native receptors.

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Abbreviations

α,β-MeATP	α,β-methylene adenosine triphosphate
β,γ-MeATP	β,γ-methylene adenosine triphosphate
βARK	β-adrenoceptor kinase
[^{3}H]InsP$_x$	total [^{3}H]inositol (poly)phosphates
2MeSATP	2-methylthio-adenosine triphosphate
ADP	adenosine diphosphate
AII	angiotensin II
AMP	adenosine monophosphate
ARL 67156	6-N,N-diethylβ,γ-dibromomethylene-D-ATP
ATP	adenosine triphosphate
ATPγS	adenosine 5'-O-(2-thiodiphosphate)
BAECs	bovine aortic endothelial cells
BSS	balanced salt solution
BzATP	benzoylbezoyl adenosine triphosphate
Ca^{2+}	calcium ion
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
DAG	sn-1,2-diacylglycerol
DMEM	Dulbeco's modified Eagles medium
DMSO	dimethylsulphoxide
dpm	disintegrations per minute
EDTA	ethylenediaminetetraacetic acid
et al	*et alia*
FCS	foetal calf serum
G protein	guanine nucleotide-binding protein
GDP	guanosine diphosphate
GS	glutathione-sepharose
GST	glutathione S-transferase
GTP	guanosine triphosphate

GTPγS	guanosine 5'-O-(3-thiophosphate)
HEPES	N-2 hydroxyethylpiperazine-N'-2-ethanesulphonic acid
hSV-SMCs	human saphenous vein smooth muscle cells
$Ins(1,3)P_2$	inositol (1,3) bisphosphate
$Ins(1,3,4)P_3$	inositol (1,4,5) trisphosphate
$Ins(1,3,4,5)P_4$	inositol (1,3,4,5) tetrakisphosphate
$Ins(1,4)P_2$	inositol (1,4) bisphosphate
InsP	inositol monophosphate
IPTG	isopropylthio-β-D-galactoside
K_D	equilibrium disociation rate constant
log	logarithm to base 10
MAPK	mitogen activated protein kinase
MEK/MAPKK	mitogen activated protein kinase kinase
NA	noradrenaline
NAD	nicotinamide adenine dinucleotide
NDPK	nucleoside diphosphokinase
NECA	5'-N-ethylcarboxanidoadenosine
NF023	di(8-benzamidonapthalene-1,3,5-trisulphonic acid)
NO	nitric oxide
PAGE	polyacrilamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PGI_2	prostacyclin
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PPADS	pyridoxalphosphate-6-azophenyl-2,4,-disulphonic acid
$PtdIns(4,5)P_2$	phosphatidylinositol (4,5) bisphosphate
PTX	pertussis toxin
SDS	sodium dodecyl sulphate

SHR	spontaneously hypertensive rat
TCA	trichloroacetic acid
TPA	12-O-tertadecanoyl phorbol 13-acetate
UDP	uridine diphosphate
UMP	uridine monophosphate
UTP	uridine triphosphate
VSMCs	vascular smooth muscle cells
WKY	Wistar Kyoto rat

Chapter 1. Introduction

1.1 History and classification of purinoceptors

The first reported biological effect of adenine compounds was the slowing of the guinea-pig heart after intravenous injection of ATP (Drury & Szent-Gyorgyi, 1929). The first evidence for ATP as a neurotransmitter came in 1959, when Holton demonstrated the release of ATP from sensory nerves, and in 1972, Burnstock proposed that ATP was the molecule responsible for the non adrenergic, non cholinergic transmission of nerves supplying the gastrointestinal tract, calling it 'purinergic' transmission.

The receptors for adenine compounds were termed 'purinoceptors' and were initially subdivided into P1 and P2 purinoceptors on the basis of a number of criteria (Burnstock, 1978). At the P1 purinoceptor, the agonists adenosine and AMP were more potent than ADP and ATP. P1 purinoceptors were also coupled to adenylate cyclase, and were competitively antagonised by low concentrations of methylxanthines. In contrast, P2 purinoceptors were activated preferentially by ATP and ADP, were not antagonised by methylxanthines, and did not alter cAMP levels.

In 1985, Burnstock & Kennedy proposed the first subdivision of P2 purinoceptors into P_{2X} and P_{2Y} subtypes, based on differences in rank order of agonist potency and nature of reponses to ATP. The P_{2X} purinoceptor was characterised as mediating vasoconstriction and contraction of visceral smooth muscle, and as being selectively stimulated by the ATP analogue α,β-methylene ATP (see section 1.4.2). Contrastingly, the P_{2Y} purinoceptor mediated vasodilation and relaxation of intestinal smooth muscle, and was potently activated by 2-methylthioATP (section 1.4.2). Since then, many studies have shown P_{2X} receptors to be ligand gated ion channels and P_{2Y} receptors to be G protein coupled, seven transmembrane domain receptors.

Gordon proposed two further P2 purinoceptor subtypes in 1986. These were the P_{2T} purinoceptor, which was ADP selective and involved in platelet aggregation, and the P_{2Z} purinoceptor, which was activated by ATP^{4-} and present on many cells of the immune system. It soon became clear that in a wide variety of tissues, uridine nuleotides were as active as adenosine nucleotides. This lead to the proposal of a P_{2U} purinoceptor that was activated equipotently by UTP and ATP (O'Connor et al, 1991;

O'Connor, 1992). As uridine is a pyrimidine and not a purine, the term 'nucleotide receptor' was also widely used. There were also reports of receptors that responded to UTP, but not ATP, e.g. in the contraction of cerebral vascular tissue (Von Kugelgen & Starke, 1990), and in 1989, Seifert & Schultz postulated the existance of a pyrimidinoceptor. Finally, the molecule diadenosine tetraphosphate was shown to be stored and released from synaptic vesicles (Flodgaard & Klenow, 1982; Pintor-Torres et al, 1991), and to bind to receptors tentatively named P_{2D} receptors (Hilderman et al, 1991; Castro et al, 1992).

However, this classification of P2 purinoceptors has become outdated for several reasons. Firstly, it does not conform to the guidelines layed down by the International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification (NC-IUPHAR), which suggests that classification should be based on mammalian systems (see Fredholm et al, 1997). Secondly, there are receptors that preferentially bind pyrimidine rather than purine nucleotides, and finally, the advent of purinoceptor cloning has paved the way for a more logical basis for classification, based on molecular structure.

The NC-IUPHAR has made a number of suggestions for a revised nomenclature. Firstly, the family of receptors should be named P2 receptors rather than P2 purinoceptors. A P2 receptor is now defined as a receptor for a purine or pyrimidine nucleotide (or dinucleotide) (Fredholm et al, 1997). The second suggestion involves a change in subtype classification. In 1986, Dubyak summerised the evidence that ATP exerts its effects through two different transduction mechanisms: intrinsic ion channels and G protein coupled receptors. This information, together with the cloning of P2 receptors, lead Abbrachio & Burnstock (1994) to propose that P2 receptors should be classified in two families: G protein coupled receptors termed P2Y and intrinsic ion channels termed P2X, and that newly cloned subtypes should be numbered sequentially e.g. $P2Y_1$, $P2Y_2$, $P2Y_3$ etc. This scheme has now become widely accepted, and the P2X receptor family now consists of 7 subtypes ($P2X_{1-7}$), and the P2Y receptors comprise up to 7 proposed subtypes ($P2Y_{1-7}$).

The nomenclature used in this thesis will follow the general principles suggested by the IUPHAR Nomenclature Committee.

1.2 Sources of extracellular nucleotides

Although ATP is present in millimolar concentrations in the cytosol of all cells, extracellular levels are normally maintained at extremely low levels by several mechanisms. Firstly, the predominant cytosolic form of ATP, MgATP, is unable to cross lipid bilayers, and secondly, ubiquitous ectonucleotidases rapidly hydrolyse extracellular nucleotides. Thus, appreciable levels of extracellular ATP occur only transiently and in response to specific physiological and/or pathological conditions. There are 3 major sources of extracellular ATP; release from ruptured cells, vesicular release and non-vesicular release.

1.2.1 Release from ruptured cells

The most obvious source of extracellular ATP is cytosolic ATP, which is present at concentrations of 3 to 5 mM in most cells. Sudden breakage of intact cells releases ATP at high concentrations into the extracellular space, for example, during blood vessel trauma. ATP released from ruptured endothelial cells can act at P2 receptors expressed by most of the cell types that must be rapidly activated to minimise blood loss and invasion of pathogens at sites of tissue damage, including platelets, neutrophils, macrophages, vascular endothelial cells and vascular smooth muscle (see sections 1.8.1 and 1.8.3).

1.2.2 Vesicular release

1.2.2.1 Vesiclular storage

Many studies have demonstrated the costorage of ATP and ADP with other transmitters. For example, ATP is stored with noradrenaline (NA) in the synaptic vesicles of adrenergic nerves in both the peripheral and central nervous system (Thureson-Klein et al, 1979; Whittaker, 1982). ATP is also stored with NA in adrenal chromaffin granules (Wiley et al, 1990) at a concentration in the 100 mM range (Hillarp & Thieme, 1959), and in a ratio from 3:1 to 20:1. Other nucleotides, including ADP, UTP, GTP and CTP are also present in chromaffin granules, albeit at

much lower concentrations. Platelet dense granules have been shown to contain ADP/ATP in the 100 mM range (Carty et al, 1981), and also UTP, although at a concentration of around one tenth of ATP (Goetz et al, 1971).

1.2.2.2 Exocytotic release from non-neuronal cells

Nonneuronal release of ATP can be stimulated by many agonists. For example, stimulation of chromaffin cells by acetylcholine at nicotinic receptors causes ATP release which is dependent upon influx of extracellular Ca^{2+} (Cheek et al, 1989). Release of ATP has been extensively studied in platelets (Born & Kratzer, 1984), and has long been utilised as an index of platelet activation (Born, 1962). It has been calculated that intact human platelets contain approximately 4.5 µmol of releasable ATP plus ADP per 10^{11} cells. Given a normal platelet count of 3×10^{8} cells ml^{-1} blood, a concerted degranulation of platelets could transiently raise the serum concentration of ATP/ADP to the 50 µM range (Meyers et al, 1981). Moreover, platelet aggregation occurs prior to degranulation, perhaps resulting in an even greater local nucleotide concentration.

1.2.2.3 Release from nerve terminals

In 1959, Holton demonstrated the neuronal release of ATP from sensory nerves following antidromic stimulation of sensory nerve fibres in the skin of rabbit ear (Holton, 1959). Since then, release of ATP from nerves has been measured directly in many different systems. Measurements have been obtained using a number of techniques including the sensitive luciferin-luciferase luminometric assay (Burnstock et al, 1978), high performance liquid chromatography with derivatised flourescently tagged nucleotides (Westfall et al, 1990), and the measurement of tritium overflow after preincubation with [^{3}H]-adenine or [^{3}H]-adenosine (White, 1982). ATP release has also been demonstrated indirectly by pharmacological means, whereby neurogenic responses are selectively blocked in parallel with those due to exogenous ATP (Sneddon et al, 1982). There is substantial evidence in support of the hypothesis that ATP is packaged and released as a cotransmitter with NA in certain sympathetically innervated smooth muscles, e.g. vas deferens and some blood velssels

(see Von Kugelgen & Starke, 1991). It appears, however, that the ratio of released ATP and NA is not consisitent. Simple changes in the frequency or chain length of stimulation can change the composition of the released co-transmitter mixture (von Kugelgen & Starke, 1991). Also, activation of prejunctional α_2-adrenoceptors preferentially reduces the release of [^{3}H]noradrenaline, and activation of prejunctional A_1 purinoceptors preferentially reduces ATP release (Starke et al, 1996), suggesting that NA and ATP are not stored in the same proportions in all vesicles in a homogeneous population of axons (see Burnstock, 1990).

1.2.3 Nonvesicular release of ATP

ATP is also released from intact, nonexcitable cells which possess no regulated exocytotic pathway for nucleotides e.g. from endothelial cells and fibroblasts in response to sheer stress (Milner et al, 1990a,b; Grierson & Meldolesi, 1995). Vascular endothelial cells have also been shown to take up uridine, and to release UTP, UDP, UMP and uridine upon increased fluid shreer stress (Saiag et al, 1995). The process by which this passage across intact membranes occurs has not yet been determined. One proposal is that ATP is pumped out of cells by members of the ABC transporter family, such as the multidrug resistance (MDR) protein or the cystic fibrosis transmembrane conductance regulator (CFTR) (Abraham et al, 1993; Reisin et al, 1994; see Brake & Julius, 1996).

Endothelial cells are able to release ATP in response to increases in perfusion flow rate and hypoxia (Milner et al, 1990a,b; Bodin et al, 1992). It is likely that ATP released by endothelial cells during periods of increased flow acts on P2 receptors further downstream to take part in the vasodilation which occurs in response to increased sheer stress (Ralevic et al, 1992).

1.3 P1 purinoceptors

Soon after Burnstock (1978) proposed the division of purinoceptors into the P1 and P2 subfamilies, Van Calker et al (1979) suggested the subdivision of the P1 family into A_1 and A_2 subtypes. This was based on work with cultured nerve cells,

where it was demonstrated that adenosine and its analogues either inhibited or stimulated adenylate cyclase, and that the order of potency in eliciting these responses was different. Adenosine was found to be more potent at the A_1 subtype, and 5'-*N*-ethylcarboxanidoadenosine (NECA) was selective for the A_2 subtype. In 1986, Bruns et al further subdivided the A_2 purinoceptors into the A_{2a} and A_{2b} subtypes, showing that NECA bound with high affinity to A_2 receptors in the striatum (A_{2a}), but with low affinity to A_2 receptors in fibroblasts (A_{2b}).

A third P1 subtype, the A_3, was cloned in 1992 by Zhou et al. It is also linked to adenylate cyclase, but unlike the other P1 subtypes, it does not generally interact with xanthine antagonists (Carruthers & Fozard, 1993). However, a new compound, 1-propyl-3-(3-iodo-4-aminobenzyl)-8-(4-oxyacetate)phenylxanthine, has been shown to antagonise A_3 receptor-mediated hypotensive responses in the rat (Fozard & Hannon, 1994).

Research in the P1 purinoceptor field has been assisted by the availability of selective, high affinity agonists and antagonists. Two selective antagonists, 8-*p*-sulphophenyltheophylline (8-SPT) and 8-phenyltheophylline, have been extensively used to block A_1 and A_2 purinoceptors. These compounds have assisted the study of P2 receptors in tissues where P1 and P2 receptors coexist, as breakdown products of P2 agonists can potentially also act at P1 receptors.

1.4 Tools used in P2 receptor classification

In order to conclusively classify a receptor pharmacologically, the affinity of agonists/antagonists for that receptor must be obtained (Kenakin et al, 1992). Functional studies using only agonists give information on potency, but not affinity. Changes in receptor number and efficacy can alter the observed agonist potency, which may result in different agonist potency values for the same receptor in different tissues. Radioligand binding studies can be utilised to obtain agonist affinity, and several attempts have been made to design a reliable binding protocol for P2 receptors.

1.4.1 Radioligand binding studies at P2 receptors

Successful radiolabelling of cell surface receptors has usually depended on the availability of ligands of high affinity, stability and specificity. These ligands are usually neutral antagonists, because unlike agonists or inverse agonists, GTP-shift (which alters the affinity of receptor binding) does not occur. Such tools are not yet available for interaction with P2 receptors. There are no selective antagonists, and the several compounds that do competitively inhibit P2 receptors (e.g. suramin) do so with only micromolar affinity and interact with many other proteins. Agonists of P2 receptors also present problems. Their binding affinities are only slightly higher than their affinities for other ATP binding proteins, and they are subject to hydrolysis by ectonucleotidase. Despite this, there have been many attempts to establish a reliable binding assay for P2 receptors.

A radioligand binding assay using [^{35}S]ADPβS was developed by Harden and coworkers (Cooper et al, 1989). They reported specific labelling of the P2Y receptor responsible for phospholipase C activation in turkey erythrocyte membranes. However, subsequent work using newly synthesised, highly selective $P2Y_1$ agonists (Fischer et al, 1993) has cast doubt on this initial assertion (see Harden et al, 1995). [^{35}S]ADPβS has also been used with endothelial cells in culture (Wilkinson & Boarder, 1995), but in this study it was found that most binding was at non-$P2Y_1$ sites.

The photoaffinity label, [^{32}P]benzoylbenzoyl ATP ([^{32}P]BzATP) has been used to competitively label, and on exposure to UV light, covalently incorporate radiolabel into $P2Y_1$ receptors on turkey erythrocyte plasma membranes (Boyer & Harden, 1989). In a study by Erb et al (1993), [^{32}P]BzATP was shown to also label $P2Y_2$ receptors transfected into K562 human leukemia cells. However, it apparently does not label the receptor at the agonist binding site, because labelling was not inhibited by UTP.

More recently, studies have been performed using [^{35}S]ATPαS as the radiolabelled ligand. Simon et al (1995a,b) have demonstrated that overexpression of cloned chick $P2Y_1$ receptor in COS-7 cells confers a [^{35}S]ATPγS high-affinity binding site that exhibits the general pharmacological specificity of a P2Y receptor. High-affinity agonist binding to G protein coupled receptors is thought to require a stoichiometric presence of the G protein to which the receptor couples. However,

Simon et al report a density of expressed receptors of 8 pmol mg^{-1} protein, which is a level that exceeds what might be expected for the presence of $G_{q/11}$ in COS-7 cells (see Harden et al, 1995). Despite this, Webb et al (1996c) and Akbar et al (1996a,b) have used [^{35}S]ATPαS binding to designate two novel P2Y receptors, the $P2Y_5$ and the $P2Y_7$. The validity of these results has recently been challenged by Schachter & Harden (1997). They show that [^{35}S]ATPαS binding to membranes from non-transfected COS-7 cells is inhibited by adenine nucleotide analogues with similar potencies to those obtained for membranes from 6H1 and $P2Y_7$ receptor-transfected COS-7 cells.

1.4.2 Agonists

To aid discrimination between P2 receptor subtypes, many analogues of ATP have been produced. Modifications include additions to the second carbon of the adenine ring, such as 2-methylthioadenosine triphosphate (2MeSATP) and modifications to the polyphosphate chain such as replacement of interphosphate linkages with a methylene link to give adenosine 5'-(α,β-methylene) triphosphate (α,β-MeATP). Also, an anionic oxygen in the polyphosphate chain has been replaced with sulphur or flourine to yield adenosine 5'-O-(1-thiotriphosphate) (ATPαS) or adenosine 5'-O-(2-flourodiphosphate) (ADPβF). Other analogues have been specifically designed to resist degradation by ectonucleotidases.

Figure 1.1 shows the structure of some commonly used P2 receptor agonists.

1.4.3 Antagonists

It has been strongly emphasised that antagonists, rather than agonists, are the prefered tools for pharmacological classification (Kenakin et al, 1992). This is because apparent agonist potency depends not only on agonist binding to the receptor, but also on the entire signal transduction machinery. The lack of specific, high affinity antagonists of P2 receptors has already been addressed. Despite this, some compounds have proved useful in the characterisation of P2 receptors (see Figure 1.2 for the structure the antagonists used in this thesis).

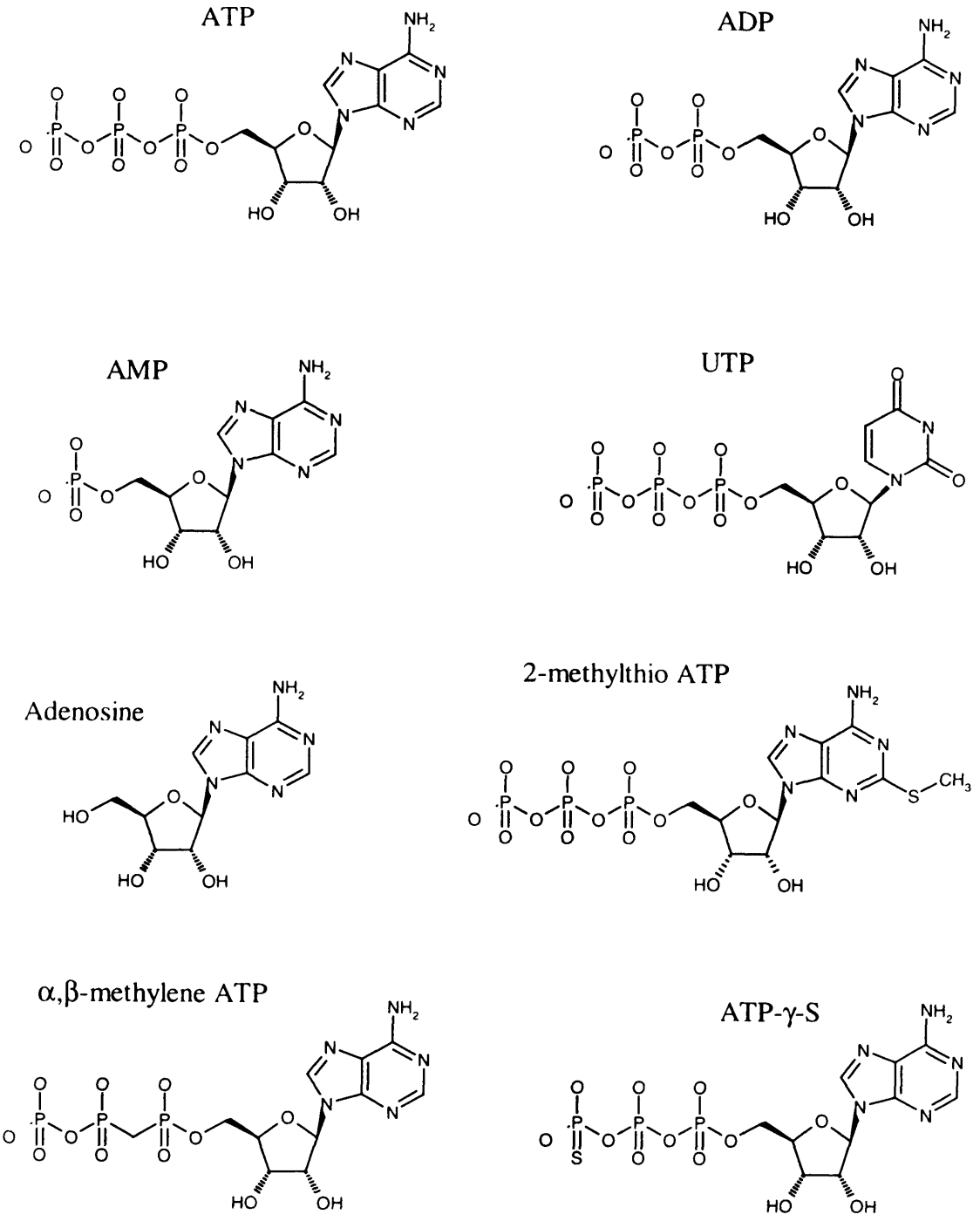
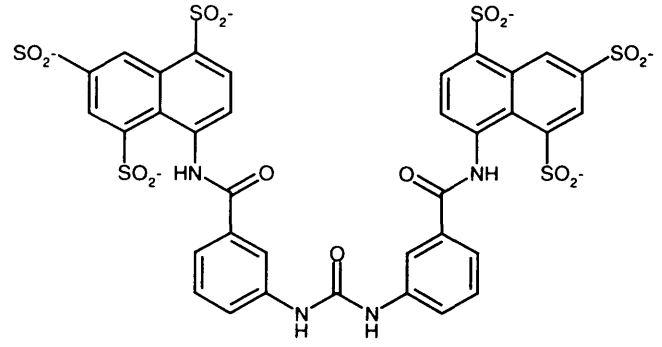
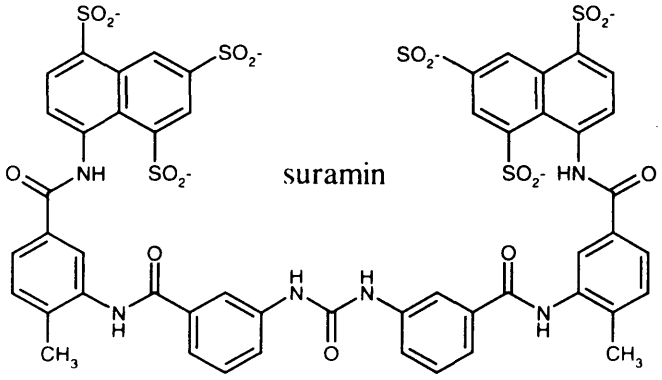


Figure 1.1
Structures of some commonly used P1 and P2 receptor agonists.

NF023





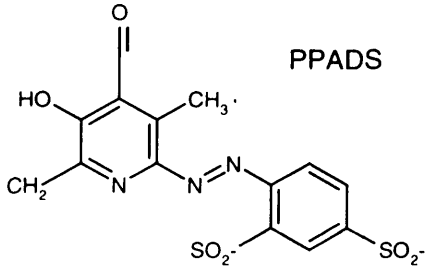


Figure 1.2
Structure of the P2 receptor antagonists used in this thesis.

1.4.3.1 Suramin

Suramin is a highly negatively charged sulphonic acid derivative of naphthalene and has a history as a trypanocidyl agent dating back to 1916. It has been used as a P2 antagonist in many studies, including visceral smooth muscle preparations, various blood vessels, platelets and cultured coeliac ganglion neurons (Dunn and Blakeley, 1988; Hoyle et al, 1990; Leff et al, 1991; Evans et al, 1992a; Hourani et al, 1992; Brake et al, 1994). Suramin has been described as non-selective for P2X and $P2Y_1$ receptors ($pA_2 \approx 5.0$; Holye et al, 1990) and has been shown to inhibit P_{2T}-mediated platelet aggregation with a similar apparent pA_2 value (Hourani et al, 1992). There have been reports in the literature of both suramin sensitive and insensitive $P2Y_2$ receptors (Dainty et al, 1994; Wilkinson et al, 1994a,b), suggesting the existence of multiple $P2Y_2$ receptor subtypes.

Suramin, however, is notoriously non-specific, and has many other biological effects (Voogd et al, 1993). It is a potent inhibitor of ectonucleotidases, the extracellular enzymes responsible for nucleotide degradation (Hourani & Chown,1989; Beukers et al, 1995; discussed in detail in section 1.7.1.5.2). It has anti-reverse transcriptase and anti-proliferative activities through inhibition of the binding of various growth factors to their cell surface receptors (e.g. fibroblast growth factor 2, platelet-derived growth factor, epidermal-derived growth factor and interleukin 3; see Nakajima et al, 1991.) Suramin has also been shown to suppress [^{35}S]GTPγS binding to purified, recombinant G protein α subunits (selective for Gsα), an effect that is due to inhibition of GDP release (Freissmuth et al, 1996; Beindl et al, 1996). In this study, it was found that suramin did not interfere with the interaction between α subunits and G protein βγ dimers but competed with binding of the effector in a competitive manner. Suramin also decreases the maximal response to isoprenaline at β-adrenoceptors, inhibiting the activation of phospholipase C in a non-competitive manner (Boyer et al, 1994). Despite this array of non-specific effects, suramin remains one of the most commonly utilised P2 receptor antagonists.

1.4.3.2 NF023

NF023, a novel suramin analogue, is the symmetrical 3'-urea of 8-(benzamido) napthalene-1,3,5-trisulphonic acid. It was introduced as a specific, directly interacting, competitive P2 receptor antagonist, being highly selective for P2X subtypes over $P2Y_1$ and $P2Y_2$ receptors with a pA_2 value of 5.96 ± 0.04 at P2X receptors in rabbit saphenous artery. (Ziyal et al, 1997; Lambrecht et al, 1996). However, like suramin, it is beset by many non-specific biological interactions. NF023 is an ecto-ATPase inhibitor with an activity similar to suramin (Beukers et al, 1995) and it also uncouples Gα subunits from their effector enzymes (Freissmuth et al, 1996; Beindl et al, 1996). Unlike suramin, which is selective for recombinant $G_{s\alpha}$ subunits, NF023 is selective for recombinant $G_{i\alpha\text{-}1}$ subunits. However, calcium current inhibition by α_2-adrenergic and muscarinic receptors was greatly reduced only when 100 μM NF023 was applied intracellularly, suggesting that uncoupling of G proteins by suramin and NF023 may have limited significance in studies using whole cells.

1.4.3.3 PPADS

Pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS), a novel compound, was introduced as a P2X antagonist in rabit vas deferens with a pA_2 of 6.4 (Lambrecht et al, 1992). Many subsequent studies have demonstrated antagonism at P2X receptors in different tissue types (e.g. Ziganshin et al, 1993; Windscheif et al, 1994). However, several examples of actions at P2Y receptors have emerged, e.g. at the phospholipase C -coupled, turkey erythrocyte $P2Y_1$ (pA_2 of 5.9 ± 0.1) (Boyer et al, 1994). PPADS has also been shown to differentiate between $P2Y_1$ and $P2Y_2$ receptors. In bovine aortic endothelial cells, PPADS antagonises responses to $P2Y_1$ agonists with a similar potency to that reported for P2X receptors, but not to $P2Y_2$ agonists (Brown et al, 1995). Since then, other groups have made this observation (e.g. Ralevic & Burnstock, 1996; Mateo et al, 1996).

PPADS, like almost all other P2 antagonists, also reduces ATP breakdown. For example, on bovine pulmonary artery endothelium, it inhibits ectoATPase with an IC_{50} of 4.0 (Chen et al, 1996).

1.4.3.4 P_{2T} antagonists

Recently, the search for selective, high affinity antagonists has produced two novel ATP analogues, 2-propylthio-D-β,γ-diflouromethylene ATP (ARL 66096, formerly FPL 66096), and 2-propylthio-D-β,γ-difchloromethylene ATP (ARL 67085, formerly FPL 67085). These compounds are potent P2T antagonists with apparent pA_2 values of 8.7 and 8.9 respectively. However, antagonism does not appear to be purely competitive, and the compounds are also weak antagonist at $P2Y_1$ receptors and P2X antagonist (Humphries et al, 1994, 1995a,b).

1.4.3.5 Adenosine-3'-phosphate-5'-phosphosulphate (A3P5PS)

Adenosine-3'-phosphate-5'-phosphosulphate (A3P5PS) has been described as a $P2Y_1$ receptor antagonist by Boyer et al (1996a), with a pA_2 value of 6.46 ± 0.17. However, it also produces small, saturable stimulatory effects at the $P2Y_1$ receptor in turkey erythrocte membranes, but not at the cloned human $P2Y_1$ in 1321N1 cells. A3P5PS also appears to be specific for the phospholipase C coupled $P2Y_1$ receptor, as no agonistic or antagonistic effects were observed on the adenylyl cyclase coupled P2Y receptor of C6 glioma cells, or on cloned $P2Y_2$, $P2Y_4$, or $P2Y_6$ receptors in 1321N1 cells.

1.4.3.6 Dyes

The anthraquinone dye, reactive blue 2, is thought to be a selective antagonist for $P2Y_1$ over P2X receptors. However, it is only effective over a narrow concentration range (10–50 μM), beyond which it has non-specific effects (Burnstock & Warland, 1987). Whereas this drug used to be of 60 % purity only, it more recently became available in a higher purity grade (Bultmann & Starke, 1994). Reactive red 2 is a potent (0.1–10 μM) $P2Y_1$ antagonist in guinea-pig taenia coli, being 15 fold selective over rat vas deferens P2X receptors (Bultmann & Starke, 1995). Evans blue (10–100 μM) and Trypan blue (3.2–320 μM), have been shown to antagonise P2X receptor mediated contractions in the rat vas deferens (Bultmann & Starke, 1993; Bultmann et al, 1994), but Evans blue has also been shown to have some non-specific effects (Khakh et al, 1994).

1.4.3.7 Irreversible antagonists

2,2'-pyriylisatogen tosylate (PIT) was originally designed as a selective antagonist of ATP receptors. PIT (10-50 μM) reduced relaxations to ATP in guinea-pig isolated taenia caeci in a time and concentration dependent manner consistent with an irreversible antagonism of postjunctional ATP receptors on smooth muscle (Spedding et al, 1975; 1976). However, subsequent studies have shown that at lower concentrations, PIT potentiates responses to ATP, but not 2MeSATP, at the cloned chick $P2Y_1$ receptor (King et al, 1996). This effect was not due to inhibition of ectonucleotidases and remains an unexplained phenomenon.

1.5 The Cloning of P2 receptors

The first P2 receptors to be cloned utilised the polymerase chain reaction (PCR) with degenerate oligonucleotide primers based on sequences in the transmembrane domains that are conserved in many G protein coupled receptors. Subsequent cloning strategies have used primers based on the new P2 sequences. Although the adenosine receptors recognise at least part of the same ligand as the P2 receptor, they have considerably less homology for P2 receptors than do peptide receptors. In fact, the P2 receptors have highest homology with receptors for interleukin-8, thrombin, vasoactive intestinal peptide, platelet activating factor, and angiotensin II. Table 1.1 shows degree of amino acid homology between the cloned human P2Y receptors.

1.5.1 P2 receptor subtypes

1.5.1.1 $P2Y_1$

In 1993, the first $P2Y_1$ clone was described (Webb et al, 1993). This 362 amino acid receptor was cloned from chick brain at around the time of hatching, as the brain undergoes an unusual burst of expression of many receptors at this time. The

$P2Y_1$	$P2Y_2$	$P2Y_4$	$P2Y_6$	
100	34	35	35	$P2Y_1$
	100	51	37	$P2Y_2$
		100	40	$P2Y_4$
			100	$P2Y_6$

Table 1.1

Percentage amino acid identities between the cloned human P2Y receptors.

hydropathicity profile of the predicted amino acid sequence revealed the typical pattern of a G protein coupled receptor, exhibiting seven hydrophobic domains.

Filtz et al (1994) cloned the turkey homologue of the chick $P2Y_1$ receptor using reverse transcriptase PCR with turkey brain and oligonucletide primers based on the chick brain $P2Y_1$ sequence, and expressed it in 1321N1 cells. They reported pertussis toxin insensitive coupling to phospholipase C and a 2-MeSATP stimulated inositol phosphate response.

The human $P2Y_1$ receptor has been cloned from a human genomic library, and the open reading frame encodes a protein of 373 amino acids (Schachter et al, 1996). The amino acid sequence is 83 % identical to the previously cloned chick and turkey $P2Y_1$ receptors, and is approximately 95 % homologous to the recently cloned rat, mouse and bovine receptors (Tokuyama et al, 1995; Henderson et al, 1995). All of the mammalian $P2Y_1$ receptors possess an 11 amino acid insert near the N-terminus that is absent in the avain sequences, with other differences occuring in TM4 and TM5.

Although only one $P2Y_1$ subtype has been cloned, there is evidence for the existence of multiple $P2Y_1$ subtypes. Boyer et al (1993) have described a $P2Y_1$ receptor linked to adenylyl cyclase in C6 glioma cells. It has a similar agonist profile to the $P2Y_1$ receptor that activates PLC in turkey erythrocyte membranes, and is blocked by pretreatment with pertussis toxin. However, they found that the P2 antagonist PPADS discriminated between the receptors, being an antagonist at the PLC-coupled $P2Y_1$ receptor on turkey erythrocytes (pA_2 of 5.9 ± 0.1), but not at the adenylate cyclase linked $P2Y_1$-like receptor on C6 glioma cells (Boyer et al, 1994). This supports the idea that different P2Y subtypes mediate coupling to adenylate cyclase and phospholipase C. Furthermore, expression of the human $P2Y_1$ receptor in C6 glioma cells conferred 2MeSATP stimulated inositol lipid hydrolysis into the cells which was sensitive to treatment with PPADS, providing more evidence that the human $P2Y_1$ receptor differs from the $P2Y_1$-like receptor expressed natively in C6 glioma cells (Nicholas et al, 1996).

1.5.1.2 $P2Y_2$

Simultaneously with Webb et al (1993), Lustig et al (1993) described the cloning of a 373 amino acid $P2Y_2$ receptor from a mouse neuroblastoma cell-line (NG108-15), that was equally sensitive to ATP and UTP and belonged to the same superfamily. This $P2Y_2$ receptor shares about 40 % sequence homology with the chick $P2Y_1$. In 1994, $P2Y_2$ receptors were also cloned from human airway and colonic epithelium (Parr et al, 1994), from rat heart (Godecke et al, 1996), and from rat alveolar type II cells (Rice et al, 1995). The rat and human $P2Y_2$ share over 95 % and 89 % homology with the mouse $P2Y_2$, respectively, with the majority of the differences concentrated in the carboxyl-terminal region.

A growing body of evidence suggests that the $P2Y_2$ receptor may be the most widely distributed of the known P2 receptor subtypes, and $P2Y_2$ mRNA has been found in the heart, liver, lung, kidney, placenta, skeletal muscle, brain, spleen and testes (see Lustig et al, 1996). However, although $P2Y_2$ receptors appear to be almost ubiquitous, only a few physiological roles have been described for them (see section 8).

The pharmacology of the cloned human $P2Y_2$ receptor has been examined by Parr et al (1994). In 1321N1 cells, ATP, UTP and ATPγS all activated the receptor, resulting in an increase in intracellular Ca^{2+}. The $P2Y_1$ and P2X specific agonists 2MeSATP and α,βMeATP had no effect. When the $P2Y_2$ receptor was transfected into K562 (human leukemia), the rise in cytoplasmic Ca^{2+} levels caused by extracellular nucleotides appeared to be largely dependent on release from intracellular stores (Erb et al, 1993).

Suggestions of the existance of $P2Y_2$ subtypes have been suggested on the basis of differential sensitivity to suramin. For example, stimulation of $P2Y_2$ receptors on PC12 rat phaechromocytoma cells are competitively inhibited by suramin (Murrin & Boarder, 1992), whilst in bovine aortic endothelial cells, responses to UTP at $P2Y_2$ receptors are not (Wilkinson et al, 1993). Also, Dainty et al (1994) have reported suramin sensitive $P2Y_2$ receptors on rat aortic rings, and suramin insensitive $P2Y_2$ receptors in canine tracheal epithelium. However, as for the $P2Y_1$, additional subtypes have yet to be cloned.

1.5.1.3 $P2Y_3$

During the cloning procedure for the $P2Y_1$ receptor, Webb et al (1996b) isolated a second clone from chick brain encoding a protein with the same structural motif and being preferentially sensitive to ADP. This receptor shared only 39 % amino acid sequence identity to the $P2Y_1$ and was termed $P2Y_3$.

When the $P2Y_3$ receptor was expressed in *Xenopus* oocytes, ADP gave the highest activity, and UTP was also strongly active. However, the $P2Y_3$ mRNA had cytotoxic effects on these cells, so data derived from this system is untrustworthy. When the coding sequence was expressed in the human T cell line Jurkat, $P2Y_3$ mediated transient increases in intracellular Ca^{2+} in response to varions nucleotides. In these cells, UDP was the most potent agonist tested, being 10-fold more potent than UTP, ADP was of relatively low activity and ATP was a partial agonist. The P2 antagonists suramin and reactive blue 2 inhibited Ca^{2+} responses to the nucleotides. The receptor transcript was found expressed in brain, spinal cord, kidney and lung, and was highly abundant in spleen.

1.5.1.4 $P2Y_4$

In 1995, two groups cloned a P2 receptor that was selective for UTP and was called the $P2Y_4$ receptor. Communi et al (1995) isolated an intronless coding sequence that was 1059-base pairs long from a human placenta cDNA library. In 1321N1 cells stably expressing this receptor, UTP and UDP stimulated the formation of inositol phosphates with equivalent potency, ATP behaved as a partial agonist, and ADP was almost inactive. The $P2Y_4$ receptor is a 365 amino acid protein and exhibits 51 % identity with the human $P2Y_2$ receptor and 35 % with the chick $P2Y_1$ receptor. Northen blot analysis has detected a 1.8 kilobase messenger RNA in human placenta. (Communi et al, 1995). Nguyen et al (1995) also isolated the sequence encoding the $P2Y_4$ receptor from human genomic DNA, and using flourescence *in situ* hybridisation, located the gene encoding it on region q13 of the X chromosome. When this $P2Y_4$ receptor was transfected into 1321N1 cells, they found that although UTP and UDP were full agonists, ATP and ADP were inactive (Nguyen et al, 1995).

However, Nicholas et al (1996b) have since reported that the observed response to UDP was due to its cell-surface conversion to UTP, and that UDP is inactive at this receptor.

1.5.1.5 $P2Y_5$

The orphan G protein coupled receptor, 6H1, present in activated chicken T cells has been proposed to be a P2 receptor (Webb et al, 1996). It displays approximately 30 % amino acid sequence identity with the $P2Y_1$ and $P2Y_2$ receptors, and has been designated $P2Y_5$. Displacement of bound [^{35}S]dATPαS has given a rank order of agonist potency of ATP > ADP > 2MeSATP >>UTP. However, a recent report by Schachter & Harden (1997) disputes the reliability of the binding data (see section 1.4.1). In addition, Li et al (1997) have cloned the turkey homologue of the $P2Y_5$ receptor and expressed the HA epitope-tagged receptor in 1321N1 cells. Despite obtaining stable expression of the receptor, no responses to any nucleotides were observed in these cells. Therefore, as no functional data have been reported to date, this putative receptor will not be discussed further in this thesis.

1.5.1.6 $P2Y_6$

Chang et al (1995) have isolated cDNA encoding a P2 receptor from a rat aortic smooth muscle cell library, which shows 44 and 38 % amino acid identity with the rat $P2Y_2$ and chick $P2Y_1$, respectively. This clone was designated $P2Y_6$. They showed that when transfected into C6 glioma cells, the $P2Y_6$ receptor was functionally coupled to phospholipase C but not adenylate cyclase. The rank order of agonist potency as judged by intracellular Ca^{2+} mobilisation responses was UTP > ADP = 2MeSATP > ADPβS > ATP = ATPγS. Both suramin and reactive blue 2 were shown to inhibit this response, but only in a very preliminary examination. $P2Y_6$ mRNA was found in various tissues in the rat including lung, stomach, intestine, spleen, mesentery, heart, and, most prominantly, aorta.

Nicholas et al (1996) have undertaken a more thorough investigation into the agonist profile of the $P2Y_6$. When the initial purity and stability of agonists were

ensured, they found that the $P2Y_6$ receptor was activated potently by UDP, but weakly by UTP, ATP and ADP. They also showed that the $P2Y_6$ receptor was natively expressed in C6-2B glioma cells, and was responsible for the responses to uridine nucleotides observed in previous studies (e.g. Lazarowski & Harden, 1994).

The $P2Y_3$ receptor has a very similar rank order of agonist potency to the $P2Y_6$ receptor, and it is possible that they are species homologues of the same receptor. However, in their cloning paper, Webb et al (1996) state that the $P2Y_3$ receptor shares only 60 % amino acid homology with the $P2Y_6$ receptor, well below the chicken-mammal correspondence in the $P2Y_1$ receptor, and so must be a different receptor.

1.5.1.7 $P2Y_7$

Finally, Akbar et al (1996b) cloned a P2 receptor from human erythroleukaemic cells, which they provisionally named $P2Y_7$. When transfected into COS-7 cells, they reported that the receptor responded to micromolar concentrations of ATP (K_i = 37 nM) and its analogues. However, in a recent paper by Yokomizo et al (1997), the same molecular structure was assigned to a G protein-coupled receptor for leukotriene B_4 that mediates chemotaxis. The authors go on to say that the COS-7 cells used by Akbar et al contain native P2 receptors that respond to 1 μM ATP. This has also been shown by Schachter & Harden (1997) (see section 1.4.1). When Yokomizo et al (1997), transfected the receptor into C6-15 glioma cells, which possess negligible amounts of intrinsic P2 receptors, ATP at concentrations of up to 300 μM caused no change in intracellular Ca^{2+} levels. However, a Ca^{2+} response was induced by exposure to 10 nM leukotriene B_4. Their results show that the receptor is not a member of the P2 family. Further evidence against the $P2Y_7$ receptor has been provided by Herold et al (1997). They demonstrated that expression of epitope-tagged $P2Y_7$ receptors in 1321N1 cells did not lead to responses to nucleotides. For these reasons, the putative $P2Y_7$ receptor will no longer be discussed in this thesis.

1.5.1.8 P2X receptors

The first P2X receptor, $P2X_1$, was cloned in 1994 from rat vas deferens and in 1995 from urinary bladder (Valera et al, 1994; 1995). When the recombinant receptor was expressed in *Xenopus* oocytes or human embryonic kidney cells, inward currents that desensitised with repeated application of agonist were observed. These currents were blocked by suramin and PPADS, and exhibited an unusual rank order of potency where 2-MeSATP was the most potent. The protein is 399 amino acids long and contains only two membrane spanning regions, with both termini located inside the cell. As such, the $P2X_1$ receptor is a member of a new family of ligand-gated ion channels.

The $P2X_2$ receptor, cloned from rat pheochromocytoma PC12 cells (Brake et al, 1994), and the $P2X_3$ receptor, cloned from rat dorsal root ganglia (Chen et al, 1995), are able to form a heteromultimer with a novel channel phenotype (Lewis et al, 1995). The $P2X_4$, cloned from rat hippocampus, has been identified as an α,β-MeATP insensitive and antagonist resistant receptor (Buell et al, 1996b). Further receptors, $P2X_5$ and $P2X_6$, have been cloned from rat ganglia (Collo et al, 1996). Finally, a receptor has been cloned from rat brain that appears to be the P_{2Z} receptor responsible for membrane pore formation in cells of the immune system (Suprenant et al, 1996). It is homologous to other P2X receptors, but has a unique carboxyl-terminal domain that is required for the lytic actions of ATP when this receptor is expressed HEK 293 cells. This receptor, designated $P2X_7$, has been shown to be a bifunctional molecule that can function in both fast synaptic transmission and the ATP-mediated lysis of antigen-presenting cells.

1.5.2 Structure of P2Y receptors

Hydropathicity analysis shows that P2Y receptors possess seven α helical transmembrane domains with an extracellular amino terminus and an intracellular carboxy terminus, and as such, belong to the G protein-coupled superfamily. They are among the shortest sequences (328-373) in this superfamily, with short N-terminal domains and a short third intracellular loop. They also possess two consensus

sequences for N-linked glycosylation on the N-terminus, with the $P2Y_1$ having two additional sites in the first and second extracellular loops. There are conserved cysteine residues in the first and second extracellular loops that are believed to form a disulphide bond that stabilises the protein structure. This bond has been shown to be critical for the function of other G protein coupled receptors (Dohlman et al, 1990). Also, serine and threonine residues in the third cytoplasmic loop and COOH-terminal domain form part of consensus sites for various protein kinases, and these may represent potential phosphorylation sites that play a role in receptor desensitisation. As with other G protein coupled receptors, the regions of highest homology are found in the transmembrane domains (TMs), particularly within TM3 and TM7. A sequence of 7 consecutive amino acids in TM3 (LFLTCIS) may represent a P2Y receptor signature (Communi et al, 1996).

The cloned murine $P2Y_2$ receptor, transfected into K562 human erythroleukemic cells, was identified as a 53 kDa plasma membrane protein in 1993 by Erb et al. They used a photoaffinity agonist, 3'-O-(4-benzoylbenzoyl)adenosine 5'-[α-^{32}P]triphosphate ([α-^{32}P]BzATP) and a histidine tagged $P2Y_2$ receptor to show labelling of a 53 kDa protein in transfected, but not wild-type, cells. Since the predicted amino acid sequence of this receptor indicates a molecular mass of approximately 42 kDa, the $P2Y_2$ receptor appears to be glycosylated on one or both of the consensus glycosylation sites in the N-terminus.

Figure 1.3 shows the predicted structure of the human $P2Y_2$ receptor.

1.5.2.1 Putative agonist binding site

Sequence comparisons between $P2Y_1$, $P2Y_2$, and the cloned adenosine A_1 receptor indicate several positively charged amino acid residues in TM3, TM6 and TM7 that are present in the P2Y subtypes, but not in the A_1 receptor. Since the fully ionised form of nucleotides has been shown to be an activating ligand for both $P2Y_1$ and $P2Y_2$ receptors, it is possible that these positive residues are involved in the binding of the negatively charged phosphate moeties of the P2 agonists. Site directed mutagenesis of several of these positively charged amino acids has been performed by Erb et al (1995) on the murine $P2Y_2$ receptor. After mutagenesis, the altered receptors were transfected into 1321N1 cells where responses to several P2 agonists were

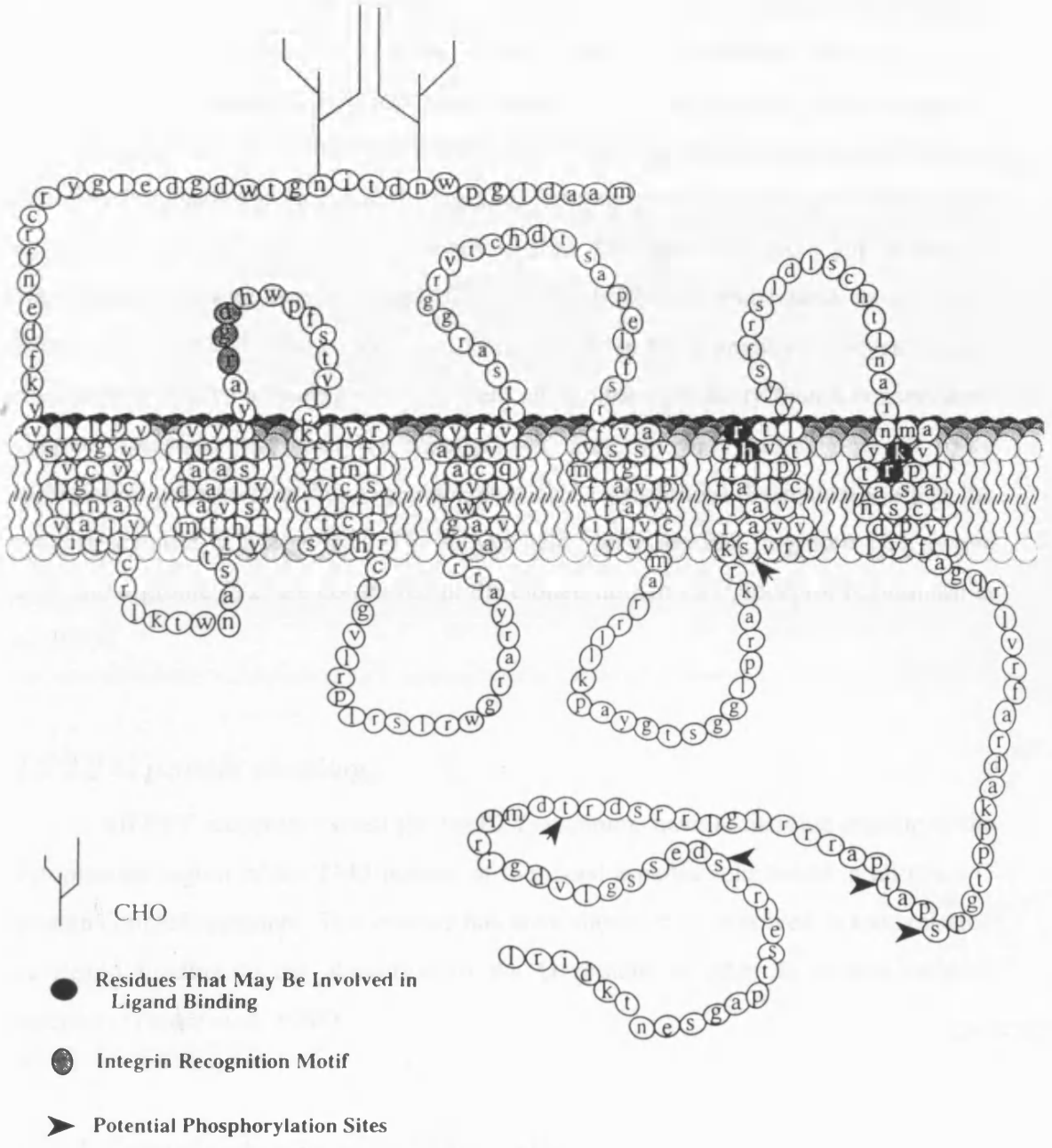


Figure 1.3

Predicted 2D structure of the human $P2Y_2$ receptor.

Taken from Boarder et al, 1995.

measured. Neutralisation of the positively charged amino acids arginine 265, arginine 292 and histidine 262 by substitution with leucine markedly decreased the potency of ATP and UTP, and rendered ADP and UDP ineffective. In contrast, neutralisation of lysine 107 or arginine 110 did not alter the agonist potency profile of the receptors. Interestingly, although neutralisation of lysine 289 did not alter receptor specificity, a conservative change from lysine to arginine (another positively charged amino acid) altered the rank order of agonist potency so that ADP and UDP were approximately 100-fold more potent than ATP and UTP. This is difficult to understand, as it is more conceivable that the substitution of a positive residue for a negative or even neutral residue would have a more profound effect on agonist specificity than a conservative change. Despite this, receptor mutagenesis may, in the future, provide more information on how minor changes in amino acid composition dramatically affect the binding specificity of agonists. The amino acids histidine 262, arginine 265, lysine 289, and arginine 292, are conserved in the cloned human $P2Y_4$ receptor (Communi et al, 1995).

1.5.2.2 G protein coupling

All P2Y receptors except the rat $P2Y_6$ receptor, have a histidine residue at the cytoplasmic region of the TM3 instead of the usual aspartic acid found in all other G protein coupled receptors. This residue has been shown to be involved in transmission of ligand binding to the activation of the G protein in other G protein coupled receptors (Fraser et al, 1988).

1.5.3 Transfection into 1321N1 cells

Many of the cloned receptors have been transfected into a 1321N1 human astrocytoma cell line that possesses no endogenous P2 receptor. Several methods have been used to achieve stable expression. For example, the cDNA encoding the $P2Y_1$ receptor was ligated into the pcDNA3 expression vector that contains a cytomegalovirus promotor for high expression levels in mammalian cells. Cells were transfected by the calcium phosphate precipitation method of Chen & Okayama (1987) (Filtz et al, 1994).

The human $P2Y_2$ receptor cloned from airway epithelium was transfected into 1321N1 cells using the retroviral expression plasmid, pLXSN. The receptor sequence was inserted into this vector, and the recombinant plasmid was transfected into PA317 amphotrophic packaging cells for production of the viral vector (see Miller & Rosman, 1989). The viral vector was then used to transfect 1321N1 cells (Parr et al, 1994). The $P2Y_4$ receptor was transfected into 1321N1 cells using the same retroviral system (Nguyen et al, 1995). All transfection systems have used a neomycin resistance gene, which confers resistance to G418, for selection of positive clones.

1.6 Transduction mechanisms of P2Y receptors

In general, P2Y receptors are linked to PLC, their activation leading to inositol phosphate production and raised cytosolic Ca^{2+} levels. However, evidence exists for their coupling to PLD, PLA_2, adenylyl cyclase, ion channels, and MAPK (see section 1.8.5). For example, the opening of K^+ channels after an increase in cytosolic Ca^{2+} evokes hyperpolarisation, and it is this mechanism that underlies relaxations after P2Y receptor activation. This section will outline the nature of some of the proteins involved in P2Y signal transduction.

1.6.1 G proteins

G proteins are so named because they comprise one sub-family of a superfamily of guanine nucleotide-binding proteins that share considerable sequence similarity of their guanine nucleotide-binding sites. They were first discovered by Rodbell *et al* (1971) as mediating cyclic AMP formation in liver membranes in response to glucagon. Since then, they have been shown to transduce signals from activated receptors to many different effector enzymes (e.g. adenylyl cyclase, GMP-phosphodiesterase, phospholipase C and phospholipase A_2) or ion channels (e.g. potassium and calcium channels) (Northup *et al*, 1980; Baylor, 1996; Pang & Sternweis, 1990; Jelsema & Axelrod, 1987; Brown & Birnbaumer, 1988). G proteins

are heterotrimeric membrane-associated proteins, composed of α, β and γ subunits, each of which has several closely related isoforms.

The receptors that activate G proteins are heptahelical, i.e. they have seven transmembrane domains plus N-terminal and C-terminal sequences and cytoplasmic loops of varying length. Interaction of the receptors with G proteins involves, to varying degrees, their cytoplasmic loops and C-terminus tails, and for many receptors, sequences in the third cytoplasmic loop play a major role (Exton, 1996; Savarese & Fraser, 1992; Strader et al, 1995).

1.6.1.1 Mechanism of action

G proteins cycle between a GTP-bound active form and a GDP-bound inactive form. The α subunits bind GTP and hydrolyse it to GDP. All isoforms of α subunit are GTPases, although the intrinsic rate of GTP hydrolysis varies greatly from one type of α subunit to another (Carty et al 1990; for review see Fields & Casey, 1997). Figure 1.4 illustrates the cycle of activation and the interaction with receptor. When GDP is bound to the α subunits, they associate with βγ subunits to form the inactive heterotrimer. In this form, the G protein is able to associate with an inactive receptor. Although GDP-bound α subunits are able to bind receptor without βγ, the association is greatly enhanced by its presence (Florio & Sternweis, 1985). Upon activation of the receptor, a conformational change is transmitted to the α subunit, reducing its affinity for GDP. GDP is released, resulting in a ternary complex consisting of agonist-bound receptor and guanine nucleotide-free G protein. In this state, the receptor has a high affinity for the agonist. However, this state is transient, and GTP, which is at much higher concentrations in the cell than GDP, binds at the active site. GTP binding activates the α subunit, which then dissociates from the receptor and βγ subunits. Also, the receptor has a decreased affinity for its agonist in this state and releases the ligand. Although G proteins are heterotrimers, functionally they are dimers because β and γ subunits do not dissociate unless they are denatured. When dissociated, the α and βγ subunits each interact with effectors (reviewed by Clapham & Neer, 1993). The signal is terminated by the intrinsic GTPase activity of the G_{α} subunit, which

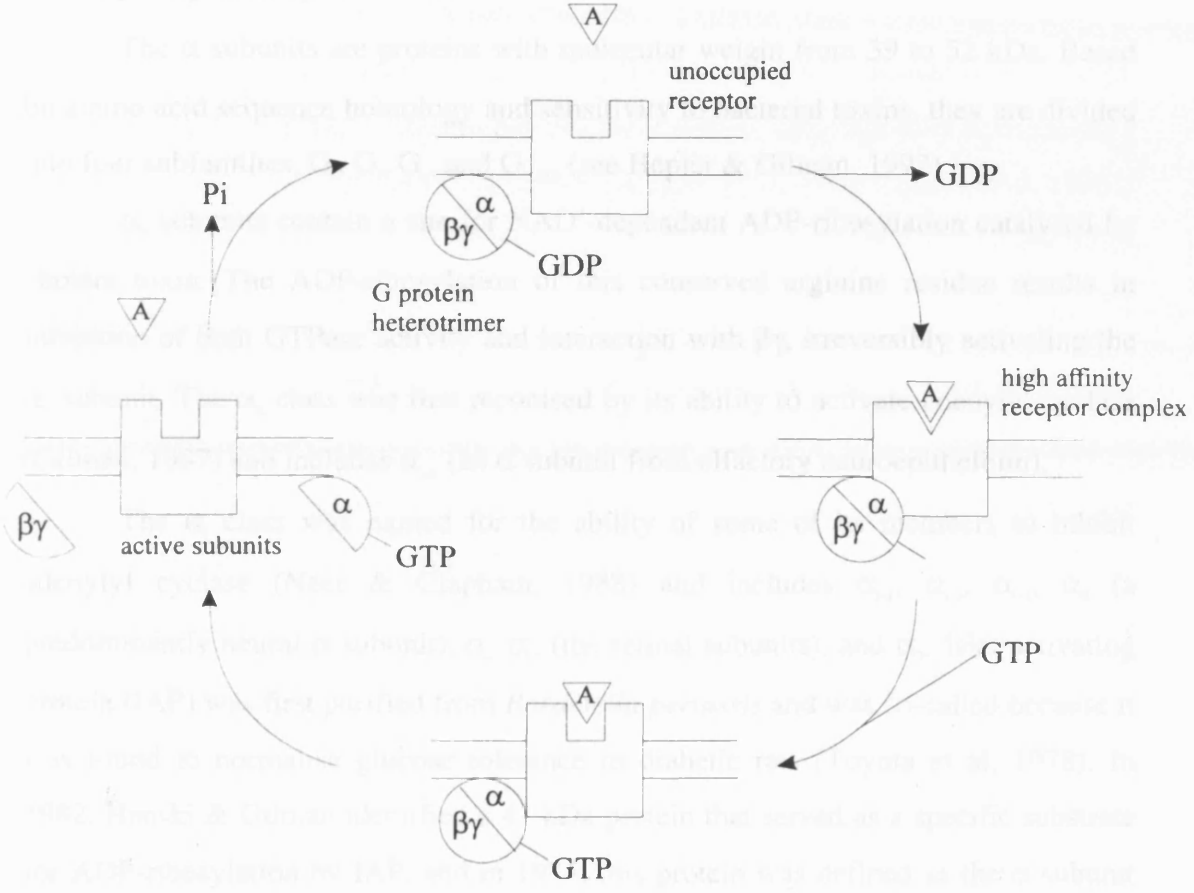


Figure 1.4
Schematic representation of the ternary complex model of the G protein cycle. See section 1.6.1.1 for details.

hydrolyses the bound GTP to GDP, and the cycle is completed as the resultant G_{α}-GDP re-associates with βγ.

1.6.1.2 Subtypes

1.6.1.2.1 Properties of α subunits

The α subunits are proteins with molecular weight from 39 to 52 kDa. Based on amino acid sequence homology and sensitivity to bacterial toxins, they are divided into four subfamilies, G_s, G_i, G_q, and $G_{12/13}$ (see Hepler & Gilman, 1992).

α_s subunits contain a site for NAD^+-dependant ADP-ribosylation catalysed by cholera toxin. The ADP-ribosylation of this conserved arginine residue results in inhibition of both GTPase activity and interaction with βγ, irreversibly activating the α_s subunit. The α_s class was first reconised by its ability to activate adenylyl cyclase (Gilman, 1987) and includes α_{olf} (an α subunit from olfactory neuroepithelium).

The α_i class was named for the ability of some of its members to inhibit adenylyl cyclase (Neer & Clapham, 1988) and includes α_{i-1}, α_{i-2}, α_{i-3}, α_o (a predominantly neural α subunit), α_t, α_{t2} (the retinal subunits), and α_z. Islet activating protein (IAP) was first purified from *Bordetella pertussis* and was so-called because it was found to normalise glucose tolerance in diabetic rats (Toyota et al, 1978). In 1982, Hanski & Gilman identified a 41 kDa protein that served as a specific substrate for ADP-ribosylation by IAP, and in 1984, this protein was defined as the α subunit of G_i (Bokock et al, 1984). Since then, pertussis toxin has been shown to ADP-ribosylate a cysteine residue in all members of the G_i family, with the exception of α_z (Fong et al, 1988).

The G_q class includes G_q, G_{11}, and G_{16}, which activate phospholipase C in a rank order of PLCβ1 > PLCβ2 > PLCβ3 (Nakamura et al, 1995; Smreka & Sternweis, 1993). Some of the sequences in $G_q\alpha$ that are involved in interaction with PLCβ isoenzymes have been identified (Venkatakrishnan & Exton, 1996). Kai et al (1996) have showed that prolonged agonist (100 nM angiotensin II) exposure causes down-regulation of $G\alpha_q$ and $G\alpha_{11}$ in cultured rat vascular smooth muscle cells to 50 % after

6 hours. In a more recent study, they have shown that phosphorylation of a tyrosine residue (Tyr^{356}) on $G_{q/11}$ is essential for activation, and that stimulation of receptors coupled to $G_{q/11}$ induces that phosphorlation (Umemori et al 1997). G_{16} is expressed exclusively in hematopoietic cells (Amatruda et al, 1991), and can interact indiscriminately with a multitude of G protein-coupled receptors (Offermanns & Simon, 1995; Zhu & Birnbaumer, 1996), including $P2Y_2$ receptors (Baltensperger & Porzig, 1997).

The fourth class includes α_{12} and α_{13} (for review see Offermanns & Schultz, 1994). The targets of this subfamily are not well defined, although they have recently been implicated in regulation of mitogenesis (Aragay, et al, 1995; Collins et al, 1996).

1.6.1.2.2 Properties of βγ subunits

There are four different β subunits and seven γ subunits, but although many different combinations are possible, not all the pairs can form. Within the structure of the β subunit is an amphipathic α helix which is involved in the coiled-coil interaction that holds β to γ, and although there are no covalent linkages (Thomas et al, 1993), the βγ subunit forms a very stable structure. βγ subunits are able to inhibit Type I adenylyl cyclase, and can act in synergy with α_s to further activate Types II and IV (Taussig et al, 1993). βγ subunits can activate the muscarinic K^+ channel in cardiac atria (Logothetis et al, 1987) and have been shown to directly bind to voltage-dependant calcium channels (DeWaard et al, 1997). As with adenylyl cyclase, the pattern of regulation of phospholipase Cβ (PLCβ) is characteristic for each isoform. βγ subunits stimulate PLCβ with a rank order of PLCβ3 > PLCβ2 > PLCβ1 (Smreka & Sternweis, 1993).

1.6.1.3 P2Y receptors and G proteins

The bacterial toxin, pertussis toxin, has been utilised to try to establish which family of G proteins each receptor couples to. In BAECs, the $P2Y_1$ and $P2Y_2$ receptors activate PLC through distinct G proteins. The $P2Y_1$ receptor couples to a pertussis toxin insensitive G protein (possibly G_q), whereas the $P2Y_2$ receptor couples to a

pertussis toxin sensitive G protein (possibly $G_{i/0}$) (Purkiss et al, 1994). This has been shown to be the case in other tissues. For example, in human skin fibroblasts and CF/T43 human airway epithelial cells, pertussis toxin reduces the $P2Y_2$ induced stimulation of inositol phosphates by around 40 %, whereas responses at $P2Y_1$ receptors in porcine coronary artery and rat liver were unaffected (Fine et al, 1989; Brown et al, 1991; Flavahan et al, 1989; Siddiqui & Exton, 1992).

The cloned $P2Y_2$ receptor has also been shown to couple to a $G_{i/0}$ protein, as pertussis toxin inhibited 40 % of the $P2Y_2$ mediated Ca^{2+} increase (Erb et al, 1993). However, Communi et al, (1995) have shown that inositol phosphate accumulation in 1321N1 cells transfected with the human $P2Y_4$ receptor was modulated by preincubation with pertussis toxin only at a 30 second stimulation time point. They suggest that the $P2Y_4$ receptor is capable of coupling to different G proteins depending on the time course of activation. Pertussis toxin pretreatment does not inhibit ADP-induced inositol phosphate production in C6 glioma cells transfected with the $P2Y_6$ receptor, suggesting coupling of this receptor to $G_{q/11}$.

ADP acts at P_{2T} receptors in platelets to stimulate PLC and increases in intracellular Ca^{2+} levels, and has also been shown to inhibit adenylyl cyclase, probably via G_{i2} (Gachet et al, 1992; Ohlmann et al, 1995).

1.6.1.4 G proteins in 1321N1 astrocytoma cells

Many studies have used 1321N1 cells to study the interaction of G proteins with receptors and effector enzymes. In a paper by Gutowski et al (1991), antisera raised against a peptide representing the 12 amino acids of the common carboxl termini of α_q and α_{11} was shown to attenuate PIP_2 hydrolysis stimulated by histamine and GTPγS.

A_1 adenosine receptors have been shown to inhibit PIP_2 hydrolysis via a pertussis toxin sensitive G protein (Nakahata et al, 1991). Nakahata et al (1995) have shown by western blotting with specific antisera that $G\alpha_s$, $G\alpha_i$, and $G\alpha_{q/11}$ are all present in the membranes of 1321N1 cells.

1.6.2 Phospholipases

Phospholipases are important enzymes in cell signal transduction since they hydrolyse membrane phospholipids to generate signalling molecules. They are named according to their specificities, i.e. where they cleave the phospholipid (see Figure1.5).

1.6.2.1 Phospholipase D

Phospholipase D (PLD) cleaves phosphatidylcholine containing membrane phospholipids resulting in the formation of phosphatidic acid (PA) and free choline. The role of PA in cells signalling has not been fully elucidated, although it may play a role in mitogenic signalling (see Boarder, 1994, for review). Interconversion between PA and DAG occurs by action of the enzymes DAG kinase and phosphatidate phosphohydrolase, and hence PLD has the potential to stimulate PKC.

Although there is biochemical evidence for the existence of PLD isoenzymes (Exton, 1994), only one clone has been reported (Hammond et al, 1995). Receptor coupling to PLD is described as being either phosphloipase C (PLC) dependant or independent. PLD activation downstream of PLC is attributed to elevated intracellular Ca^{2+} concentration and activation of protein kinase C (PKC) (Pfeilschifter & Merriweather, 1993). PLC independent activation of PLD have been described as those either directly coupled to G proteins or those that are linked to tyrosine kinase receptors (Malcolm et al, 1995; Thompson et al, 1991).

P2Y receptors have been shown to couple to PLD in a variety of studies. For example, Purkiss et al (1993) showed that $P2Y_2$ receptor activation in bovine adrenal medullary endothelial cells lead to PLD activation, and concluded that this activation was probably downstream of PLC. Malcolm et al (1995) have demonstrated PLC independent PLD activation by P2 agonists, although the specific receptor subtype was not determined.

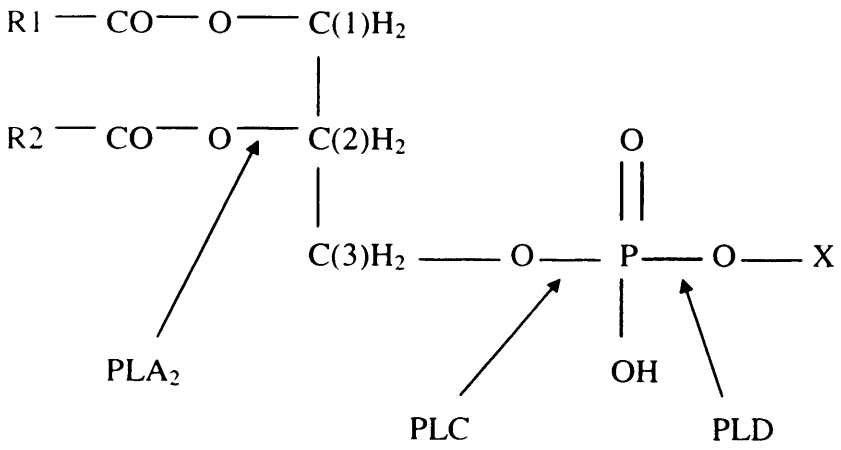


Figure 1.5

Sites of cleavage of membrane phospholipids by phospholipases.

PLA_2, phospholipase A_2; PLC, phosphoinositide specific phospholipase C; PLD, phospholipase D. R1 and R2 are fatty acid chains. Unsaturated fatty acids (normally arachidonyl) are usually located at R2. X can be inositol, ethanolamine, choline, serine or hydrogen.

1.6.2.2 Phospholipase A_2

Phospholipase A_2 (PLA_2) cleaves membrane phospholipids resulting in the formation of arachidonic acid, a twenty carbon, polyunsaturated fatty acid. There are two isozymes, secreted PLA_2 and cytosolic PLA_2 ($cPLA_2$), both of which are regulated by calcium concentration. The enzyme can also be regulated by phosphorylation by protein kinase C, cAMP dependant kinases, and receptor tyrosine kinases (Glaser et al, 1993; Wightman et al, 1982; Goldberg et al, 1990).

P2Y receptor activation of PLA_2 has been demonstrated in endothelial cells and has been interpreted as reflecting PLC-mediated elevation of cytosolic Ca^{2+} activating $cPLA_2$ (Carter et al, 1988). However, subsequent studies have shown that P2-mediated PLA_2 activation is subject to modulation by PKC (Carter et al, 1989), with involvement of tyrosine kinases and MAPK (Lin et al, 1993; Patel et al, 1996a, b).

1.6.2.3 Phosphoinositide specific phospholipase C (PLC)

PLC isoenzymes that are specific for inositol phospholipids have been purified and cloned from several animal species and are divided into three types, β, γ and δ (Rhee & Choi, 1992). All isoforms have two regions of close sequence similarity (X and Y) that probably represent the catalytic domain, and have a pleckstrin homology domain (PH) in the N-terminus (Lee & Rhee, 1995). Although the PH domain can bind PIP_2, the enzyme is still catalytically active without the domain, and it is probably involved in anchoring the enzyme to the membrane surface (Cifuentes et al, 1993). All three types also have a C2 domain near the C-terminus that binds phospholipids in a calcium-dependant manner, a site that is also present in protein kinase C.

1.6.2.3.1 PLCδ and PLCγ

The δ isozymes are of a lower molecular weight and are regulated only by Ca^{2+}. This form has recently been crystallized and has yielded much useful information about its membrane association and catalytic activity (Essen et al, 1996). PLCγ is stimulated directly by tyrosine kinase receptors (e.g. platelet-derived growth factor receptors and epidermal growth factor receptor) and has Src homology domains

(SH2 and SH3). In unstimulated cells, PLCγ is largely cytosolic but translocates to the membrane as its SH2 domain binds to the activated receptor. Binding of $PLC\gamma_1$ to the receptor results in phosphorylation of specific tyrosine residues and activation of $PLC\gamma_1$. Once in the membrane, $PLC\gamma_1$ is in contact with its substrate, $PtdIns(4,5)P_2$.

1.6.2.3.2 PLCβ

There are four subtypes of PLCβ, PLCβ1-4. It was originally believed that phosphoinositide specific PLC was activated only by the G_q subfamily of G proteins, which are insensitive to pertussis toxin (PTX). However, it was noted that some responses were inhibited by PTX (Cockcroft & Thomas, 1992) and this was only resolved when it was discovered that G_i subtypes (a PTX sensitive subfamily) could control PLC via their βγ subunits. $G_q\alpha$ subunits activate PLCβ in a rank order of responsiveness of β1≥ β2 > β4 > β3 (Lee & Rhee, 1995). The third α helix in the C-terminus of PLCβ may be important for interaction with $G_q\alpha$ subunits (Arkinstall et al, 1995). βγ subunits activate the PLCβ isozymes in the rank order β3 > β2 > β1 (Lee & Rhee, 1995), but the β4 isozyme is not affected (Jiang et al, 1994). The region in PLCβ2 that interacts with βγ differs from that of $G_q\alpha$, and is located in a sequence that encompasses part of the X and Y domains (Kuang et al, 1996).

Tissue distribution of PLCβ isozymes varies. β1 and β2 are fairly widespread (Jhon et al, 1993), whereas the β3 isozymes is limited to haemopoetic cells (Park et al, 1992) and β4 is preferentially expressed in retina (Lee et al, 1993).

1.6.2.3.3 P2Y receptor activation of PLC

The inositol lipid signalling pathway was the first second messenger cascade to be associated with P2Y receptors. Examples of some of the tissues studied include hepatocytes (Charest et al, 1985), vascular endothelial cells (Pirotton et al, 1987), and turkey erythrocytes (Boyer et al, 1989). The $P2Y_1$ receptor in turkey erythrocytes couples to the PLC-β class of isoenzymes (Waldo et al, 1991), via the G_q family protein, G_{11} (Maurice et al, 1993). Since then, cloned $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$

receptors have all been shown to couple to PLC when transfected into 1321N1 cells (Filtz et al, 1994; Parr et al, 1994; Communi et al, 1995; Chang et al, 1995).

1.6.2.3.4 Inositol 1,4,5-trisphosphate

Activation of PLC catalyses the hydrolysis of phosphatidyl (4,5) bisphosphate (PtdIns(4,5)P_2). The two products of this reaction are inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) and *sn* 1,2-diacylglycerol (DAG), both of which are second messengers. DAG is lipophilic and remains within the plasma membrane where it is able to stimulate protein kinase C (PKC). Ins(1,4,5)P_3 is soluble and so diffuses across the cytosol where it bind to specific Ins(1,4,5)P_3 receptors on intracellular calcium stores. Binding of Ins(1,4,5)P_3 to these tetrameric calcium channels causes them to open, releasing the sequestered calcium into the cytosol (for review see Berridge, 1993).

Breakdown of Ins(1,4,5)P_3 is achieved by two different pathways. In the first, Ins(1,4,5)P_3 is further phosphorylated to inositol 1,3,4,5-tetrakiphosphate (Ins(1,3,4,5)P_4) by the action of a 3-kinase. This compound may also act as a cell signalling molecule, stimulating influx of calcium into cells (Luckhoff & Clapham, 1992; Cullen et al, 1990). Alternatively, Ins(1,4,5)P_3 is sequentially dephosphorylated to inositol which is reincorporated into the membrane phospholipid pool.

1.6.2.3.5 The effects of lithium on inositol phosphate metabolism

Lithium is an uncompetitive inhibitor of inositol monophosphates (Hallcher & Sherman, 1980), that is, the degree of inhibition increases with increased phosphoinositide hydrolysis (Nahorski et al, 1991). The resulting accumulation of inostitol monophosphates means that inositol is no longer available for recycling into the inositol containing membrane phospholipid pool and substrate availability for PLC is reduced. Therefore, receptor-mediated PLC activity may be selectively impaired by lithium as a secondary consequence of inositol monophosphatase inhibition (Batty & Downes, 1994). This action of lithium is thought to underlie its therapeutic use in the treatment of manic depressive dissorder. At higher concentrations of lithium, typically above 5mM, the Ins(1,4)P_2 and Ins(1,3,4)P_3 1-phosphatase enzymes are also inhibited, resulting in the accumulation of higher inositol phosphates. The use of lithium as an experimental tool was first suggested by

Berridge et al (1982), and since then it has been used extensively in the study of PLC activity and the phosphoinostide cycle (Nahorski et al, 1991). Figure 1.6 shows the phosphoinositide cycle and the points at which lithium has its action.

1.6.3 Protein Kinase C

Protein kinase C (PKC) is a family of serine/throenine protein kinases. It is activated by *sn* 1-2 diacylglycerol (DAG), generated from phosphatidylinositol 4,5-phosphate (PIP_2) hydrolysis by phospholipase C. Activation by DAG is highly stereospecific, as neither 1-3, nor 2-3 DAG have any affect (Bonser et al, 1988).

1.6.3.1 Subtypes and structure

The PKC family comprises at least 11 subspecies. The enzymes can be classified into Ca^{2+}-dependant and Ca^{2+}-independent groups and further subdivided on the basis of conserved structural features and their requirements for lipid activators. Ca^{2+}-dependant PKC consists of α, β_I, β_{II} and γ subspecies that are activated by Ca^{2+}, phosphatidylserine and DAG. Ca^{2+}-independent PKC consists of δ, ε, η, and θ subspecies that do not require Ca^{2+}, but are activated by phosphatidyl serine and DAG. A third group, the atypical PKC isoforms, consist of ζ, λ and ι subtypes that are activated only by phosphatidylserine and not Ca^{2+} or DAG. The structure of PKC can be divided into two domains; a kinase (catalytic) domain and a regulatory domain that contains the binding sites for Ca^{2+} and lipids (Andrea & Walsh, 1992).

1.6.3.2 Action of phorbol esters

PKCs are also activated by tumor promoting phorbol esters such as 12-O-tetradecanoyl phorbol 13-acetate (TPA) (Nishizuka, 1992). However, phorbol esters are not readily metabolised by the cell, causing chronic stimulation of PKC, and this may underlie the tumor promoting effects of these compounds. The use of phorbol esters has shown that PKC is involved in the regulation of PLC linked receptors.

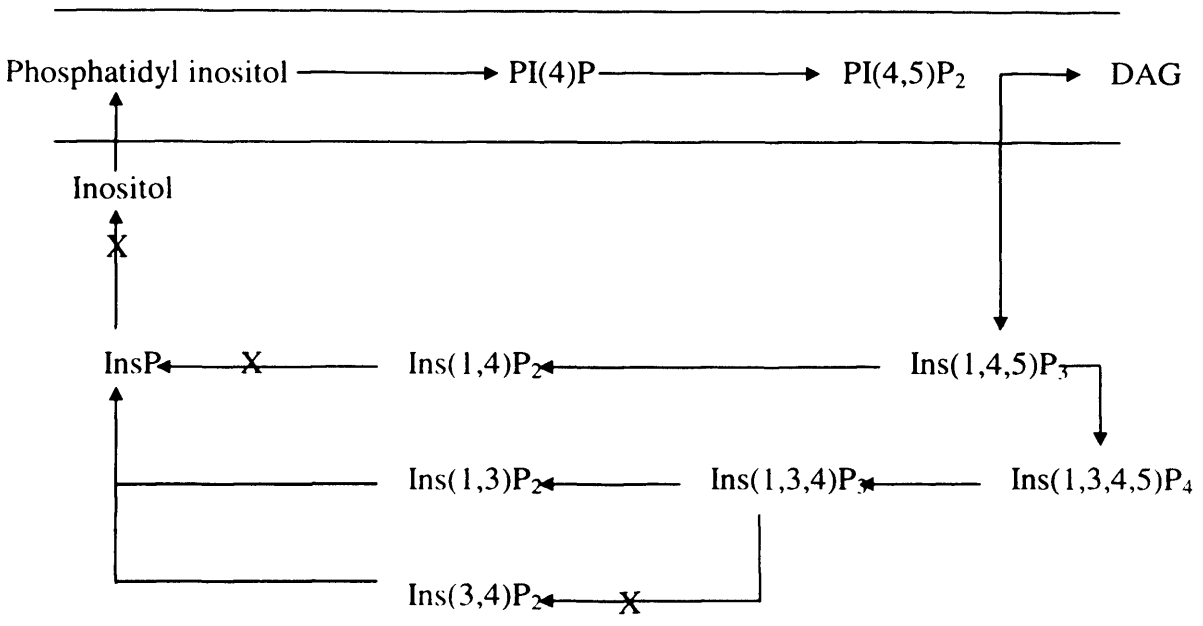


Figure 1.6

The phosphoinositide cycle.

PI(4)P, phosphatidylinositol 4-phosphate; $PI(4,5)P_2$, phosphatidyl 4,5-bisphosphate; DAG, *sn*-1,2-diacylglycerol; $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate; $Ins(1,3,4,5)P_4$, inositol 1,3,4,5-tetrakisphosphate; $Ins(1,3,4)P_3$, inositol 1,3,4-trisphosphate; $Ins(1,3)P_2$, inositol 1,3-bisphosphate; $Ins(1,4)P_2$, inositol 1,4-bisphosphate; $Ins(3,4)P_2$, inositol 3,4-bisphosphate; InsP, inositol (1), (3) and (4) monophosphates. X indicates where lithium inhibits the reaction.

1.6.3.3 PKC inhibitors

The first PKC inhibitors to be used included staurosporin and H7 (Tamaoki et al, 1986), but these compounds also inhibit other protein kinases. Interest in the possible anti-cancer actions of PKC inhibitors promoted the search for new, selective inhibitors. Among these, a group of compounds based on staurosporin were developed, including Ro 31-8220, which was 100 to 1000 times more selective for PKC (Davis et al, 1989). This compound, however, was not selective for PKC isozymes (e.g. Harris et al, 1996), and has also been shown to have non-specific effects.

1.6.3.4 PKC Regulation of the PLC response

Short term (5–10 minutes) preincubation of phorbol esters with cells has been shown to attenuate the responses to agonists in some systems (Helper et al, 1988; Purkiss et al, 1994), and this has been related to a short feedback loop. However, long term exposure to phorbol esters can result in potentiation of agonist induced PLC activation, e.g. with angiotensin II in vascular smooth muscle cells (Pfeilschifter et al, 1989). This has been attributed to down regulation of PKC. Varying the exposure times to phorbol ester can selectively down regulate different PKC isoforms, e.g. in rat messangial cells, 8 hour exposure decreases levels of α and δ isoforms, whilst 24 hour exposure also causes reductions in the ε isoform (Patel et al, 1996a; Pfeilschifter & Merriweather, 1993).

Responses to different P2 receptors have been shown to be differentially regulated by PKC. Purkiss et al (1994) have shown that in bovine aortic endothelial cells, the inositol 1,4,5-trisphosphate response to $P2Y_1$ agonists is significantly inhibited by TPA, whilst the response to $P2Y_2$ agonists is not altered. Also, inhibition of PKC by Ro 31-8220 enhanced responses to $P2Y_1$ agonists, but not to $P2Y_2$ agonists. Contrastingly, in C6-2B rat glioma cells, $P2Y_2$ receptor stimulation of PLC is inhibited by over 80 % by PKC activation (Munshi et al, 1993). Although this indirect evidence appears to show that P2Y receptor activation results in an inhibitory feedback loop involving PKC, there is no direct evidence for the activation of PKC by P2Y receptors (see Boarder et al, 1995). Further work on cloned and transfected

receptors was undertaken in this present study to elucidate the relationship between receptor subtype and PKC inhibition.

1.6.3.5 PKC in 1321N1 cells

PKC has been extensively studied in 1321N1 cells (Johnson & Toews, 1990), and PKC activation has been shown to inhibit internalisation and downregulation of muscarinic receptors (Hoover & Toews, 1990). PKC is also necessary for the activation of PLD by muscarinic receptors (Martinson et al, 1990). Trilivas et al (1991) have demonstrated the translocation of PKCα from the cytosol to the membrane of 1321N1 cells during muscarinic receptor activation.

1.6.4 Adenylyl cyclase

1.6.4.1 Structure and subtypes

Adenylyl cyclase is a family of proteins that synthesise adenosine 3,5-cyclic monophosphate (cAMP), an important second messenger molecule which regulates certain cAMP-dependant protein kinases. All isoforms have a common structure, consisting of 12 transmembrane domains spanning the plasms membrane in two cassettes, each followed by a single cytosolic catalytic sequence. However, the isoforms show diversity in their regulatory characteristics, and can be divided into three groups on this basis. Group A consistes of types I, III, and VIII. They are positively modulated by Ca^{2+}/calmodulin in a manner which is synergistic with any input from $G\alpha_s$. In the absence of activation by Ca^{2+}/CaM, $G\alpha_s$ is a relatively ineffective stimulator, while both $G\alpha_i$ and βγ subunits are inhibitory. Group B consists of types II and IV, which are effectively stimulated by $G\alpha_s$ and PKC. Both $G\alpha_i$ and Ca^{2+} have little influence. Group C consists of types V and VI which are stimulated by $G\alpha_s$ and inhibited by $G\alpha_i$. Ca^{2+} also has an inhibitory influence, but this is of less consequence.

The isoforms also vary in location of expression e.g. types V and VI are widely expressed, whereas type I is most abundant in the brain. This differential

expression explains why the same receptor can have differing influences on cAMP in different tissues.

Adenylyl cyclase has several regulatory domains. These consist of domains for $G\alpha_s$, $G\alpha_i$, and $\beta\gamma$ subunits (Wayman et al, 1994; Wong et al, 1991; Taussig et al, 1993), Ca^{2+}/CaM binding sites (Oldenburg et al, 1992), and the so-called P site, at which certain analogues of adenosine act in a non-competitive manner to inhibit the enzyme (Johnson et al, 1989).

1.6.4.2 The control of adenylyl cyclase by P2Y receptors

A growing number of reports have demonstrated P2Y receptor mediated changes in cAMP levels. Both the inhibition and stimulation of adenylyl cyclase have been described, and several examples of each will be discussed here.

The ability of extracellular nucleotides to decrease cellular cAMP accumulation has been reported in a number of tissues e.g. rat hepatocytes (Okajima et al, 1987) and C6 glioma cells (Boyer et al, 1993), but has been best characterised in response to P_{2T} receptor activation in platelets (Cooper & Rodbell, 1979; Cristalli & Mills, 1993). Accompanying the reduction in cAMP is an activation of PLC and subsequent elevation in Ca^{2+} (Hallam & Rink, 1985). Although the elevation in Ca^{2+} could possibly result in inhibition of Group C adenylyl cyclase enzymes, the inhibition of adenylyl cyclase, but not the mobilisation of intracellular Ca^{2+}, is pertussis toxin sensitive, suggesting direct regulation of adenylyl cyclase via $G_{i/o}$ proteins. Boyer et al (1993) have shown the receptor mediating the inhibition of adenylyl cyclase in C6 glioma cells has a similar agonist profile to the $P2Y_1$ receptor that activates PLC in turkey erythrocyte membranes, and is also blocked by pretreatment with pertussis toxin.

Increases in cAMP formation due to stimulation of P2 receptor for extracellular ATP have also been reported e.g. in bovine aortic smooth muscle cells (Tada et al, 1992) and mouse C2C12 myotubules (Henning et al, 1993). The mechanisms by which P2Y receptors stimulate cAMP production are poorly understood. However, it has long been known that adenosine, acting via A_2 receptors, potently increases cAMP levels (see Stiles, 1986), and it is therefore possible that ATP acts indirectly through its hydrolysed metabolites. This issue has been addressed

by Matsuoka et al (1995) who showed that in NG108-15 cells, ATP acted directly (and not through its metabolism) to increase cAMP production. They also showed that two P2Y receptors were present, one that stimulated adenylyl cyclase, and one that stimulated PLC. Evidence for indirect stimulation of adenylyl cyclase has come from Post et al (1996). Using the cyclo-oxygenase inhibitor indomethacin, they showed that P2Y receptor stimulation lead to the PLC mediated release of PGE_2, which then activated a $G\alpha_s$ coupled receptor in an autocrine fashion.

1.6.5 Mitogen activated protein kinases (MAPK)

MAPK is a key element in the signal transduction pathways involved in cell growth (reviewed by Avruch et al, 1994). It is a family of proline-directed, serine/threonine kinases including ERK1 (p44), ERK2 (p42), and JNK. They are activated by phosphorylation of threonine and tyrosine residues, catalysed by MEK (MAPK kinase), and have a wide range of substrates, including the transcription factors c-Jun, c-Fos and c-Myc.

ATP has been shown to activate astrocyte MAPK via P2 receptors (Neary & Zhu, 1994). Treatment with 100 μM ATP for 15 minutes caused a three to fourfold increase in MAPK activity, which was inhibited by suramin. The time course of activation was rapid (1.5 minutes), peaked at 10–15 minutes and declined to near baseline by 1 hour. Activation was also observed with 2MeSATP and UTP, suggesting that both $P2Y_1$ and $P2Y_2$ receptors are linked to MAPK in astrocytes (Neary et al, 1995). Patel et al (1996b) have also shown that both $P2Y_1$ and $P2Y_2$ receptors are coupled to MAPK in endothelial cells, and that this is required for the stimulation of PGI_2 production. Graham et al (1996) have demonstrated the activation of MAPK by ATP and UTP via a $P2Y_2$ receptor in the Eahy 926 endothelial cell line.

1.6.5.1 Upstream events

A role for PKC in coupling the P2 receptor to MAPK signalling pathways has been suggested following the observation that the PKC inhibitor Ro 31-8220 reduced ATP stimulated MAPK activation (Neary et al, 1995; Graham et al, 1996). This observation has also been reported for other G protein coupled receptors, e.g.

angiotensin II activation of MAPK in vascular smooth muscle cells derived from spontaneously hypertensive rat (Wilkie et al 1996).

Fibroblast growth factor-2 (FGF-2) also activates MAPK in astrocytes, via a Ras/Raf pathway; demonstrated by inhibition of Raf by protein kinase A (Neary & Zhu, 1994). Raf is a serine/threonine kinase and is a member of the family of kinases called MAP3K (MAPK kinase kinase) that phosphorylate MEK, activating it. Raf activation is dependent upon the association of Ras (a small GTP binding protein) with a tyrosine kinase receptor. However, it has been argued that extracellular ATP does not promote MAPK activation through this pathway, as protein kinase A has no effect on ATP induced MAPK phosphorylation (Neary & Zhu, 1994).

The mechanism by which G protein coupled receptors stimulate MAPK activation remains unclear. However, there have been reports of activation via G protein βγ subunits (Crespo et al, 1994; 1995), possibly mediated by phosphoinositide 3-kinase γ (Lopez-Ilasaca et al, 1997) and/or Pyk2 (Lev et al, 1995). Pyk2 is a tyrosine kinase located in neurons, but it seems likely that Pyk2-like activity is more widely distributed.

1.6.5.2 Downstream events

MAP kinases have been reported to activate transcription factors (Pulverer et al, 1991), which bind to DNA, exerting long-term changes in gene expression. Levels of c-Fos and c-Jun, two well-studied transcription factors, have been shown to be increased by 2-MeSATP, α,β-MeATP and FGF-2 (Abbracchio et al, 1995). c-Fos and c-Jun can form functional, heterodimeric transcription complexes known as activator protein-1 (AP-1) which is responsible for many of the long term effects of mitogens (Angel & Karin, 1991). Binding of AP-1 to DNA has been shown to be increased by extracellular ATP and FGF-2 (Neary et al, 1996). This study also used the protein synthesis inhibitor, cyclohexamide, to show that FGF-2 induced increases in AP-1 is mainly dependant upon protein synthesis, whereas the ATP evoked AP-1 binding is mediated by both protein synthesis dependent and independent pathways. This suggests that extracellular ATP can promote the post-translational activation of AP-1, possibly by phosphorylation catalysed by MAPK.

These aspects appear to be related to the long term consequences of MAPK activation. However, recent work, including that on P2Y receptors in our lab (Patel et al, 1996b), have shown that MAPK is also involved in some short term responses, e.g. phosphorylation of PLA_2 (Patel et al, 1996b).

1.7 Nucleotide metabolism and interconversion

It has long been known that extracellular nucleotides are rapidly hydrolysed by ectonucleotidase enzymes on the surface of all cells, and this downstream conversion of nucleoside triphosphates to diphosphates has had a large impact on the study of P2 receptors. However, it is becoming apparent that upstream events also occur i.e. the phosphorylation of nucleoside diphosphates to triphosphates. These two separate fates of nucleotides will be discussed in turn.

1.7.1 Downstream nucleotide interconversion

Although ATP is present in all cells at a concentration of about 5 mM, to be a successful signalling agent, appreciable levels of extracellular ATP must occur only transiently and in response to specific physiological and/or pathological conditions. A specific group of enzymes, the ectonucleotidases, remove extracellular ATP, sequentially dephosphorylating it to ADP, AMP and finally to adenosine. The metabolism of ATP to adenosine is an important pathway, not only because it removes unwanted transmitter, but because it can be the route by which adenosine is produced. Adenosine is an important transmitter in its own right, and has many actions at P1 receptors throughout the body. For example, in cholinergic nerve terminals from the striatum, adenosine prevents further acetylcholine release, whereas at cortical cholinergic terminals, adenosine is not effective. This difference is accompanied by an incomplete ectonucleotidase pathway in the cortical terminals, suggesting that adenosine is not released directly into the synapse, but is rather produced by the degradation of released ATP (Richardson et al, 1987).

Three separate enzymes, ectoATPase, ectoADPase and ecto-5'-nucleotidase sequentially dephosphorylate ATP to ADP to AMP to adenosine, and these enzymes are closely associated with the sites of nucleotide release (Maienschein & Zimmermann 1996).

1.7.1.1 EctoATPase

EctoATPase splits off the terminal phosphate group of a broad range of nucleoside triphosphates, although ATP is the prefered substrate (see Ziganshin et al 1994a). There are Ca^{2+} /Mg^{2+} -dependant and –independent forms, and in most tissues the optimum pH for activity is about 7.5. cDNA sequences encoding ectoATPase have been cloned and were found to belong to the immunoglobulin superfamily of cell adhesion molecules (Najjar et al 1993). The deduced amino acid sequence of ectoATPase consists of 519 amino acids encompassing an extracellular catalytic domain, a transmembrane region that anchors the glycoprotein to the lipid bilayer, and a cytoplasmic region that contains two potential tyrosine phosphorylation sites. Interestingly, these sites undergo insulin- and growth factor-stimulated phosphorylation (Rees-Jones & Taylor 1985), which may indicate regulation of ectoATPase by these factors.

1.7.1.2 EctoADPase

EctoADPase performs the removal of the β-phopsphate group from ADP, forming AMP. It is selectively inhibited by ATP and its non-hydrolysable analogues β,γ-imidoATP and β,γ-methyleneATP (Pearson & Gordon 1985; Pearson et al 1980).

1.7.1.3 Ecto-5'-nucleotidase

Catalysis of the final step from AMP to adenosine is performed by ecto-5'-nucleotidase (ecto-monophosphatase), an enzyme linked to the plasma membrane via a phosphatidylinositol-glycan anchor. Immunocytochemical localisation of ecto-5'-nucleotidase shows a broad distribution of the enzyme at the surface of neurons (Maienchein & Zimmermann, 1996). The enzyme can be selectively inhibited by the synthetic ADP analogue α,β-methylene ADP, which has no effect on ATP or ADP

catabolism (Pearson et al 1980), or by an antiserum to the purified enzyme (Stanley et el 1980).

Ecto-5'-nucleotidase is also inhibited by ADP and ATP, thus feed-forward inhibition from high concentrations of ATP and/or ADP decreases the efficiency of adenosine production, creating a build-up of AMP. However, in a study using aortic smooth muscle cells, AMP produced at the cell surface by ADPase was hydrolysed more efficiently than predicted from its bulk phase concentration (Gordon et al, 1989). This suggests there is preferential delivery of AMP from ADPase to ecto-5'-nucleotidase, that is, that ADPase and ecto-5'-nucleotidase are sufficiently close and correctly orientated to facilitate the direct passage of AMP from ADPase to the active site of ecto-5'-nucleotidase without it entering the bulk phase (see Figure 1.7). Similarly, with ventricular myocytes, when ADP was supplied by ATPase, the apparent K_m values were much lower than those observed when ADP was supplied as the initial substrate, suggesting that the concentration of ADP derived from ATP at the cell surface was much higher than that in the bulk phase (see Meghji et al, 1993). Thus, in aortic smooth muscle this combined preferential delivery of both ADP and AMP outweighs the effect of feed-forward inhibition by ATP of ectoADPase and ecto-5'-nucleotidase, and adenosine is produced rapidly on addition of ATP.

However, these events are dependent upon the relative V_{max} values of the enzymes in the tissue being studied. For example, both ADP and AMP would be expected to build up in striatal cholinergic synapses where the V_{max} values for ATPase, ADPase and 5'-nucleotidase present in are in a ratio of 30:14:1 (James & Richardson; 1993).

1.7.1.4 Structure activity of ectonucleotidases

Naturally occurring purine and pyrimidine nucleoside triphosphates (ATP, GTP and UTP, CTP, respectively) are sequentially dephosphorylated at approximately the same rate (Welford et al 1986; Welford et al 1987), and substitutions at the 2, 8 and N^6 positions on the purine ring do not affect the rate of removal of the terminal phosphate. The phosphorothioate analogues ATPγS and ATPβS are dephosphorylated more slowly than ATP, but ATPαS is dephosphorylated at the same rate as ATP. The effect of replacing a bridging oxygen in the phosphate chain by methylene depends on

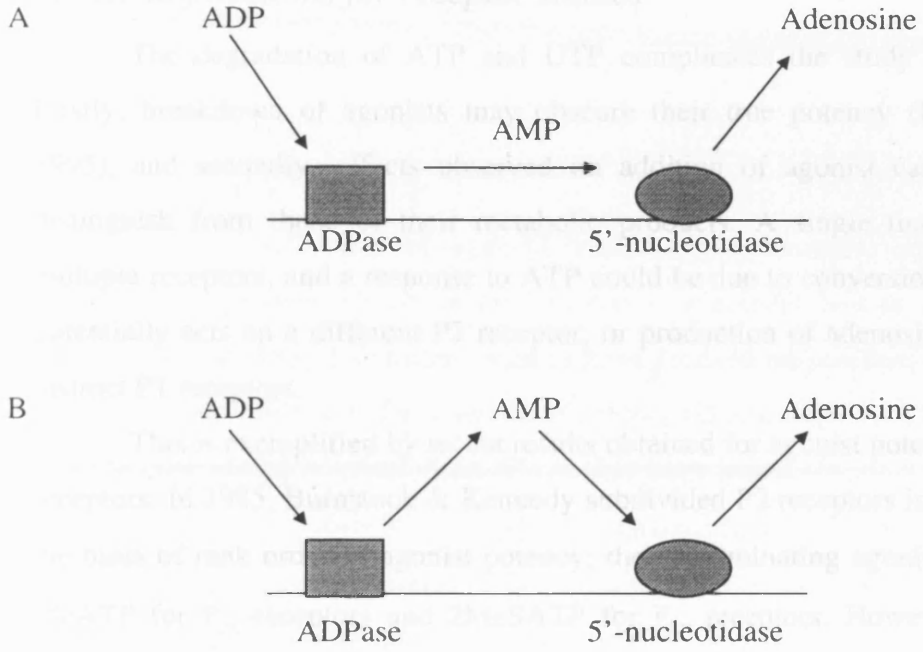


Figure 1.7

Two models of the degradation of adenosine 5'-diphosphate (ADP).

A. Preferential delivery of substrate from ADPase to 5'-nucleotidase. **B.** Delivery of substrate to 5'-nucleotidase from the bulk phase.

the position of its insertion. Homo-ATP is degraded at the same rate as ATP, but α,β-MeATP and β,γ-MeATP are very stable. Analogues in which one of the oxygen atoms on the terminal phosphate had been replaced are also resistant to degradation (Welford et al 1987).

1.7.1.5 Implications for receptor studies

The degradation of ATP and UTP complicates the study of P2 receptors. Firstly, breakdown of agonists may obscure their true potency (Kennedy & Leff 1995), and secondly, effects observed on addition of agonist can be difficult to distinguish from those of their metabolic products. A single tissue may express multiple receptors, and a response to ATP could be due to conversion to ADP, which potentially acts on a different P2 receptor, or production of adenosine, which acts at distinct P1 receptors.

This is exemplified by recent results obtained for agonist potency orders on P_{2X} receptors. In 1985, Burnstock & Kennedy subdivided P2 receptors into P_{2X} and P_{2Y} on the basis of rank order of agonist potency; the discriminating agonists included α,β-MeATP for P_{2X} receptors and 2MeSATP for P_{2Y} receptors. However, studies (e.g. Trezise et al, 1994) have showed that in some tissue, 2-MeSATP is a potent P_{2X} agonist. In a paper by Kennedy & Leff (1995) using the ectonucleotidase inhibitor ARL 67156, it was concluded that the selective breakdown of 2MeSATP compared to α,β-MeATP had disguised its potency, and that 2MeSATP was in fact more potent at P_{2X} receptors than α,β-MeATP. The problem of nucleotide breakdown persists, and has been tackled using hydrolysis resistant nucleotides and ectonucleotidase inhibitors.

1.7.1.5.1 Resistant nucleotides

Although there is a paucity of hydrolysis-resistant agonists that can distinguish between the various P2 subtypes, several nucleotides have been successfully utilised in the study of P2 receptors. α,β-MeATP and β-γ-MeATP are selective for certain P2X receptors and are very stable agonists. ATPγS has also been widely used and is equipotent to ATP in most tissues where ectonulceotidase activity is low. UTPγS, a

hydrolysis resistant pyrimidine nucleotide, can be synthesised using the enzyme nucleoside 5'-diphosphate kinase (NDPK). NDPK exibits a broad substrate specificity and can utilise either GTPγS of ATPγS as the γ-phosphorothioate donor molecule, and UDP as the acceptor substrate. UTPγS is equipotent with UTP and ATP at 1321N1 cells expressing the cloned human $P2Y_2$ receptor and is not hydrolysed by alkaline phosphatase, acid phosphatase, apyrase, nor after a 1 hour incubation with human nasal epithelium cells (Lazarowski et al., 1996).

1.7.1.5.2 Inhibitors

Classical inhibitors of endoATPases such as ouabain, sodium azide and oligomycin have been shown to be ineffective at ectoATPase in *Xenopus* oocytes (Ziganshin et al, 1995), and many studies have focused on potential ectonucleotidase inhibitors to investigate the true structure-activity relationships of ATP analogues. To date only one useful inhibitor, ARL 67156, has been found.

ARL 67156 (6-N,N-diethyl-β,γ-dibromomethylene-D-ATP) (formerly FPL 67156) is a newly synthesised analogue of ATP. It is a potent ectoATPase inhibitor, and in a human blood cell assay, the compound inhibited [γ^{32}P]-ATP degradation with a pIC_{50} of 4.6 (Crack et al, 1995). It does, however, have weak actions at some P2 receptors. It has low potency agonist effects at $P2Y_2$ receptors ($p[A_{50}] \approx 3.5$) and weak antagonist effects at P2X and P2T receptors ($pA_2 \approx 3.3$ and 3.5 respectively). ARL 67156 has been shown to greatly enhance sympathetic purinergic neurotransmission in the guinea-pig isolated vas deferens in a manner consistent with inhibition of hydrolyis of released ATP (Westfall et al, 1996).

Another compound, cyclopiazonic acid (CPA), has been described as a selective inhibitor of Ca^{2+} -ATPase in the sarcoplasmic reticulum of skeletal and smooth muscles (Kurebayashi & Ogawa, 1991; Uyama et al 1992). However, Ziganshin et al (1994b) showed that while CPA did reduce ATP degradation by 18 % in the guinea-pig urinary bladder, the potentiation of response by CPA was non-specific, since contractile responses to KCl (30mM) were also increased.

An added complication in the study of P2 receptors is that many of the compounds used as P2 antagonists have been shown to also inhibit nucleotide degradation. Thus, limited effects of antagonists on concentration-response curves to hydrolysable nucleotides may be the result of 'self-cancellation' of the potentiating (ectonucleotidase inhibitory) and rightward-shifting (receptor antagonistic) properties (see Crack et al, 1994). It has been suggested that ATPases and P2 receptors have very similar ATP-binding domains at which P2 antagonists also bind, causing inhibition of ATP degradation, antagonism of purinergic responses or both (Hoyle et al 1990).

Suramin is renowned for having many biological actions, and there are many examples in the literature of its inhibition of ectonucleotidases. Suramin has been shown to inhibit ectoATPase in a non-competitive manner in the guinea-pig urinary bladder (Hourani and Chown 1989), and in *Xenopus* oocytes (Ziganshin et al, 1995). In a study by Beukers et al (1995), suramin at a concentration of 100 μM reduced ATP (initial concentration of 10 μM) breakdown by human blood cells to 46.3 ± 4.2 % of the control value. Inhibition was again shown to be non-competitive, with a Ki value of 43.5 ± 15.5 μM.

NF023, a structural analogue of suramin, has also been shown to inhibit ATP breakdown. At concentrations of 100 μM, NF023 reduced ATP (initial concentration of 10 μM) breakdown by human blood cells to 51.0 ± 3.5 % of the control value (Beukers et al 1995).

In a study by Bultmann and Starke (1995), degradation of ATP (10 μM initial concentration) by the vas deferens and taenia coli was reduced by up to 95 % by reactive red 2, another P2 antagonist. Acid red 33, a structural analogue of reactive red 2, reduced degradation in the same system by only 20-30 %. The dichloro-triazine residue that distinguishes reactive red 2 from acid red 33 therefore appears to be very important for activity at ectonucleotidases.

Pyridoxalphosphate-6-azophenyl-2', 4'-disulphonic acid (PPADS) has been shown to inhibit the ectoATPase enzyme in several tissues, e.g. on bovine pulmonary artery endothelium, where the IC_{50} for PPADS was 4.0 (Chen et al, 1996), and in folliculated *Xenopus* oocytes (Ziganshin et al, 1996). PPADS and pyridoxal 5'-phosphate (P5P) have also been shown to reduced ATP breakdown to 80.7 ± 10.2 and 76.5 ± 10.0 % of the initial level in rat vas deferens (Khakh et al,1995).

One P2 antagonist, reactive blue 2, does not inhibit ectonucleotidases. In the guinea-pig urinary bladder, ATP breakdown was unaffected by reactive blue 2 pretreatment (Hourani & Chown, 1989). A summary of some well known ectonucleotidase inhibitors is presented in Table 1.2.

1.7.1.6 Fate of adenosine

Adenosine produced by the degradation of ATP can be deaminated by an extracellular adenosine deaminase. However, this enzyme is restrictively distributed (Meghji 1993), and uptake into cells is more common. Adenosine is transported by a specific, bidirectional uptake carrier, which is inhibited by dipyriamole and *p*-nirtobenzyl-6-thioguanosine (Jacobson 1990). Once in the cell, adenosine is inactivated either by phosphorylation by adenosine kinase forming AMP, or by deamination to inosine by adenosine deaminase.

1.7.2 Upstream nucleotide interconversion

There is growing evidence that nucleotides are not only sequentially degraded, but that interconversion occurs at the surface of cells. Several ecto-enzymes which catalyse the transfer of phosphate groups between nucleotidase have been described.

1.7.2.1 Adenylate kinase

Adenylate kinase is a family of closely related enzymes of approximately 22.5 kDa (Tsubio & Chervenka, 1975). It is dependant upon Ca^{2+} or Mg^{2+} (Kluge et al, 1975), and catalyses the reaction: 2 ADP $\leftrightarrow$ ATP + AMP.

Adenylate kinase exists predominantly as an intracellular, membrane bound protein where it is an important contributor of ATP for mitochondrial hexokinase. However, it is also found in the extracellular environment as a soluble enzyme, e.g. in plasma (see Lindena et al, 1986) and synapses (Kluge et al, 1975), released from many cells. Kluge et al (1975) also demonstrated the rapid metabolism of exogenous extracellular ATP by adenylate kinase.

Inhibitor	Normal use	Tissue studied	Degree of inhibition	Reference
Suramin	P2 antagonist	Guinea-pig urinary bladder Xenopus oocytes Human blood cells	 46.3 ± 4.2 % reduction in breakdown Ki = 43.5 ± 15.5 μM	Hourani & Chown (1989) Ziganshin et al (1995) Beukers et al (1995)
NF023	P2 antagonist	Human blood cells	51.3 ± 3.5 % reduction in breakdown	Beukers et al (1995)
Reactive red 2	P2 antagonist	Rat vas deferens	95 % reduction in ATP breakdown	Bultmann & Starke (1995)
Ethacrynic acid		Guinea-pig urinary bladder		Hourani & Chown (1989)
PPADS	P2 antagonist	Rat vas deferens	80.7 ± 10.2 % reduction in breakdown	Khakh et al (1994)
ARL 67156	EctoATPase inhibitor	Human blood cells	$pIC_{50} = 4.6$	Crack et al (1995)

Table 1.2. Summary of the properties of some ectonucleotidase inhibitors.

1.7.2.2 Nucleoside diphosphokinase

Nucleoside diphosphokinase is an enzyme that transfers the terminal phosphate group from a nucleotide triphopshate to a nucleotide diphosphate molecule. When studying the possible inhibitory effect of ATP on ADP metabolism by pig aortic endothelial cells, Pearson et al (1980) discovered that the increment in [^{3}H]ADP metabolism was exactly balanced by the formation of [^{3}H]ATP. This showed that endothelial cells possess an ectoenzyme with nucleoside diphosphokinase activity.

More recently, Nicholas et al (1996) reported the presence of ecto-nucleoside diphosphokinase activity on 1321N1 astrocytoma cells. This enzyme exhibits a larger V_{max} and lower K_m for UDP than the 1321N1 cell-associated hydrolase activity does, thus addition of UDP to the medium in the presence of ATP favours the metabolic formation of UTP rather than UMP (see Harden et al, 1997). Approximately 3% of added UDP (100nM) was converted to UTP in a 10 minute incubation. This was increased to 16 and 17 % if the medium was changed before the addition of UDP (resulting in release of endogenous ATP) or if 100 nM ATP was added concomitantly with the UDP. This activity has masked the true rank order of agonist potencies for several cloned P2 receptors, but the use of hexokinase and glucose to convert any triphosphates produced by nucleoside diphosphokinase back into diphosphates can circumvent this problem. Figure 1.8 summarises the effect nucleotide interconversion has on P2Y receptors.

1.8 Physiological roles of P2Y receptors

Receptors for extracellular nucleotides are virtually ubiquitously expressed, and as such play important roles in many physiological and pathophysiological functions. The following examples represent some of the most thoroughly studied, and give an idea of the diversity of roles of extracellular nucleotides.

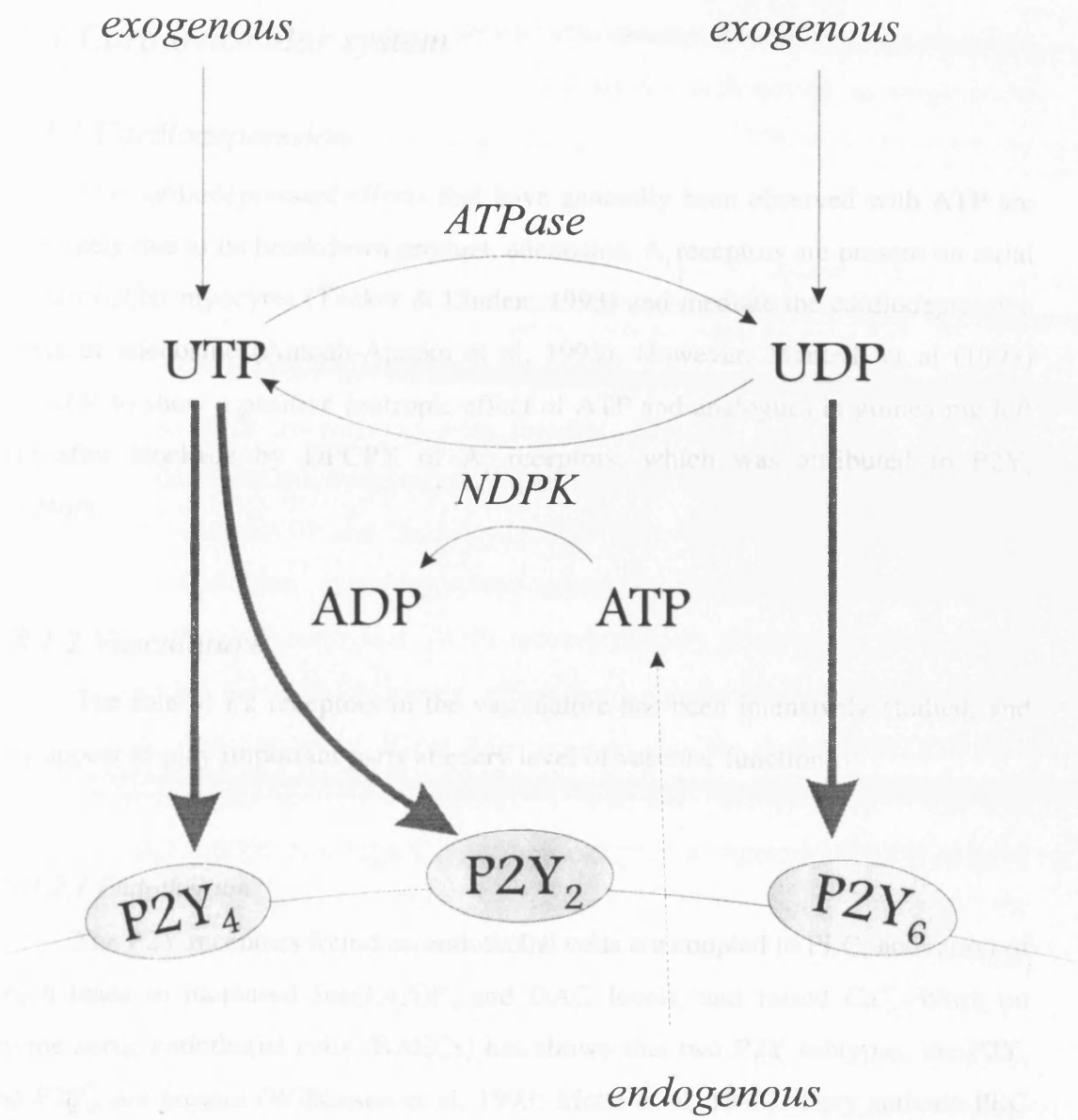


Figure 1.8

The possible consequences of nucleotide interconversion.

A cartoon demonstrating the problems posed by a system with high levels of ATPase and/or nucleoside diphosphokinase (NDPK) acitivity. The exogenous addition of either UTP or UDP to the cells may result in the stimulation of receptors not normally activated by these specific agonists. Refer to section 1.7.

1.8.1 Cardiovascular system

1.8.1.1 Cardiodepression

The cardiodepressant effects that have generally been observed with ATP are more likely due to its breakdown product, adenosine. A_1 receptors are present on atrial and ventricular myocytes (Tucker & Linden, 1993) and mediate the cardiodepressive effects of adenosine (Amoah-Apraku et al, 1993). However, Mantelli et al (1993) were able to show a positive inotropic effect of ATP and analogues in guinea-pig left atria after blockade by DPCPX of A_1 receptors, which was attributed to $P2Y_1$ receptors.

1.8.1.2 Vasculature

The role of P2 receptors in the vasculature has been intensively studied, and they appear to play important parts at every level of vascular function.

1.8.1.2.1 Endothelium

The P2Y receptors found on endothelial cells are coupled to PLC, activation of which leads to increased $Ins(1,4,5)P_3$ and DAG levels, and raised Ca^{2+}. Work on bovine aortic endothelial cells (BAECs) has shown that two P2Y subtypes, the $P2Y_1$ and $P2Y_2$, are present (Wilkinson et al, 1993; Motte et al, 1993). They activate PLC through distinct G proteins, the $P2Y_1$ receptor couples to G_q, and the $P2Y_2$ receptor couples to $G_{i/0}$. The regulation of this coupling also varies, with PKC providing inhibitory feedback for $P2Y_1$ but not for $P2Y_2$ (Purkiss et al, 1994). Action of ADP at $P2Y_1$, and ATP and UTP at $P2Y_2$ receptors on the vascular endothelium causes synthesis and release of nitric oxide (NO) and prostacyclin (PGI_2) which diffuse to the smooth muscle, causing relaxation (Boeynaems & Pearson, 1990; Carter et al, 1988; Wilkinson et al, 1994). The activation of $cPLA_2$ appears to involve both PKC and tyrosine phosphorylation and activation of MAPK (Carter et al, 1988; Patel et al, 1996a,b).

Endothelial cells are able to release ATP in response to increases in perfusion flow rate and hypoxia (Milner et al, 1990a,b; Bodin et al, 1992). It is likely that ATP

released by endothelial cells during periods of increased flow acts on P2 receptors further downstream to take part in the vasodilation which occurs in response to increased sheer stress (see Ralevic et al, 1993).

1.8.1.2.2 Platelet function: the P2T receptor

The discovery that ADP could cause platelet aggregation, and that this response was antagonised by ATP, was made by Born in 1962. The receptor responsible has been named the P_{2T} receptor (Gordon 1986), and has been extensively studied. At this receptor, analogues of ADP with 2-substitution increases potency, with 2-chloroADP and 2-methylthioADP both being more potent than ADP itself, while substitution of a methylene group for the linking oxygen in the diphosphate chain (α,β-methylene ADP) reduces potency (Hourani & Hall, 1994). The same applies for ATP analogues, which act as pure competitive antagonists. Suramin is also an antagonist at the P_{2T} receptor, roughly potent with ATP, but appears to have a non-specific component to its action (Hourani et al, 1992). The compound ARL 66096 is a highly potent, competitive antagonist of ADP-induced aggregation (Humphries et al, 1994). As with other P2 receptor subtypes, the uncomplexed form of agonist i.e. ADP^{3-} is the prefered form (Hall et al, 1994). ADP acts at P_{2T} receptors in platelets to stimulate PLC and increases intracellular Ca^{2+} levels, and has also been shown to inhibit adenylyl cyclase, probably via G_{i2} (Gachet et al, 1992; Ohlmann et al, 1995). Increased intracellular Ca^{2+} levels causes platelet activation, leading to shape change (from a biconvex disc to a spiny sphere), aggregation and exocytotic release of local mediators of inflamation, including ATP and UTP (see Hourani & Hall, 1994).

1.8.1.2.3 Vascular smooth muscle

Release of ATP from sympathetic nerves in vascular smooth muscle causes vasoconstriction via activation of P2X receptors (Benham, 1990). The receptors involved are likely to be the $P2X_1$ subtype, with evidence from both pharmacological and immunocytochemical studies (Evans & Kennedy, 1994; Vulchanova et al, 1996). UTP has been shown to raise cytosolic Ca^{2+} levels, causing vasoconstriction in a variety of tissues, e.g. rabbit ear artery (Saiag et al, 1990) and rat aorta (Garcia-

Velasco et al, 1995), with evidence presented for the presence of both $P2Y_2$ and $P2Y_4$ receptors. The $P2Y_6$ receptor was first cloned from rat aorta VSM cells, and the mRNA for both $P2Y_2$ and $P2Y_6$ have been found in cultured VSM cells (Chang et al, 1995). Nucleotides have also been shown to elicit a mitogenic response, either alone or in synergy with PDGF (see section 8.5 for more detail).

1.8.1.2.4 P2 receptor regulation of vascular function

ATP and UTP released from platelets (Ingerman et al, 1979) act at $P2Y_1$ and $P2Y_2$ receptors on the surface of the endothelium, causing the release of NO and PGI_2. These mediators diffuse to the vascular smooth muscle, causing vasodilation, and feedback on the platelets, inhibiting activation. However, if the endothelium is absent or damaged, the nucleotides act directly at P2 receptors on the vascular smooth muscle, causing vasoconstriction and proliferation. Thus, if platelets start to aggregate in an intact blood vessel, the vessel will dilate and aggregation will be limited, whereas if they aggregate in a cut or damaged blood vessel, the vessel will constrict and platelet aggregation will be enhanced and will spread (see Hourani & Hall, 1996). Figure 1.9 shows the role of P2Y receptors in vascular modulation.

1.8.2 The nervous system

1.8.2.1 Central nervous system

The role of ATP as an excitatory neurotransmitter mediating fast transmission in the CNS has been unequivocally demonstrated (Benham, 1992; Edwards et al, 1992). These effects are mediated primarily through the activation of P2X receptors, which are ATP-gated cation-selective channels. Upon opening, these channels allow sodium and potassium ions to flow across the membrane, causing rapid membrane depolarisation and action potential firing. The channels exhibit little or no selectivity among small monovalent cations. Receptors for ATP are present on neurones and glial cells (Zimmermann, 1994), and have been shown by autoradiography to be widely distributed in rat brain and spinal cord (Bo & Burnstock, 1994). ATP has been shown to participate in sensory neurotransmission (Hoyle et al, 1991). In particular, the generation of pain signals is via $P2X_1$ and $P2X_2$ receptors in the dorsal horn of the

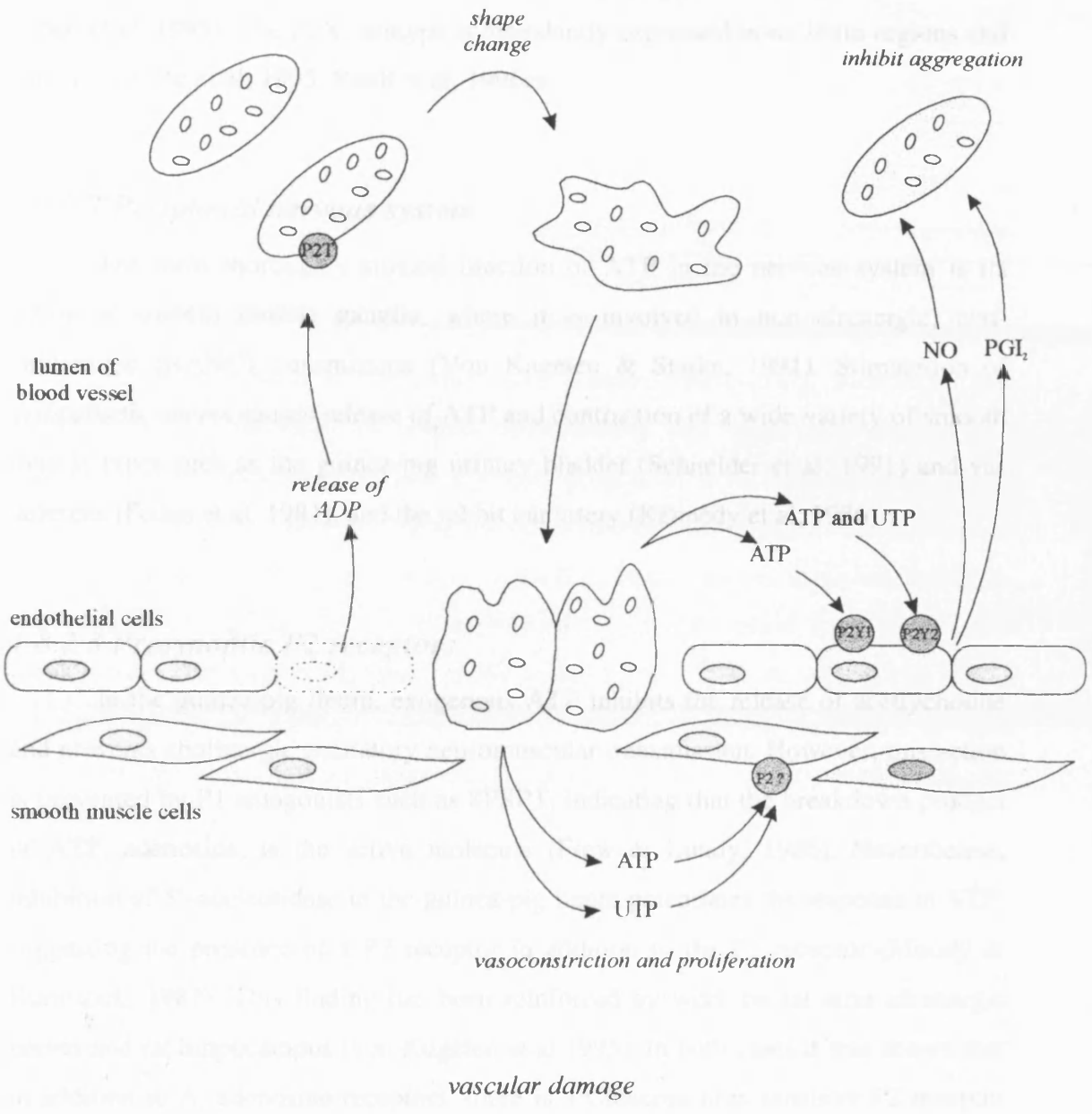


Figure 1.9
P2 receptor regulation of vascular function.
See section 1.8.1.2 for a detailed account of the role of P2 receptors in vascular modulation.

spinal cord (Kennedy & Leff, 1995), and the $P2X_3$ subtype in sensory C fibre nerves (Chen et al, 1995). The $P2X_4$ subtype is abundantly expressed in rat brain regions and spinal cord (Bo et al, 1995; Buell et al, 1996a).

1.8.2.2 Peripheral nervous system

The most thoroughly studied function of ATP in the nervous system is its action at smooth muscle ganglia, where it is involved in non-adrenergic, non-cholinergic (NANC) transmission (Von Kugelen & Starke, 1991). Stimulation of sympathetic nerves causes release of ATP and contraction of a wide variety of smooth muscle types such as the guinea-pig urinary bladder (Schneider et al, 1991) and vas deferens (Fedan et al, 1981), and the rabbit ear artery (Kennedy et al, 1986).

1.8.2.3 Presynaptic P2 receptors

In the guinea-pig ileum, exogenous ATP inhibits the release of acetlycholine and prevents cholinergic excitatory neuromuscular transmission. However, this action is prevented by P1 antagonists such as 8PSPT, indicating that the breakdown product of ATP, adenosine, is the active molecule (Frew & Lundy, 1986). Nevertheless, inhibition of 5'-nucleotidase in the guinea-pig ileum potentiates the response to ATP, suggesting the presence of a P2 receptor in addition to the P1 receptor (Moody & Burnstock, 1982). This finding has been reinforced by work on rat atria adrenergic nerves and rat hippocampus (von Kugelen et al 1995). In both cases it was shown that in addition to A_1 adenosine receptors, there is a Cibacron blue sensitive P2 receptor that inhibits the electrically evoked release of [^{3}H]noradrenaline. It remains to be proven that ATP can inhibit its own release in this way. ATP has also been shown to inhibit acetylcholine release from rat cerebral cortex (Cunha et al, 1994) and to inhibit glutamate release and action in rat hippocampus (Motin & Bennett, 1995).

1.8.3 The immune system

Extracellular ATP is known to act on a variety of cells within the immune system, including mast cells, macrophages, and lymphocytes (Wiley et al, 1994;

Zambon et al, 1994; DiVirgilio, 1995). One effect of ATP in the immune system is modulation of mast cell function. During mast cell degranulation, ATP is co-released from secretory granules together with histamine and other mediators of anaphylaxis. This extracellular ATP then functions as a paracrine signalling molecule, activating P2 receptors on nearby mast cells and potentiating further degranulation through mobilisation of intracellular Ca^{2+} (Osipchuk & Cahalan, 1992). Because this response is blocked by suramin, or by desensitising concentrations of ATP, the authors conclude that histamine and other signalling agents are not involved in this process.

Stimulation with extracellular ATP causes Ins(1,4,5)P_3 formation and intracellular Ca^{2+} increases in several inflammatory cell types (e.g. neutrophils, monocytes and macrophages) and myeliod progenitor cells (see Dubyak & El Moatassim, 1993, for review). These responses are dependent upon activation of G protein-coupled P2Y receptors for ATP and UTP. Stimulation of these receptors has also been linked to activation of phospholipases A_2 (and therefore the production of local inflamatory mediators), and protein kinase C (Cockroft & Stutchfield, 1989; Balazovich & Boxer, 1990). In addition, P2Y receptors may also facilitate migration of inflammatory cells by up-regulating expression of adhesion molecules on monocyte and granulocyte plasma membrane (Freyer et al, 1988).

Many cells of the immune system, including mast cells, lymphocytes, macrophages, and epidermal Langerhan's cells, express the $P2X_7$ receptor (DiVirigilo, 1995). Activation of $P2X_7$ receptors induces the formation of large, nonselective plasma membrane pores, the function of which remains unanswered. Speculation on the links between P2 ionotropic receptors and programmed cell death (apoptosis) (Steinberg & DiVirgilio, 1991) has been reinforced by the observation that extracellular ATP acting at the $P2X_7$ receptor is a powerful activator of IL-1β converting enzyme, a key player in the apoptotic process (Griffiths et al, 1995).

1.8.4 Gastrointestinal tract

Innervation of the gastrointestinal tract is complex and involves branches of the sympathetic, parasympathetic, and enteric nervous system. ATP has long been recognised as the non-adrenergic, non-cholinergic (NANC) transmitter, released on nervous stimulation of the gut (Burnstock et al, 1970), and the receptors involved with

nucleotide signalling have been well characterised. In the rat duodenum, P2X and $P2Y_2$ receptors mediate contraction, while $P2Y_1$ receptors mediate relaxation (Johnson et al, 1996). While at first this seems contradictory, it appears that different layers of muscle in the duodenum express different populations of P2 receptors, and are innervated separately. A_1 and A_{2b} receptors for adenosine are also present on rat duodenum (Nicholls et al, 1992). Recently, moderate levels of the $P2Y_6$ subtype mRNA have been detected in rat stomach and intestine (Chang et al, 1995).

1.8.5 Trophic actions of ATP

It has long been known that polypeptide growth factors, such as platelet derived growth factor (PDGF), are able to stimulate neural regeneration, e.g. cause astrocyte proliferation by binding to specific tyrosine kinase receptors. However, recent studies have suggested that nucleotides acting through G protein coupled receptors, may exert trophic effects in the brain during development and/or following injury (Neary et al, 1996a). For example, extracellular ATP has been shown to increase DNA synthesis and astroglial proliferation in primary cultures of rat stiatum (Abbracchio et al, 1994).

Extracellular ATP can also act synergistically with polypeptide growth factors. For example, ATP greatly enhances mitogenesis induced by polypeptide growth factors in astrocytes (Neary et al, 1994). In these cells, ATP increases DNA synthesis (as measured by [^{3}H]thymidine incorporation) approximatley twofold, fibroblast growth factor (FGF-2) increases DNA synthesis by 14 fold, but ATP and FGF-2 added concurrently cause a 52-fold increase (Neary et al, 1994). The synergistic mode of action between nucleotides and growth factors is not fully understood. However, in a study on cultured VSM it was shown that PDGF stimulated the movement of quiescent serum-starved cells from G_0 to G_1, but no further, and that while ATP and UTP did not influence the cell cycle of cells in G_0, they did stimulate progression of PDGF treated cells in G_1 into the completion of the cell cycle through S and M phases (Miyagi et al, 1996).

Several studies examining the trophic actions of ATP have been performed in cultured rat aortic smooth muscle cells (VSMCs). Yu et al (1996) have shown that

ATP triggers mitogenesis by activation of a G protein–coupled $P2Y_1$ receptor leading to the formation of inositol triphosphate and activation of PKC. PKC and, in turn, Raf-1 and MAPK are then activated, leading eventually to DNA synthesis and cell proliferation. MAPK is a key element in the signal transduction pathways involved in cell growth, and the mechanisms by which it stimulates mitogensis are discussed in section 1.6.5. In a separate study, Erlinge et al (1995) have demonstrated the activation of mitogenesis through a $P2Y_2$ receptor in VSMC. The production of $Ins(1,4,5)P_3$ and the influx of Ca^{2+} appear to be important (Erilnge et al, 1993; 1995).

1.9 Therapeutic uses

The nucleotides ATP and UTP, and the P_{2T} receptor antagonist ARL 67085 are all currently being evaluated as novel treatments for a variety of disease states. This section will outline several of the potential therapeutic uses of P2 receptor ligands, focusing on those areas of greatest potential.

ATP has been tested for multiple uses during surgery. It has been used as an adjunct to inhalation anaesthetics, reducing the doses of traditional anaesthetics required, improving the margin of safety and recovery time after surgery (Fukunaga et al, 1995). Given intravenously, ATP has been used to decrease the risks of bleeding during surgery (Muruyama et al, 1979).

Also, recent studies have suggested that nucleotides acting at P2Y receptors, may exert beneficial trophic effects in the brain during development and/or following injury (Neary et al, 1996a). For example, extracellular ATP has been shown to increase DNA synthesis and astroglial proliferation in primary cultures of rat stiatum (Abbracchio et al, 1994).

1.9.1 Cystic fibrosis

The $P2Y_2$ receptor on airway epithelial cells has attracted interest for its ability to stimulate Cl^- transport, and is of potential therapeutic benefit for patients with

cystic fibrosis. Mutations in the primary airway epithelial chloride transpoter, the cystic fibrosis transmembrane conductance regulator (CFTR), result in defective regulation of Cl^- transport, leading to dehydrated and thickened mucous secretions in the lung that are not cleared effectively (Boucher et al, 1986). Activation of the $P2Y_2$ receptor leads to an increase in Cl^- secretion through Ca^{2+} dependent Cl^- channels distinct from the CFTR, alleviating the symptoms of the disease (Jetten et al, 1989; Mason et al, 1991). Extracellular nucleotides, acting on alveolar Type II cells, can also stimulate the release of surfactant, which is important in lowering the surface tension of liquids that coat the lungs, enhancing lung clearance (Rice & Singleton, 1986).

An airway epithelial cell line (CF/T43) has been developed from a patient with cystic fibrosis (Jetten et al, 1989), which has since been used to clone a human $P2Y_2$ receptor (Parr et al, 1994).

1.9.2 Vascular effects

1.9.2.1 Proliferation of vascular smooth muscle cells

In the normal, healthy state, nucleotides released from platelets interact with endothelial P2Y receptors causing release of mediators such as prostacyclin and nitric oxide. These act to inhibit platelet activation, and also have relaxant and antiproliferative effects on the vascular smooth muscle cells. A healthy endothelium also presents a barrier between the platelet-derived nucleotides and the VSM cells, limiting their influence (see section 8.12). However, when the endothelium is damaged, due to disease or mechanical trauma, the barrier function is lost. This results in a deficit in the production of PGI_2 and NO, and increased exposure of the VSM to platelet derived nucleotides. In addition, platelet activation and nucleotide release will increase, resulting in the increased contraction and proliferation of VSM cells.

Smooth muscle proliferation plays a role in atherosclerotic plaque formation, and restenosis following balloon angioplasty or coronary bypass surgery. A more detailed understanding of the nature of the P2Y receptors responsible for the stimulation of VSM proliferation would provide information vital for potential therapeutic intervention.

1.9.2.2 Antithrombotic actions of ARL 67085

ADP released from ruptured or damaged endothelial cells can cause platelet activation via action at the P_{2T} receptor. ARL 67085 functions as a potent P_{2T} antagonist that shows greater than 30 000-fold selectivity over $P2Y_1$ receptors and P2X (Humphries et al, 1994, 1995a,b). In a canine model of thrombosis, the compound was able to abolish thrombosis and block ADP-induced platelet aggregation without changes in blood pressure and heart rate. Preclinical data showed that ARL 67085 had a superior margin of safety to fibrinogen receptor antagonists, with a separation between those doses inhibiting thrombosis and those increasing bleeding time of 28-fold (see Williams, 1996).

1.9.3 Anticancer effects

In a detailed review, Rapaport (1994) discussed the utilisation of ATP administration for the treatment of cancer and AIDS. In murine and initial human trials, the administration of adenine nucleotides has been show to produce cytostatic and cytotoxic effects on tumours, as well as many other beneficial effects such as enhancement of superoxide anion (O^{2-}) production by phagocytes. The effective and long-lasting cytostatic effects of ATP have been postulated to occur via permeabilisation of tumour cell membranes as well as the opening of plasma membrane Ca^{2+} and Na^{2+} channels (Rapaport, 1993). These cytotoxic actions of ATP appear to involve the gap junction protein, connexin 43, and may thus be mechanistically related to the ATP-stimulated, $P2X_7$ receptor-mediated apoptosis described in macrophages (DiVirgilio, 1995).

1.10 Objectives

The primary objective of this thesis was the detailed characterisation of cloned $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors transfected into 1321N1 cells. Because these receptors were all transfected into the same host cell type, direct comparisons between each of the receptors could be made. This information would then aid the classification of native P2Y receptors in other tissues and cell types. Initially, the effect of several different agonists and antagonists were to be tested, and then a more detailed examination of the transduction coupling of these receptors was to be carried out. Many studies examining agonist potency and antagonist affinity in the past have been compromised by the effect of nucleotide breakdown by cell surface ectonucleotidases. Therefore, in this study the effect of nucleotide hydrolysis was to be carefully monitored and controlled for.

Secondly, the information gained from this initial study on cloned receptors was to be used to characterise the P2Y receptors present on the surface of vascular smooth muscle cells. Also, the possible effects of P2Y receptor stimulation on vascular smooth muscle cell proliferation were to be examined.

Finally, an attempt at antibody production against the bovine $P2Y_1$ receptor was to be made. Specific antisera would have several uses. Firstly, it would help in the assessment of receptor expression number. It would also enable an examination of potential receptor phosphorylation by immunoprecipitation of the receptor protein. Finally, it would have many applications in the field of immunocytochemistry, allowing cellular distribution and localisation of the $P2Y_1$ receptor to be examined.

Chapter 2: Methods

2.1 Cell culture

2.1.1 Preparation of transfected 1321N1 cells

The proceedure used in our laboratory to produce 1321N1 cells expressing the bovine $P2Y_1$ receptor is described in section 2.2. Cloned turkey $P2Y_1$, human $P2Y_2$, and human $P2Y_4$ receptors transfected into a 1321N1 human astrocytoma cell line that possesses no endogenous P2 receptor were gifts from Dr. G. Weisman, University of Columbia-Missouri, U.S.A. The methods used in the transfection of each of these receptors are outlined below.

cDNA encoding the turkey $P2Y_1$ receptor was ligated into the pcDNA3 expression vector that contains a cytomegalovirus promotor for high expression levels in mammalian cells. Cells were transfected by the calcium phosphate precipitation method of Chen & Okayama (1987) (Filtz et al, 1994).

The human $P2Y_2$ receptor cloned from airway epithelium was transfected into 1321N1 cells using the retroviral expression plasmid, pLXSN. The receptor sequence was inserted into this vector, and the recombinant plasmid was transfected into PA317 amphotrophic packaging cells for production of the viral vector (see Miller & Rosman, 1989) (Parr et al, 1994). The viral vector was then used to transfect 1321N1 cells. The human $P2Y_4$ receptor was transfected into 1321N1 cells using the same retroviral system (Nguyen et al, 1995).

All transfection systems used a neomycin resistance gene, which confers resistance to G418, for selection of positive clones.

2.1.2 Preparation of SHR and WKY VSM cells

Cells were prepared by Dr. L. L. Ng at the Department of Medicine in Leicester Royal Infirmary using the method described by Davies et al (1991). Briefly, 12 week old Spontaneously Hypertensive rats (SHR) and Wistar Kyoto rats (WKY) were monitored for high and low blood pressure respectively. They were then decapitated and the thoracic aorta removed and stripped of adventitia. The media was

then cut into 1 mm pieces and enzymatically digested with trypsin. Cells were then pelleted by centrifugation and resuspended in growth medium consisting of (Dulbeco's modified eagles medium (DMEM), supplemented with 10 % foetal calf serum, 25 iu/ml penicillin, 25 µg/ml streptomycin, and 2 mM glutamine). Clonal cultures were established and after five days those colonies with smooth muscle morphology were combined.

2.1.3 Preparation of human saphenous vein smooth muscle (hSVSM) cells

Cells were prepared by Dr. K. Porter at the Department of Medicine in Leicester Royal Infirmary. Briefly, healthy ends were excised from human saphenous veins recovered after coronary artery bypass operations. The smooth muscle was stripped of endothelium and connective tissue and was coarsely minced using scissors. It was then placed in an 80 cm^2 flask in 15 ml whole growth medium (RPMI 1640, supplemented with 10 % foetal calf serum, 25 iu/ml penicillin, 25 µg/ml streptomycin, and 10 mM glutamine). After 2 weeks, the smooth muscle cells had grown out from the tissue and were passaged as described below. Cells from two separate donors were used in parallel for the experiments presented in this thesis.

2.1.4 Maintenance of cultured cells

All cell culture work was performed under Class II containment, in a microbiological cell culture cabinet. Cells were maintained in full growth medium, comprising DMEM with D-valine for VSM cells, or high (4.5 mg/ml) glucose DMEM for 1321N1 cells, supplemented with 10 % foetal calf serum, 25 iu/ml penicillin, 25 µg/ml streptomycin, and 2 mM glutamine. When cells reached confluence, they were passaged by splitting into 4 times the volume of medium. The monolayer was washed three times with 10 ml of Earles Balanced Salt Solution (EBSS) without Ca^{2+} and Mg^{2+}, and 4 ml 0.05 % trypsin, 0.53 mM EDTA solution was added. The flask was gently agitated until all the cells were detached. The activity of the trypsin was quenched by the addition of 10 ml of full growth medium, and the cell suspension was transferred to a 30 ml sterile tube. After centrifugation at 200 g for 5 minutes, the

supernatant was discarded and the cell pellet was resuspended in 2 ml fresh medium. The cells were separated by gentle trituration through an 18 g needle, and added to the required volume of medium. Cells were then aliquoted into 24 well multiwell plates at 1 ml/well, or into 180 cm^2 flasks in a volume of 50 ml. 1321N1 cells were not passaged more than 25 times, and smooth muscle cells were not maitained past passage 5.

2.1.5 Storage of 1321N1 cells and VSM cells

Cells to be stored for future use were grown to confluence in a 180 cm^2 flask, then washed and harvested as described above. After pelleting, the cells were resuspended by trituration in 2 ml freezing medium (45 % DMEM, 45 % foetal calf serum, and 10 % dimethyl sulphoxide (DMSO)). 1 ml of cells in freezing medium were pipetted into a sterile cryotube and placed into the gaseous phase of a liquid nitrogen cylinder for 4 hours. The cryotube was then placed in liquid nitrogen for storage.

Cells were retrieved from storage by placing the cryotube in warm water for rapid thawing. As soon as the contents were thawed they were transferred to an 80 cm^2 flask containing 25 ml full growth medium. When the cells had plated down (usually after 4 hours) the medium was changed to remove DMSO.

2.2 Transfection of the bovine $P2Y_1$ receptor into 1321N1 cells

2.2.1 Isolation of the bovine $P2Y_1$ receptor cDNA

The bovine $P2Y_1$ cDNA was isolated by Henderson et al (1995), by hybridisation of a ^{32}P-radiolabelled chick $P2Y_1$ (ChR803) receptor probe to plaques of a bovine cultured aortic endothelial cell cDNA library (Stratagene #936705). Positive clones were purified through three more rounds of screening, before ligation into pBluescript for sequencing and extension. Once the whole sequence was isolated, it was cloned into the *Not* I and *Hin*dIII polylinker cloning sites of the vector pRc/CMV

(Invitrogen), downstream of the immediate early gene promoter from cytomegalovirus.

2.2.2 Transfection by electroporation

Electroporation is the use of pulsed electric fields to introduce DNA in to cells in culture. The efficiency of transfection is influenced by a number of factors including the strength of the applied field, the length of electric pulse, temperature, conformation and concentration of DNA, and the ionic composition of the medium.

2.2.2.1 Transfection procedure

The transfection procedure was adapted from Erb et al (1993). Wild type 1321N1 cells (a generous gift from G. Weisman, University of Missouri-Columbia, U.S.A.) were cultured as described above. Confluent cells in a 180 cm^2 flask were washed four times with EBSS (without Ca^{2+} and Mg^{2+}), and were harvested rapidly with 4 ml 0.05 % trypsin, 0.53 mM EDTA solution. The trypsin activity was quenched by the addition of 10 ml complete medium, and the cells were pelleted by centrifugation at 200 g for 5 minutes. The cells were resuspended in 25 ml whole medium and were counted using a haemocytometer. They were then pelleted and resuspended in PBS at a concentration of 4×10^7 cells /ml. Cells were chilled on ice for 10 minutes in a pre-chilled 0.4 cm gap electroporation cuvette (Biorad) with 10 µl of *Sca* I linearised DNA (1 µg/µl). Cells were pulsed once in a Gene Pulser electroporation chamber (Biorad) at 1 kV, 25 µF. The cuvette was returned to ice for 10 minutes, then cells were resuspended in 15 ml whole growth medium and left for 48 hours at 37 °C. The medium was replaced with fresh medium supplemented with 0.7 mg/ml G418 (Sigma). This was performed every 2-3 days for 2 weeks to select stable transfectants expressing neomycin resistance, during which time the cells were split into two 80 cm^2 flasks.

2.2.2.2 Assaying the mixed population

Cells from both flasks were plated into a 24 well multiwell and grown to confluence. They were then assayed for [^{3}H]inositol (poly)phosphate ([^{3}H]InsP$_x$) accumulation using the method described in section 3.3.1B. Briefly, the cells were labelled overnight with 0.5 μCi/ml [^{3}H]inositol. In a water bath at 37 °C, 50 μl 110 mM LiCl in BSS was added to the labelling medium and left for 10 minutes. The cells were then stimulated by the addition of 50 μl 2MeSATP in BSS to give a final concentration of 30 μM. Stimulations were terminated by aspiration of the medium and addition of 0.5 ml TCA (0.5 M). The results are shown in figure 2.1A. Cells from both flasks gave large stimulations, with fold over non-stimulated counts of 9.4 and 7.9 for flasks 1 and 2, respectively (basal: 3160 ± 64 dpm, stimulated: 29780 ± 2039 dpm for cells from flask 1).

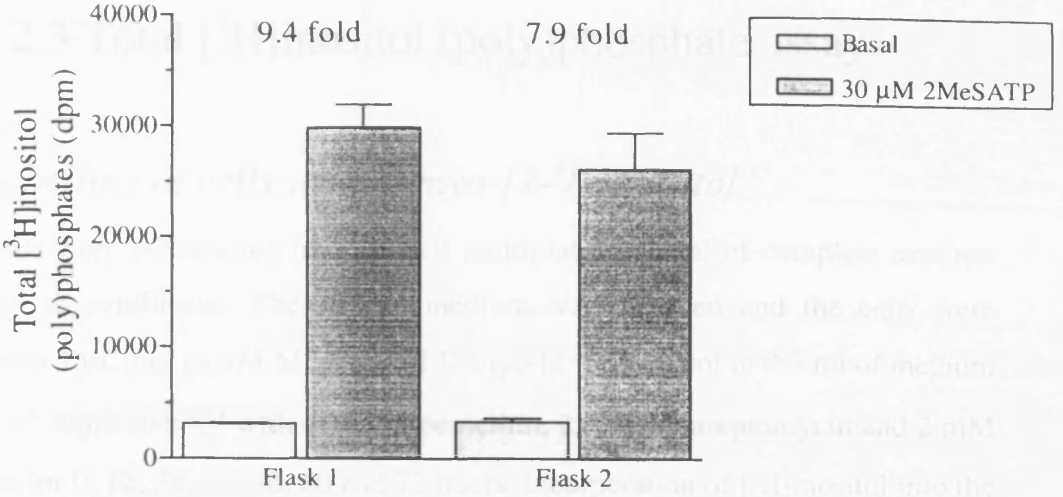
2.2.3 Cloning the transfected cells

Cells were cloned by limiting dilution. Cells were plated out in a 96 well multiwell at a density of 0.3 cells/well in 200 μl of 50 % fresh medium and 50 % conditioned medium (filtered from 1-2 day-old wild type 1321N1 stock cultures), supplemented with G418 at 0.4 mg/ml. Wells that contained only one cell were noted, and they were left to grow for approximately 2 weeks, with medium changes every 2-3 days. When sufficient numbers of clonal cells were available, they were harvested and grown to provide stocks.

2.2.3.1 Assaying the clonal cell lines

The accumulation of [^{3}H]InsP$_x$ in response to 30 μM 2MeSATP was measured as before in six clones: B2, C3, F2, F4, E4, and H2. Figure 2.1B shows that all clones gave a response to 2MeSATP, with C3 giving the largest stimulation (basal: 2630 ± 116 dpm, stimulated: 19845 ± 685 dpm: fold stimulation: 7.5). Therefore, clone C3 was used in all further studies with the bovine P2Y$_1$ receptor.

A



B

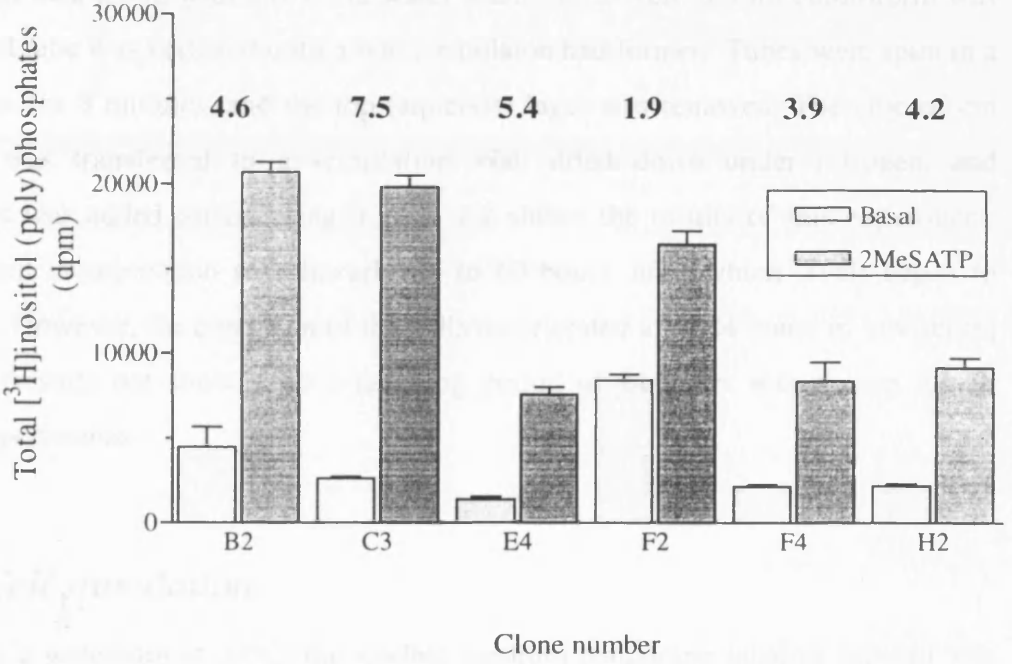


Figure 2.1

Total [^{3}H]inositol (poly)phosphates ([^{3}H]InsP$_X$) assay on mixed populations and clones of 1321N1 cells transfected with the bovine P2Y$_1$ receptor.

Mixed populations (**A**) or cloned (**B**) 1321N1 cells transfected (as in section 2.2) with the bovine P2Y$_1$ receptor were assayed using the [^{3}H]InsP$_X$ assay (section 2.3). Cells were preincubated with 10 mM LiCl before challenging with 30 µM 2MeSATP for 15 minutes. Numbers above each set of columns represents the fold over basal stimulation in each flask/clone. Data are mean ± SEM from one experiment performed in triplicate.

2.3 Total [^{3}H]inositol (poly)phosphate assay

2.3.1 Labelling of cells with D-myo-[2-^{3}H]inositol

Cells were subcultured into 24 well multiplates in 1 ml of complete medium and grown to confluence. The growth medium was removed and the cells were labelled with 1 μCi/ml (0.074 MBq/ml) of D-myo-[2-^{3}H] inositol in 0.5 ml of medium 199 (Gibco) supplemented with 25 iu/ml penicillin, 25 μg/ml streptomycin and 2 mM Glutamine for 0, 12, 24, 36, 48, 60 and 72 hours. Incorporation of [^{3}H]inositol into the lipid fraction of the 1321N1 cells was determined using the following method. Labelling medium was aspirated and cells washed twice with 1 ml water. 0.5 ml methanol was added, the cells were scraped from the plate, and were transferred to 1.5 ml capped tube along with a 0.45 ml water wash of the well. 0.5 ml chloroform was added and tube was vortexed until a white emulsion had formed. Tubes were spun in a microfuge for 3 minutes, and the top (aqueous) layer was removed. The chloroform fraction was transferred to a scintilation vial, dried down under nitrogen, and scintillant was added for counting. Figure 2.2 shows the results of this experiment. [^{3}H]inositol incorporation rose linearly up to 60 hours, after which levels began to decrease. However, the condition of the cells deteriorated after 24 hours in low serum medium (results not shown), so a labelling period of 24 hours was chosen for all future experiments.

2.3.2 Cell stimulation

In a waterbath at 37°C, the loading medium containing labelled inositol was aspirated and the cells were washed with 1 ml balanced salt solution (BSS, composition in mM: NaCl 125, KCl 5.4, $NaHCO_3$ 16.2, HEPES 30, NaH_2PO_4 1, $MgSO_4$ 0.8, $CaCl_2$ 1.8 glucose 5.5, pH 7.4). 1 ml BSS containing 10 mM lithium chloride (BSS+Li) was added and left for 10 mins. BSS+Li was aspirated and the cells were stimulated by the addition of 0.5 ml BSS+Li containing the drug solutions or a vehicle control. Where appropriate, antagonist was added to the 10 min BSS+Li incubation and again to the stimulating solution. The reaction was stopped by aspirating the stimulating solution and adding 0.5 ml ice cold 0.5M trichloroacetic

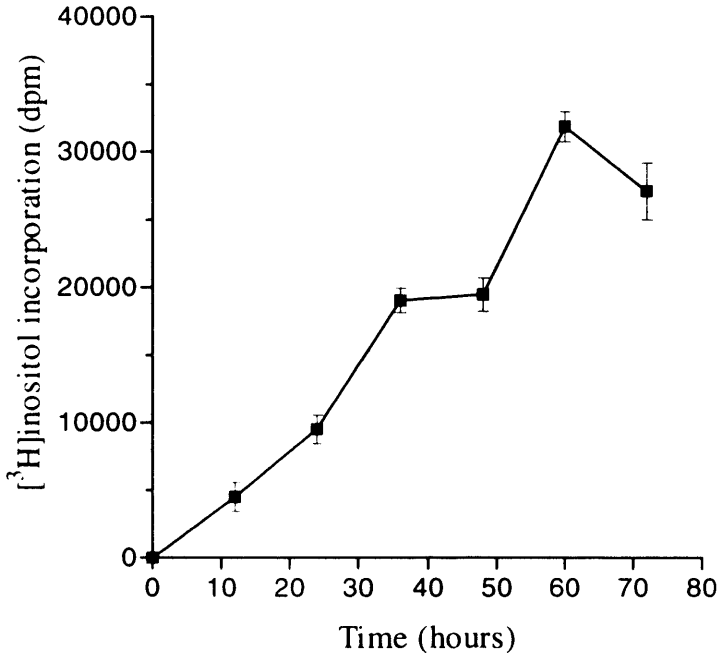


Figure 2.2

Time course of [^{3}H]inositol incorporation into the lipid fraction of 1321N1 cells.

Cells were incubated with [^{3}H]inositol for the specified times, and incorporation into the lipid fraction of 1321N1 cells was measured as described in section 2.3.1. Data are the mean ± SEM of one representative experiment of three, each performed in triplicate.

acid (TCA). The plates were then transferred to ice and left to extract for at least 1 hr. Each sample was removed to a 5 ml polypropylene tube and was washed three times with 2 ml of water saturated diethyl ether to extract the TCA. 125µl of 60 mM $NaHCO_3$ was added to each tube, at which stage they could be stored overnight at 4°C. Inositol phosphate were separated using the ion exchange resin Dowex-I Cl^- packed into plastic 10 ml chromatography columns.

2.3.3 Preparation of Dowex columns

The chloride ions were stripped from the Dowex by washing in 1 mM NaOH, followed by washing in dH_2O until neutral. 1 ml of 50 % Dowex, 50 % dH_2O slurry was packed into each column and 5 ml of 1M formic acid was added to charge the Dowex with formate ions. The columns were washed with 20 ml water and stored at 4°C until use.

2.3.4 Separation of inositol phosphates

The samples were added to the columns and washed with 20 ml water. 10 ml of 60mM ammonium formate was run through the columns to elute the contaminating glycerophosphinositols. The remaining inositol phosphates were eluted with 5 ml of 1 M formic acid and 2.5 ml of this fraction was placed in a scintillation vial with 10 ml FLO-SCINT IV scintillation cocktail. This scintillation cocktail was used as it has an aqueous capacity of 1:3 and was designed especially for use with ammonium formate. The vials were vortexed to ensure the two phases were thoroughly mixed before counting. The Dowex was regenerated by washing with 5 ml formic acid (1M) and 20 ml water.

2.4 Mass IP_3 assay

2.4.1 Cell stimulations

Cells were plated into 24 well multiplates and were grown to confluence. 24 hours before they were used, the culture medium was replaced with 1 ml serum-free medium. The cells were transferred to a water bath at 37 °C, and were washed once with 1 ml BSS. The wash BSS was aspirated, and stimulations were started by the addition of agonist in 0.5 ml BSS. Stimulations were terminated by aspiration of the agonist and addition of 0.5 ml trichloroacetic acid (TCA) (0.5 M). Multiwells were transfered to ice and left to extract for 2 hrs. The samples were placed in polypropylene tubes and the TCA was extracted by three 2 ml washes with water-saturated diethyl ether. Samples were buffered by the addition of 62.5 µl 60mM $NaHCO_3$ and 62.5 µl of 30 mM EDTA, and were stored at 4°C.

2.4.2 IP_3 binding protein preparation

Ten to twelve bovine adrenal glands were collected from a local abbatoir and transported to the laboratory in ice cold buffer (20 mM $NaHCO_3$, 1 mM dithiothreitol, pH 8.0). Each gland was cut in half and the cortex removed. Approximately 50 g of cortical tissue was collected from ten glands. The tissue was divided into 8 RC-5 centrifuge tubes, 5 ml ice cold buffer was added to each, and was homogenised for 20 –30 seconds. The tubes were topped up with ice cold buffer, and the samples were spun for 10 minutes at 3000 g at 4 °C. The supernatant was removed and kept on ice, a further 5 ml buffer was added to the tubes, and the tissue was re-homogeised. After a second 10 minute centrifugation at 3000 g, the supernatant was decanted and added to that previously produced. The combined supernatants were spun at 38 000 g for 20 minutes, after which the supernatant was discarded and the pellets were re-suspended in buffer to give a final concentration of 15–25 mg/ml protein (as determined by the Lowry assay, see section 2.6). Aliquots of 1 ml were frozen and stored at –20 °C.

2.4.3 Determination of mass inositol trisphosphate ($Ins(1,4,5)P_3$) levels

The mass $Ins(1,4,5)P_3$ levels were determined using a radioreceptor assay (Challiss *et al* 1988), using adrenal cortex membranes to provide binding sites for the $Ins(1,4,5)P_3$. A standard curve of increasing $Ins(1,4,5)P_3$ concentrations (see Figure 2.3, for example) was prepared in a buffer blank, obtained by the removal of TCA from BSS using diethyl ether in the same way as for each sample. A 30 µl aliquot of sample or standard was taken and to this was added 30 µl [^{3}H]$Ins(1,4,5)P_3$ (44 Ci/mmol) in water and 30 µl of assay buffer composed of 100 mM Tris-HCl and 4 mM EDTA. The incubation was initiated by the addition of 30 µl bovine adrenal cortical membranes and was allowed to proceed on ice for 30 minutes. The incubation was terminated by the addition of 3 ml ice cold washing buffer (25 mM Tris-HCl, 1 mM EDTA, 5 mM $NaHCO_3$, buffered to pH 8.0 with HCl) followed by immediate vacuum filtration through Whatman GF-B filters. This was followed by two further 3 ml washes to each tube and a final 3 ml wash of the filter. Filters were transferred to scintillation vials, 5 ml of scintillation fluid was added, and samples were left overnight to extract before vortexing and counting.

2.5 Procedure to measure [^{3}H]thymidine incorporation

[^{3}H]thymidine incorporation was used to measure synthesis of DNA as an index of cell growth. Cells were grown to 90 % confluence in 24 well multiwells, and the medium was replaced 24 hours before experimentation by serum free medium (0.5 ml/well). In a Class II cell culture cabinet, 50 µl agonist at 11 fold final concentration in serum free medium was added to the cells, which were then returned to the incubator for 1 hour. The medium was replaced with fresh serum free medium (1 ml) and cells were incubated for 19 hours. [^{3}H]thymidine in serum free medium was added to each well to give a final concentration of 1 µCi/ml, and the cells were icubated for a further 4 hours. Cells were placed on ice, the medium was aspirated, and the cells were washed (carefully to avoid cell detachment) for 6 minutes with 0.5 ml ice cold 5

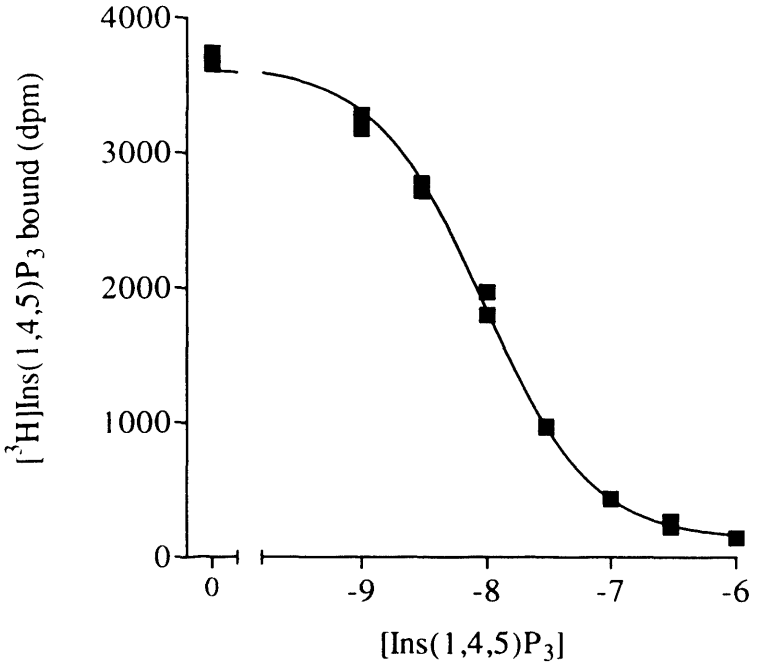


Figure 2.3

Standard curve for [^{3}H]Ins(1,4,5)P$_3$ binding.

Concentration dependent competition curve for displacement of [^{3}H]Ins(1,4,5)P$_3$ binding by Ins(1,4,5)P$_3$. The curve is one representitive example, performed in duplicate.

% TCA. This was repeated. Cells were then washed twice with 0.5 ml ice cold ethanol for six minutes each. Finally, 0.5 ml cold NaOH (0.1 M) was added to each well and cells were placed at –4 °C until frozen. Cells were then thawed, scraped from the well and transferred to scintillation vials with 5 ml Emulsifier Safe scintillation fluid, before counting.

2.6 The Lowry protein assay

Protein concentration of samples was read from a standard curve made up using bovine serum albumin: 200 µl of 0, 40, 60, 100, and 200 µg/ml bovine serum albumin in water were added to 3.5 ml polythene tubes in duplicate. Each sample to be tested was made up to 200 µl with water, and also placed in a 3.5 ml tube. To all the tubes was added 1 ml of a solution consisting of: Na_2CO_3 (2 %) in NaOH (1 M), with 1 % Na/K tartrate (2 %), and 1 % $CuSO_4.5H_2O$ (1 %). The tubes were vortexed and left for 10 minutes. 100 µl Folin solution (1:2 dilution in water) was added to the tubes, which were vortexed, covered, and left for 30 minutes. 1 ml water was added to each tube and the absorption was measured at 750 nm using a Beckmann Du-64 Spectrophotometer.

2.7 SDS Polyacrylamide Gel Electrophoresis (SDS PAGE)

All gels were run under reduced and denaturing conditions using the BioRad minigel system. A 12% resolving gel was prepared [12 % Acrylamide: bisacrylamide (29:1 %) 375 mM Tris-HCl (pH 8.8), 1 % SDS, 0.004 % tetramethylenediamine (TEMED), and 0.1 % ammonium persulphate (APS)], and cast between the gel plates. 200 µl ethanol was layered over each gel which was left to polymerise for 30 minutes. Once polymerised, ethanol was aspirated and the gel was washed with 0.1 % SDS solution to remove all ethanol. A 5% stacking gel [5 % Acrylamide: bisacrylamide (29:1 %) 125 mM Tris-HCl (pH 6.8), 0.1 % SDS, 0.001 % TEMED, and 0.1 % APS]

was cast above each running gel, combs were positioned and the gels left to polymerise for one hour at room temperature before use. Electrophoresis cassettes were assembled and placed in a Biorad electrophoresis tank with enough running buffer [25 mM Tris, 190 mM glycine, and 0.66 mM SDS] to cover the lower electrode. The cassette was then filled with running buffer to cover the upper electrode. Samples were prepared by the addition of 2x SDS sample buffer [100 mM Tris-HCl (pH 6.8), 4 % SDS (M), 20 % glycerol, 5 % 2-mercaptoethanol, and 0.01 % bromophenol blue], and were boiled at 100°C for 5 minutes before loading. Gels were run at 12 mA per gel, and were stopped once the bromophenol blue had just run off the gel.

The proteins in the gel were then directly visualised by Coomassie staining, or by Western blotting and probing with antibodies.

2.7.1 Coomassie staining of SDS PAGE gels

Gels were stained for 1 hour in coomassie blue stain [50 % methanol, 10 % acetic acid, 0.25 % Coomassie Blue], and destained for several hours in destain solution [10 % methanol, 5 % acetic acid] until protein bands were visualised.

2.7.2 Western Blotting

The proteins separated using SDS PAGE were transferred onto a nitrocellulose membrane by Western blotting. A piece of nitrocellulose was pre-soaked in blotting buffer [48 mM Tris base, 39 mM glycine, 20 % methanol, 0.037 % SDS] for 2 minutes. The gel and membrane were sandwiched between 8 pieces of 3MM paper (Whattman) and 2 pieces of glass wool cloth, and were placed in the blotting apparatus such that the negatively charged proteins moved towards the anode thus passing from the gel onto the nitrocellulose membrane. A cooling coil was placed in the blotting tank which was filled with blotting buffer and run at 100 V for 1 hour. Successful blotting was checked by Ponceau S staining of the proteins.

2.7.3 Probing the western blot with antibodies

Nitrocellulose blots were washed for 20 minutes in PBS-Tween [80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, pH 7.5, 1 % Tween-20 (polyoxyethelene-sorbitan monolaurate)], with frequent changes of buffer. Non-specific binding sites on the nitrocellulose were blocked by 10 % Marvel in PBS-Tween for one hour, after which, the nitrocellulose was washed for 20 minutes in PBS-Tween. Primary antibody was added in 5 % marvel in PBS-Tween (5 ml) at dilutions specified in the main text, for 1 hour. Nitrocellulose was washed as before, and was then incubated for 1 hour with secondary antibody in 5 % marvel in PBS-Tween. Following a final set of washes, 500 µl ECL reagent (Amersham) was added to the nitrocellulose for 1 minutes. Excess reagent was drained off, and the nitrocellulose was wrapped in Saran film and transferred to a dark room where it was exposed to photographic film for varying periods of time. The film was developed in a Hyper-processor (Amersham).

2.8 Data analysis

2.8.1 Normalisation

In most cases, the raw data from experiments performed on different days could not be directly compared. Therefore, unless otherwise stated, data were normalised as a percentage of the response achieved by the maximal agonist concentration in the absence of other treatments. Data were processed using Excel 5.0 (Microsoft Corporation, U.S.A.).

2.8.2 Curve fitting

Concentration-response curves were fitted and log EC_{50} values determined using GraphPad Prism 2 (GraphPad Software Incorporated, U.S.A.).

2.8.3 Analysis of antagonism

The effect of antagonists at the P2Y receptors was determined by calculating the pA_2 value using the equation of Arunlakshana & Schild (1959);

$$Log_{10}(r-1) = n\log_{10}[A] + pK_A$$

where r is the dose ratio (the ratio of EC_{50} values for each concentration of antagonist compared to the EC_{50} in the absence of antagonist), [A] is the concentration of antagonist, and pK_A is the affinity of the antagonist for the receptor. A Schild plot was constructed by plotting $\log_{10}(r-1)$ against $\log_{10}$ antagonist concentration, the intercept on the abscisa (antagonist concentration) was the pK_A. Competitive antagonism was tested by comparing the Schild slope parameter (n) with unity, using Student's t-test.

Where the affinity of antagonist was calculated using a single concentration of antagonist, the pA_2 value was determined according to Furchgott (1972);

$$pA_2 = \log_{10}(r-1) + p[A]$$

where p[A] was the $-\log_{10}$ of the molar antagonist concentration.

2.8.4 Statistical analysis

Significant differences, where appropriate, were determined using Student's t test with $P<0.05$ being considered significant. Where two concentration response curves were compared, two-way analysis of variance was used, and one-way analysis of variance was used with Dunnett's post test for multiple comparisons with a single control. All analysis of variance was performed using GraphPad Software Incorporated, U.S.A., and Students t-tests were calculated using SPSS (Version 6.0; SPSS Inc., U.S.A.).

2.9 Materials and suppliers

Media and tissue culture plastic ware were purchased from Gibco, Paisley, U.K. [^{3}H]inositol, Ins(1,4,5)P_3 and [^{3}H]thymidine were from Amersham Life Science, and [^{3}H]Ins(1,4,5)P_3 was from NEN Life Science Products, Boston, MA, U.S.A.. Nucleotides were from Sigma Chemical Company Ltd., Poole, U.K., except 2MeSATP which was from Research Biochemicals Inc. (RBI) St. Albans, Herts. U.K. Suramin was from RBI, and PPADS and NF023 were from Sigma. Ro 31-8220 was a kind gift from Dr. G. Lawton, Roche Products, Herts. U.K.. Electrophoresis equipment and electroporator were from BioRad Herts, U.K.. Nitocellulose was from Scheicher & Schuell, London, U.K., and the acrylamide was from Flowgen Instruments Ltd., Staffs, U.K.. Molecular weight markers and ponceau S concentrate were from Sigma. Anti-rabbit peroxidase-linked Ig was from New England Biolabs, Hitchin, Herts., U.K..

Chapter 3: Preliminary characterisation

3.1 Introduction

Recent advances in the use of molecular genetic techniques in the field of P2 receptors has lead to the cloning of a number of G protein coupled P2Y receptors. This has enabled the expression of these receptors in cell lines with no native P2 receptors, so that they can be studied in isolation, thus avoiding complications derived by their multiple expression and differences in tissue studied.

Two major considerations must be made when choosing a cell line for transfection of P2 receptors. Firstly, it must have no endogenous receptor for nucleotides, and secondly it must have a broad array of transduction components so that transfected receptors can link into the host cells signal pathways. 1321N1 astrocytoma cells have been extensively used to characterise receptor systems, predominantly muscarinic receptors (e.g. Martinson et al, 1990; Trilivas et al 1991), and do not respond to P2 receptor agonists (Parr et al, 1994). They also possess many G protein subtypes (Nakahata et al, 1995), and have been used to study PLC, PLD, PLA_2 and PKC (Batty et al, 1993; Nieto et al 1994; Bayon et al, 1997; Johnson & Toews, 1990). 1321N1 cells are also adherent, offering an advantage for many studies over other cells that do not possess an endogenous nucleotide receptor (such as Jurkat cells) which are grown in suspension.

The suitability of 1321N1 cells for P2Y transfection studies was first recognised by G. Weisman and J. Turner (University of Columbia-Missouri, U.S.A.) who produced stable transfectants of human $P2Y_2$ (Parr et al, 1994). Subsequently, several other groups have utilised this cell type to transfect P2Y receptors, including the turkey $P2Y_1$ receptor (Filtz et al, 1994), the human P2Y4 receptor (Nguyen et al, 1995), and the bovine $P2Y_1$ receptor (this thesis).

These P2 receptors in 1321N1 cells are all are coupled to phospholipase C (PLC) via G proteins. Activation of PLC leads directly to the formation of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) and *sn*-1,2-diacylglycerol (DAG) from the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) (see Berridge, 1993; section 1.6.2.3, this thesis).

The work in this chapter represents preliminary investigations into the use of these transfected systems to characterise the cloned turkey and bovine $P2Y_1$ receptors, and the human $P2Y_2$ and $P2Y_4$ receptors. Issues such as endogenous ATP release and nucleotide breakdown by ectonucleotidases are addressed, and concentration response curves to some frequently used agonists are presented.

3.2 Total [^{3}H]inositol (poly)phosphate accumulation

Before any receptor characterisation could be performed, it was essential to determine that any responses seen to P2 nucleotide agonists were via interaction with the transfected receptors, and not through non-specific or native receptor interactions. It was also necessary to carry out some preliminary investigations to determine the conditions for subsequent assays measuring either total [^{3}H]inositol (poly)phosphates ([^{3}H]$InsP_x$) or mass inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$), both used as an index of phospholipase C (PLC) activation by these receptors.

3.2.1 Wild type versus transfected cells

The first clone to be received was the human $P2Y_2$ receptor described by Parr et al (1994). The cells transfected with cDNA encoding the $P2Y_2$ receptor were tested against wild type cells and cells into which only the plasmid vector was transfected. 1321N1 cells possess an endogenous M3 muscarinic receptor linked to PLC (Batty et al, 1993), so in each case, the muscarinic receptor agonist carbachol (1mM) was used as a reference stimulation. The method used for stimulation of cells was as follows: in a waterbath at 37°C, the loading medium containing labelled inositol was aspirated and the cells were washed with 1 ml BSS. 1 ml BSS containing 10 mM lithium chloride (BSS+Li) was added and left for 10 mins. BSS+Li was aspirated and the cells were stimulated by the addition of 0.5 ml BSS+Li containing the drug solutions or a vehicle control. The reaction was stopped by aspirating the stimulating solution and adding 0.5 ml ice cold 0.5M trichloroacetic acid (TCA).

Figure 3.1 shows that carbachol produces a response that is not significantly different in each of the cells types. UTP at 300 μM gave no increases [^{3}H]InsP$_x$ accumulation in the wild type and vector only cells. Also, other P2 agonists ATP, ADP, 2MeSATP and α,βMeATP had no effect on the wild type and vector only cells (data not shown). However, in the P2Y$_2$ transfected cells, UTP produced a large stimulation, which over 4 separate experiments was 178.6 ± 15.8 % of the response to carbachol. Moreover, the basal accumulation of [^{3}H]InsP$_x$ was also dramatically increased by the transfection of the P2Y$_2$ receptor, to a level of 5.16 ± 0.65 fold over vector only cells. Thus, although there was a large stimulation to UTP in the P2Y$_2$ cells, this only appeared as a 2.32 ± 0.64 fold over basal increase in [^{3}H]InsP$_x$. If the P2Y$_2$ stimulations are compared to the vector only basal, then the fold over basal stimulation would be 11.64 ± 2.31. Table 3.1 shows raw data from one typical experiment.

Using this method, high levels of basal accumulation of [^{3}H]InsP$_x$ were also observed with the P2Y$_1$ 1321N1 cells, which resulted in a fold over basal stimulation with 30 μM 2MeSATP of just 1.5 (3272 ± 247 dpm basal and 4934 ± 327 dpm with 30 μM 2MeSATP).

At around the same time as these preliminary experiments were performed, Lazarowski et al (1995) described the release of ATP from 1321N1 cells after sheer stress induced by culture medium changes. This was not due to cell lysis, as there was no detectable extracellular lactate dehydrogenase (LDH) activity. Therefore, one explanation for the increased basal levels observed in the transfected cells is that the method used (which involve several medium changes) leads to the sheer stress-induced released of ATP from the 1321N1 cells. This released ATP would have had no effect on the basal [^{3}H]InsP$_x$ levels in the wild type and vector only cells, because they do not possess a receptor for ATP. However, when a P2 receptor was transfected into the cells, the endogenously released nucleotide would cause stimulation of PLC, and therefore an increased apparent basal level of inositol phosphates.

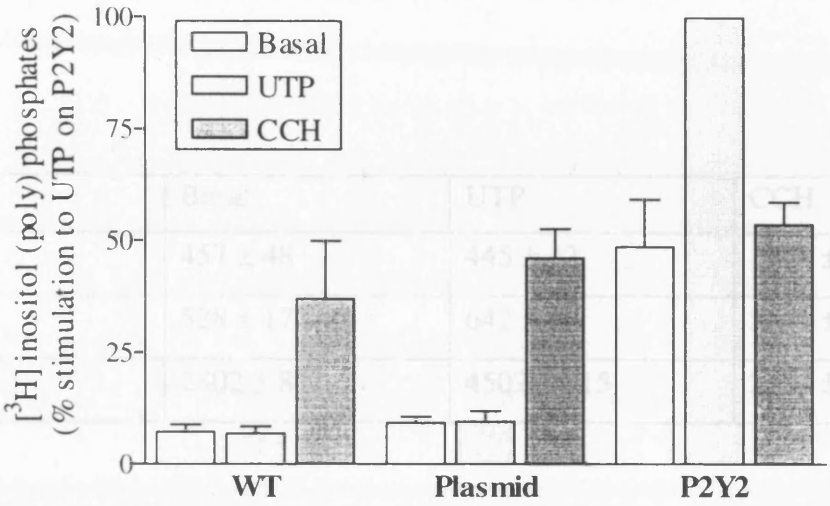


Figure 3.1

The effect of UTP on wild type (WT), vector only (plasmid) and P2Y2 13321N1 cells.

Total [^{3}H] inositol (poly) phosphates assay on wild type (WT) 1321N1 cells, 1321N1 cells transfected with just the plasmid vector (plasmid), and 1321N1 cells transfected with the $P2Y_2$ receptor cDNA (P2Y2). The cells were incubated for 10 minutes with 10 mM LiCl, and were stimulated with 300 μM UTP or 1mM carbachol (CCH). Each bar is the mean ± SEM of three triplicate experiments and are expressed as a percentage of the response to 300 μM UTP on the $P2Y_2$ 1321N1 cells.

	Basal	UTP	CCH
WT	457 ± 48	445 ± 42	1984 ± 333
Plasmid	528 ± 17	642 ± 42	2234 ± 81
$P2Y_2$	2802 ± 89	4502 ± 815	2147 ± 98

Table 3.1

[^{3}H]InsP$_x$ responses to UTP and carbachol (CCH) at wild type, plasmid, and P2Y$_2$ 1321N1 cells. A comparison of the [^{3}H]InsP$_x$ response to the addition of UTP and carbachol between wild type 1321N1 cells (WT), 1321N1 cells transfected with plasmid vector only (plasmid) and 1321N1 cells transfected with the $P2Y_2$ receptor. Data are mean ± SEM of raw dpm counts from one representative experiment of three, each performed in triplicate.

3.2.2 Method development

Other cells are able to release ATP in response to sheer stress. For example, ATP release and increase in second messenger production in the absence of added agonist have been observed in endothelial cells (Milner et al, 1990a,b; Purkiss et al, 1994). In previous studies, stimulation procedures have been modified to reduce the mechanical stress produced during medium changes (Purkiss et al, 1994; Lazarowski et al, 1995), and two different modified procedures were tested for use in this thesis.

Method A. The overnight labelling medium was aspirated and replaced with 300 µM BSS for 30 minutes. Then 50 µl of LiCl at 7 fold final concentration (70mM) in BSS was added and given a 10 minute incubation. Where appropriate, suramin was also added at 7 fold final concentration with LiCl in BSS, for 10 minutes. Agonist was then added in 50 µl BSS at 8 fold final concentration for a further 15 minutes, after which 100 µl of 2.5 M trichloroacetic acid (TCA) was added to stop the stimulation.

Method B. (modified from Lazarowski et al, 1995) The medium was not changed following labelling overnight. To the 0.5 ml of labelling medium was added 50 µl of LiCl at 11 fold final concentration (110mM) in BSS (± suramin at 11 fold final concentration, where appropriate). After 10 minutes, agonist was added in 50 µl BSS at 12 fold final concentration. After a further 15 minutes, the medium was aspirated and 0.5 ml ice cold TCA (0.5 M) was immediately added to stop the reaction. In both methods, the TCA was extracted by ether washing as described before (see section 2.3).

To assess the two methods, the turkey $P2Y_1$ and the human $P2Y_2$ cells were used, with and without the P2 antagonist suramin (100 µM) (Figure 3.2). Very preliminary studies indicated that the basal levels of $[^3H]InsP_X$ in the $P2Y_1$ cells were sensitive to suramin, so a more detailed examination of the effect of suramin with the two methods was undertaken with the $P2Y_1$. The experimental design and full results are presented in section 3.311, but are also summarised in Figure 3.2 for direct comparison with the $P2Y_2$ receptor. Figure 3.2 shows the increases in $[^3H]InsP_X$ accumulation when the $P2Y_1$ cells were stimulated with 30 µM 2MeSATP and the

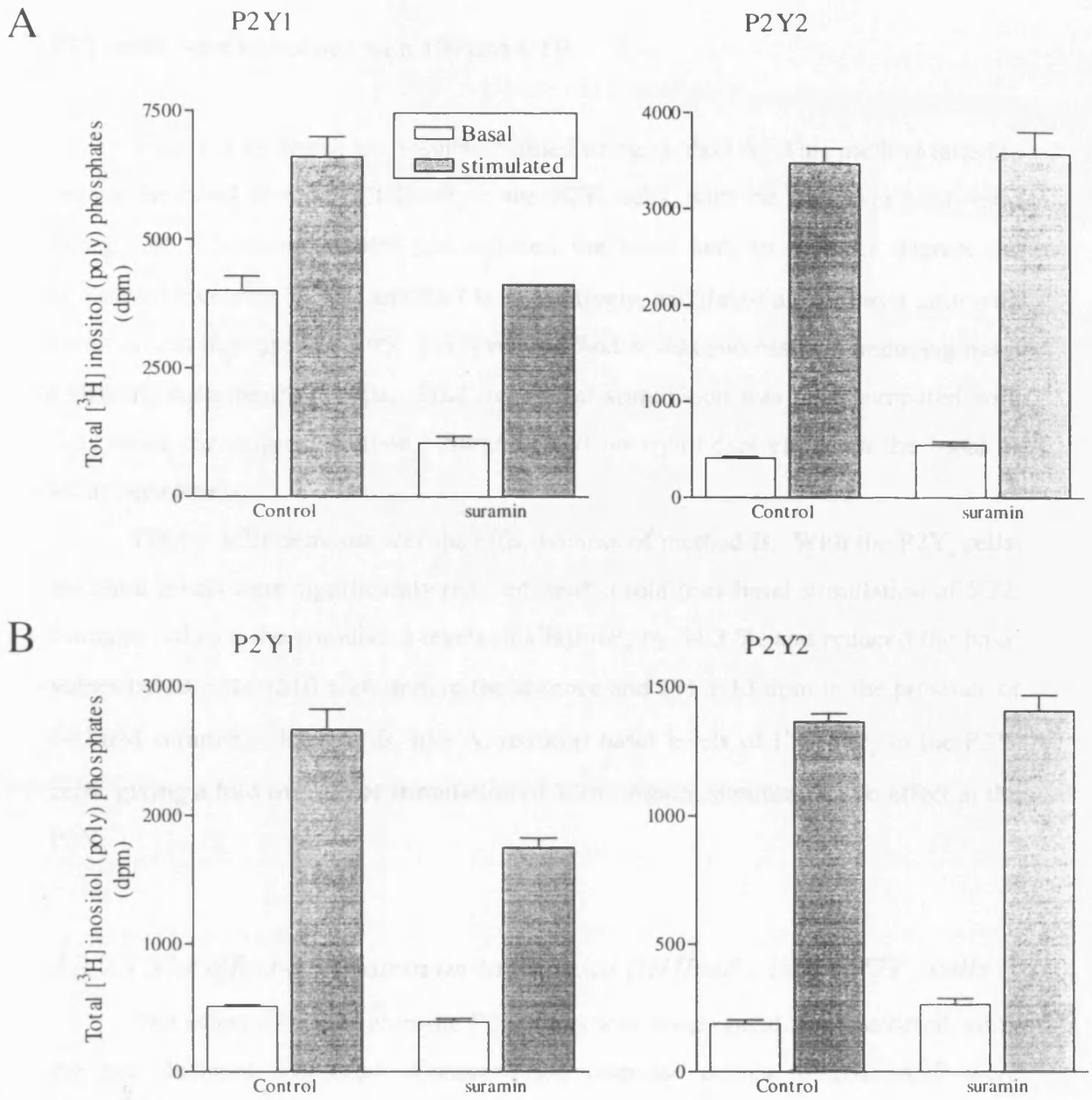


Figure 3.2

Stimulation of [^{3}H]InsP$_X$ accumulation in turkey P2Y$_1$ and human P2Y$_2$ 1231N1 cells using two modified procedures.

Stimulation of [^{3}H]InsP$_X$ accumulation in turkey P2Y$_1$ cells to 2MeSATP (30 μM) and in human P2Y$_2$ cells to UTP (100 μM), using (**A**) method A, or (**B**) method B, section 3.22. Suramin was used at 100 μM in both cases, and data is mean ± SEM from one experiment, performed in triplicate.

$P2Y_2$ cells were stimulated with 100 μM UTP.

Figure 3.2A shows the results obtained using method A. This method failed to reduce the basal levels of [^{3}H]$InsP_x$ in the $P2Y_1$ cells, with the fold over basal ratio being 1.65. Suramin at 100 μM reduced the basal and, to a lesser degree, the stimulated levels by 73.9 % and 37.7 % respectively, and the fold over basal ratio with suramin was increased to 3.95. However, method A was successful at reducing basal [^{3}H]$InsP_x$ with the $P2Y_2$ cells. Fold over basal stimulation was 8.51 compared with 2.32 using the original method. Suramin had no significant effect on the basal or stimulated levels.

Figure 3.2B demonstrates the effectiveness of method B. With the $P2Y_1$ cells, the basal levels were significantly reduced, with a fold over basal stimulation of 5.22. Suramin reduced the stimulated levels of [^{3}H]$InsP_x$ by 34.3 %, and reduced the basal values by 23.3 %. (510 ± 26 dpm in the absence and 391 ± 13 dpm in the presence of 100 μM suramin). Method B, like A, reduced basal levels of [^{3}H]$InsP_x$ in the $P2Y_2$ cells, giving a fold over basal stimulation of 7.26. Again, suramin had no effect at the $P2Y_2$.

3.2.2.1 The effect of suramin on high basal [^{3}H]$InsP_x$ in the $P2Y_1$ cells.

The effect of suramin on the $P2Y_1$ cells was investigated in more detail using the two different methods. Concentration response curves to 2MeSATP were constructed using method A and method B with and without suramin (100 μM). Using method A, 2MeSATP alone produced a shallow, almost linear response curve (Figure 3.3). When suramin was present, the whole curve was lowered, and concentrations of up to 100 nM 2MeSATP had no effect on [^{3}H]$InsP_x$ accumulation. At 300 nM 2MeSATP, the curve began to rise up to 4103 dpm with 30 μM, but it did not reach a plateau. With suramin, the basal levels were reduced from 4010 ± 287 dpm to 1045 ± 138 dpm, and the stimulated levels were reduced from 6629 ± 368 dpm to 4103 dpm (n=2), equivalent to 73.9 and 37.7 % reduction by suramin for basal and stimulated cells respectively.

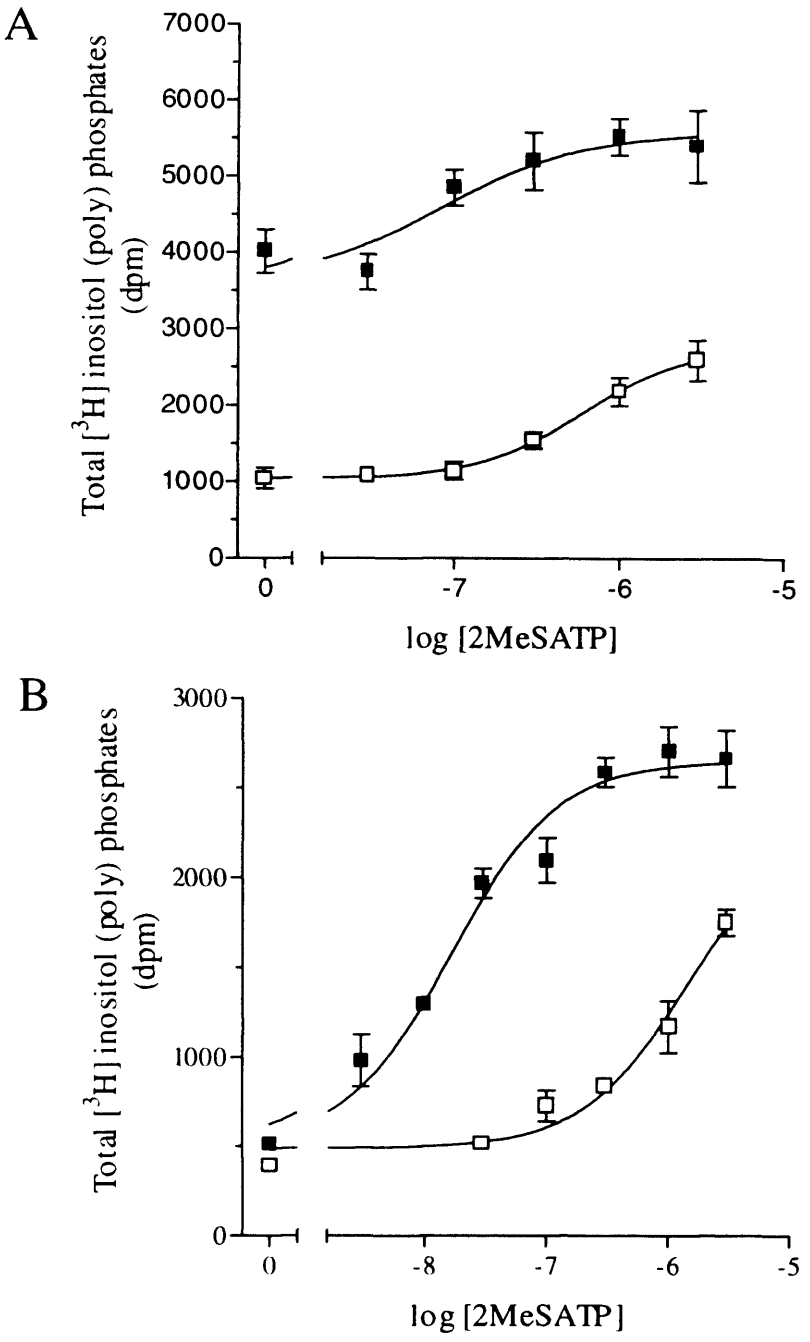


Figure 3.3

The effect of two modified stimulation procedures and suramin on the concentration-response curve to 2MeSATP on turkey $P2Y_1$ 1321N1 cells.

Stimulation of [^{3}H]$InsP_X$ accumulation in turkey $P2Y_1$ cells by increasing concentrations of 2MeSATP in the absence (■) and presence (❑) of suramin (100 μM), using (**A**) method A and (**B**) method B, section 3.22. Data are mean ± SEM from one experiment, performed in triplicate

With method B, the basal level of [^{3}H]InsP$_x$ was reduced and a full concentration response curve was revealed, with a -log EC_{50} value of 7.78 (16.6 nM). When suramin was included, 2MeSATP produced a similar response to that seen for method A, with the curve shifted to the right of that without suramin, and not reaching a plateau.

The ability of suramin to reduce the level of basal [^{3}H]InsP$_x$ accumulation provides more evidence that the high levels seen with method A are due to nucleotide release which then stimulates the $P2Y_1$ receptor. Suramin at 100 μM is able to completely antagonise the response to the endogenously released nucleotide.

When the raw data were normalised, the two methods could be directly compared to determine the approximate concentration of endogenously released nucleotide. When each point was normalised, the basal level of [^{3}H]InsP$_x$ with method A was 74.4 ± 5.33 % of the maximal response to 2MeSATP. The equivalent concentration of 2MeSATP required to give the same stimulation with method B was 36.6 nM (calculated using the concentration response curve fitted by Graph Pad Prism).

3.3 Time course of mass Ins(1,4,5)P_3 production

The mass Ins(1,4,5)P_3 assay was used to provide a second measurement of PLC activity in the transfected 1321N1 cells. Importantly, measurement of Ins(1,4,5)P_3 production is made over a much shorter time period than [^{3}H]InsP$_x$ accumulation, providing less time for nucleotide hydrolysis by enzymes potentially present on the surface of 1321N1 cells. Before agonist concentration response curves were constructed, the time point of peak Ins(1,4,5)P_3 production was determined.

Figure 3.4A shows the time course of Ins(1,4,5)P_3 production in $P2Y_2$ transfected cells during stimulation with UTP (100 μM). Culture medium was aspirated and 100 μM UTP in 0.5 ml BSS was added (or 0.5 ml BSS alone for the

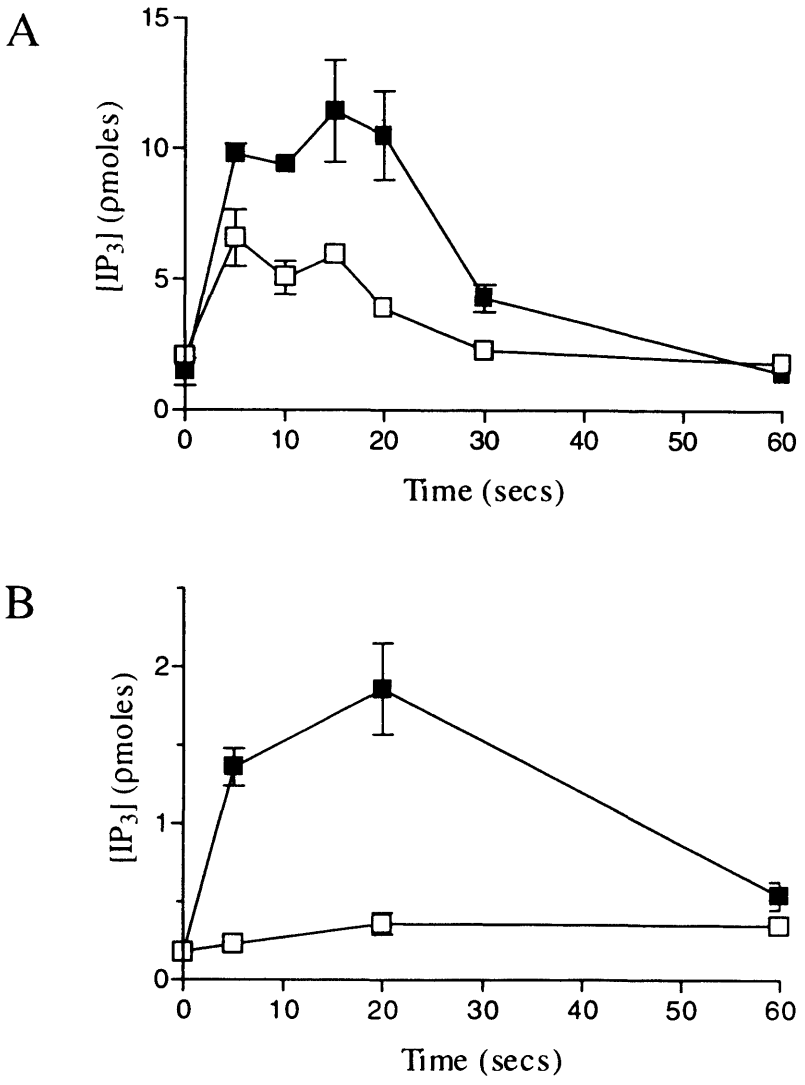


Figure 3.4

Mass inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) time course on human $P2Y_2$ 1321N1 cells.

60 second time course of Ins(1,4,5)P_3 production in $P2Y_2$ 1321N1 cells in response to 100 μM UTP in BSS (■) or to BSS alone (❑). Stimulations were performed using the original method (**A**), or the modified procedure (**B**), section 3.13. Data is mean ± SEM from one representative experiment of two, each performed in triplicate.

controls). Incubations were terminated at the specified time points by aspiration of the BSS and the addition of 0.5 ml TCA (0.5 M). There was a rapid increase in Ins(1,4,5)P_3 by 5 seconds (the shortest measurable time point) to a sustained plateau between 5 and 20 seconds, after which the levels dropped to basal. However, as was observed with [^{3}H]InsP_x accumulation, there was a basal increase in Ins(1,4,5)P_3, which followed a similar time course as with UTP. This similar profile of basal and stimulated Ins(1,4,5)P_3 production may be explained by the basal endogenous release of a nucleotide that acts at the same receptor as UTP, as discussed earlier. Again, the increased basal levels of Ins(1,4,5)P_3 reduced the observed fold over basal stimulations from approximately 8 fold to less than 2 fold. To eliminate this problem, stimulation procedures involving fewer medium changes were developed.

3.3.1 Method development

To try to reduce the basal levels of Ins(1,4,5)P_3, a procedure similar to method A (section 3.22) was employed. Medium was replaced with 300 µl BSS and left for 30 minutes at 37 °C to equilibrate. Agonist was then added in 100 µl BSS at 4 fold final concentration. The stimulations were terminated by the addition of 100 µl ice cold TCA (2.5 M). The result of this new method is shown in Figure 3.4B. Stimulations were obtained with UTP, but addition of BSS alone (control) did not produce the rise in Ins(1,4,5)P_3 levels observed with the previous procedure.

As with the [^{3}H]InsP_x assay, reduction of sheer stress by the improved stimulation procedure for the mass Ins(1,4,5)P_3 time course lowered the control levels of Ins(1,4,5)P_3 in the $P2Y_2$ 1321N1 cells. The difference in Ins(1,4,5)P_3 levels obtained using the different methods is probably due to differences in cell number between the two separate experiments.

3.4 Total [^{3}H]inositol (poly)phosphates time courses

Before any further studies were performed using the total [^{3}H]InsP$_x$ assay, an optimal time point for the termination of incubations with agonists was determined. Time courses were performed using method B described in section 3.3.1.

3.4.1 20 minute time courses

Preliminary time courses were constructed to 2MeSATP (30 μM) for the turkey P2Y$_1$, and to UTP (100 μM) for the human P2Y$_2$ over 20 minutes (Figure 3.5A and B, respectively). Levels of total [^{3}H]InsP$_x$ rose linearly in both the P2Y$_1$ and P2Y$_2$ 1321N1 cells, at a rate of 1312 dpm/min and 1562 dpm/min, respectively. The controls obtained by adding BSS alone did not increase the level of [^{3}H]InsP$_x$ in either of the cell types at any point in the time course.

3.4.2 2 hour time courses

Longer time courses were performed over 2 hours. 2MeSATP (30 μM) produced a steady accumulation of [^{3}H]InsP$_x$ over 60 minutes, after which the rate of accumulation was reduced (Figure 3.6A). Time matched control points showed that there was no basal accumulation of inositol phosphates over 2 hours. Figure 3.6B shows the same experiment performed on P2Y$_2$ cells. UTP at 100 μM gave a very similar response to 2MeSATP at the P2Y$_1$ receptor, and again, time matched controls remained at basal levels.

Extracellular inositol uptake by 1321N1 cells has been shown to be rapid, with a large capacity (Batty et al, 1993). This large intracellular pool of inositol has been estimated as being capable of sustaining receptor stimulated PIP$_2$ hydrolysis for a period of several hours (Batty et al, 1993). However, when Batty & Downes (1994) used Li$^+$ to prevent the recycling of [^{3}H] inositol after muscarinic receptor activation, the rate of [^{3}H]InsP$_x$ accumulation declined over 30–60 minutes as a direct consequence of inositol depletion. This suggests that the reduction in rate of [^{3}H]InsP$_x$ accumulation observed after 60 minutes is due to the depletion of inositol in

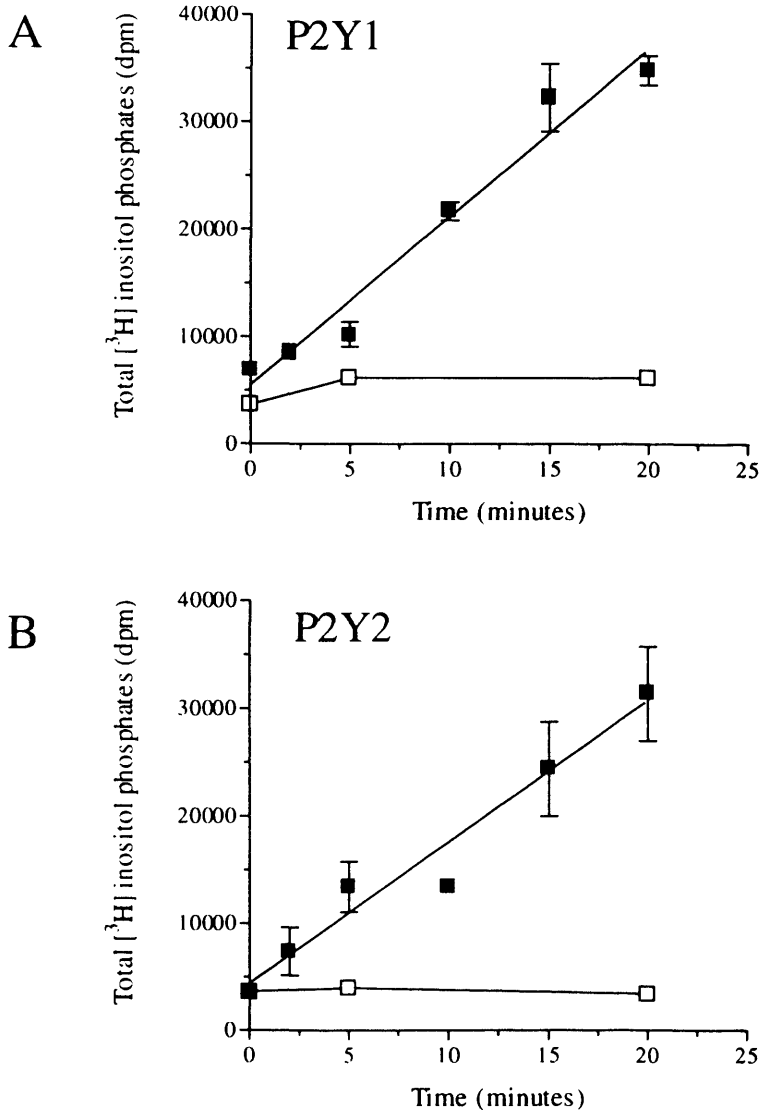


Figure 3.5

20 minute time course of [^{3}H]InsP$_X$ accumulation in turkey P2Y$_1$ and human P2Y$_2$ 1321N1 cells.

Time course of total [^{3}H]InsP$_X$ accumulation in (**A**) turkey P2Y$_1$ 1321N1 cells in response to 30 μM 2MeSATP in BSS (■) or BSS alone (❑), or (**B**) human P2Y$_2$ 1321N1 cells to 100 μM UTP in BSS (■) or to BSS alone (❑). Cells were preincubated with 10 mM LiCl for 10 minutes before addition of agonist or control BSS. Incubations were terminated at specified time points by aspiration of medium and addition of 0.5 ml TCA (0.5 M). Data are mean ± SEM from one experiment performed in triplicate.

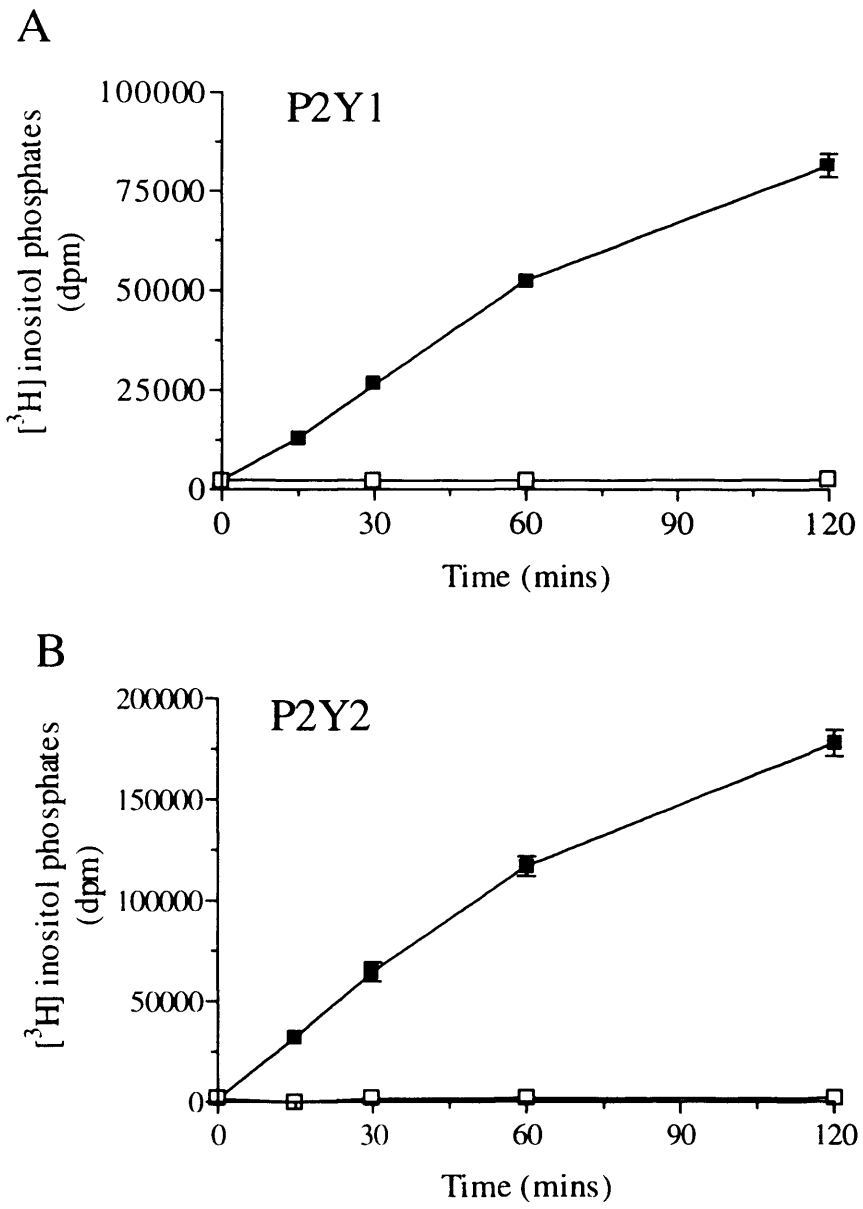


Figure 3.6

120 minute time course of total [^{3}H]InsP$_X$ accumulation in turkey P2Y1 and human P2Y2 1321N1 cells.

Time course of total [^{3}H]InsP$_X$ accumulation in (**A**) turkey P2Y$_1$ 1321N1 cells in response to 30 μM 2MeSATP in BSS (■) or BSS alone (❑), and (**B**) human P2Y$_2$ 1321N1 cells in response to 100 μM UTP in BSS (■), or BSS alone (❑).Cells were preincubated with 10 mM LiCl for 10 minutes before addition of agonist or control BSS. Incubations were terminated at specified time points by aspiration of medium and addition of 0.5 ml TCA (0.5 M). Data is mean ± SEM from one experiment performed in triplicate.

these cells, and not to the desensitisation of the receptor or other members of the transduction pathway leading to PIP_2 hydrolysis.

Although the levels of [^{3}H]$InsP_x$ continued to rise linearly over 60 minutes, the stimulation period of 15 minutes was retained. This was because there was still a sufficient stimulation at this time to carry out the desired experiments, without any [^{3}H]inositol depletion, and also because in 15 minutes, there would be less potential nucleotide hydrolysis than at longer time points. Unless stated otherwise, all further [^{3}H]$InsP_x$ assays were performed using 15 minute stimulation times.

3.5 Agonist concentration response curves

Having established a suitable method for the measurement of [^{3}H]$InsP_x$ accumulation in 1321N1 cells, a more detailed examination of the structure-activity relationships of the P2Y receptors was undertaken.

3.5.1 Turkey $P2Y_1$

Figure 3.7 shows the concentration response curves for stimulation of [^{3}H]$InsP_x$ by 2MeSATP, UTP, ATP, ADP and ATPγS, acting at the turkey $P2Y_1$ receptor. The overall rank order of agonist potency was 2MeSATP > ADP > ATPγS = ATP, with UTP having no effect. 2MeSATP was the most potent agonist, with a -log EC_{50} value of 7.55 (28.1 ± 3.6 nM). ADP, ATP and ATPγS were all full agonists, with –log EC_{50} values of 6.74 (0.18 ± 0.01 μM), 5.91 (1.2 ± 0.7 μM), and 6.3 (0.49 ± 0.7 μM), respectively.

3.5.1.1 The hydrolysis resistant ATP analogue ATPγS on the turkey $P2Y_1$

There have been reports in the literature of $P2Y_1$ receptors at which ATP acts as a partial agonist (Feolde et al, 1995, Henderson et al, 1995), or even an antagonist (Leon et al, 1997). It is possible that the full agonist response observed to ATP at the

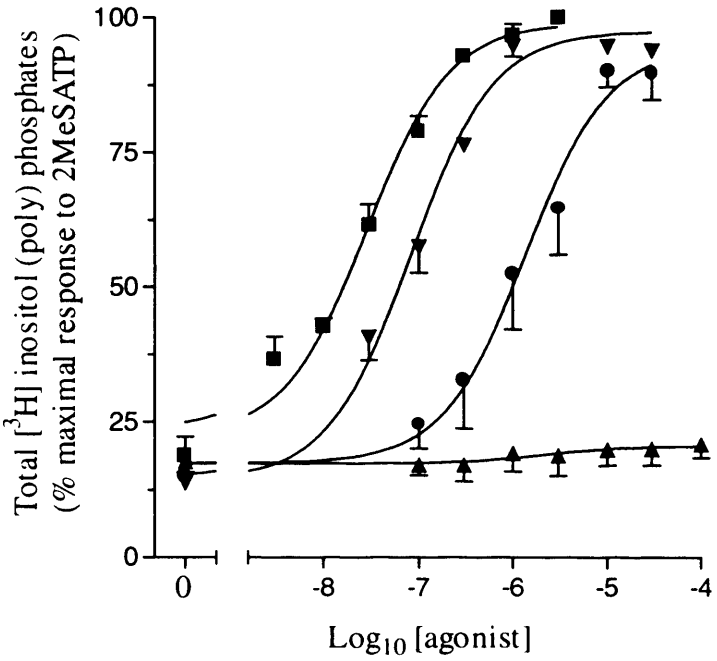


Figure 3.7

Agonist concentration response curves for [^{3}H]InsP$_X$ accumulation in turkey P2Y$_1$ 1321N1 cells.

Cells, prelabled overnight with [^{3}H] inositol were incubated for 10 minutes at 37 °C in 10 mM LiCl. Cells were then incubated for 15 minutes with increasing concentrations of 2MeSATP (■), UTP (▲), ADP (▼), ATP (●), before the aspiration of stimulating medium and addition of 0.5 ml TCA (0.5 M). Data are mean ± SEM of 3 separate experiment, each performed in triplicate, and are expressed as a percentage of the stimulation to 3 μM 2MeSATP.

turkey $P2Y_1$ receptor was actually due to the action of ADP, formed by the hydrolysis of ATP by ectoATPase. To test this hypothesis, a concentration response curve was constructed to ATPγS, a hydrolysis resistant analogue of ATP (Welford et al, 1987), and was directly compared to the curve to ATP (figure 3.8). ATP and ATPγS had very similar potencies, with $-\log EC_{50}$ values of 5.9 (1.2 ± 0.7 μM) and 6.3 (0.49 ± 0.6 μM) respectively. Although the curves were not statistically different by two-way anova, the curve to ATPγS lay slightly to the left of that to ATP.

A possible explanation for this result is that ATPγS is a more potent agonist at this receptor. Alternatively, a small amount of ATP (but not ATPγS) degradation may have occurred during the 15 minutes stimulation period. However, if this were the case, the degradation product was less potent that ATP (because the curve is shifted to the right), and was therefore unlikely to be ADP. These results are difficult to reconcile with the view that ATP is a partial agonist at the cloned turkey $P2Y_1$ receptor.

3.5.2 Bovine $P2Y_1$

Figure 3.9 shows the concentration response curves for stimulation of [^{3}H]$InsP_x$ by 2MeSATP, UTP, ATP and ADP, acting at the bovine $P2Y_1$ receptor. 2MeSATP and ADP produced curves with a plateau, and ATP reached the same maximum, but did not achieve a plateau at the concentrations used. The overall rank order of agonist potency was 2MeSATP > ADP > ATP, with UTP having no effect. The resulting $-\log EC_{50}$ values were 6.41 (387 ± 22 nM) for 2MeSATP, 5.96 (1.10 ± 0.52 μM) for ADP, and 5.05 (8.87 μM) for ATP (assuming that the curve would reach a plateau at the same level of response as 2MeSATP).

3.5.3 Human $P2Y_2$

Concentration response curves to UTP, 2MeSATP, ATP, ADP and UDP on the human $P2Y_2$ receptor are shown in Figure 3.10. UTP, ATP and ADP all produced curves with a plateau that reached the same maximal response, and UDP and 2MeSATP were less potent, failing to achieve the maximal response. UTP and ATP

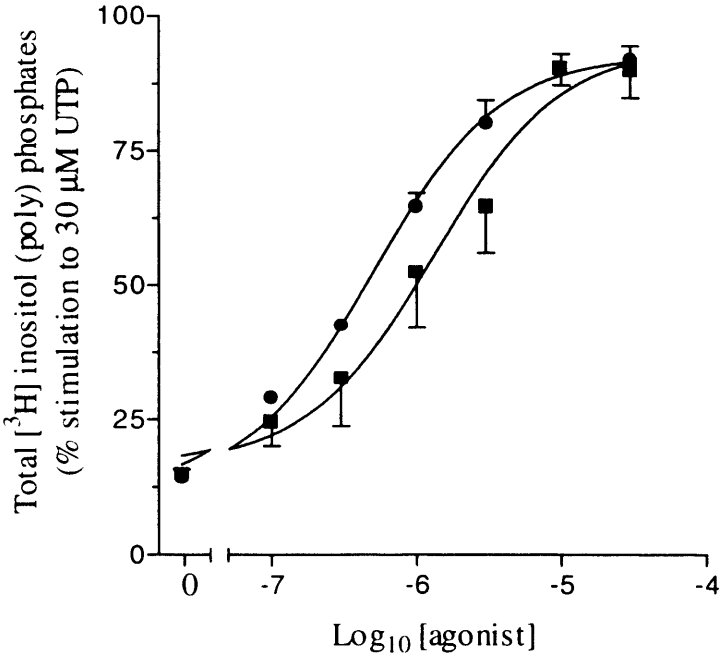


Figure 3.8

ATP and ATPγS concentration response curves for [^{3}H]InsP$_X$ in turkey P2Y$_1$ 1321N1 cells. Cells, prelabled overnight with [^{3}H] inositol were incubated for 10 minutes at 37 °C in 10 mM LiCl. Cells were then incubated for 15 minutes with increasing concentrations of ATP (■) or ATPγS (●), before the aspiration of stimulating medium and addition of 0.5 ml TCA (0.5 M). Data are mean ± SEM of 3 separate experiment, each performed in triplicate, and are expressed as a percentage of the stimulation to 3 μM 2MeSATP (not shown).

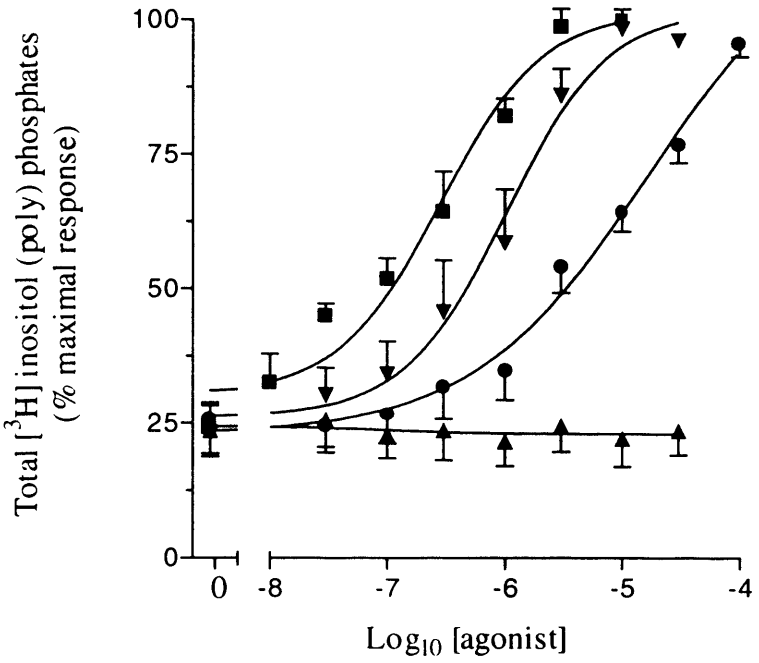


Figure 3.9

Agonist concentration response curves for [^{3}H]InsP$_X$ accumulation in bovine P2Y$_1$ 1321N1 cells.

Cells, prelabled overnight with [^{3}H] inositol were incubated for 10 minutes at 37 °C in 10 mM LiCl. Cells were then incubated for 15 minutes with increasing concentrations of 2MeSATP (■), UTP (▲), ADP (▼), ATP (●), before the aspiration of stimulating medium and addition of 0.5 ml TCA (0.5 M). Data are mean ± SEM of 3 separate experiment, each performed in triplicate, and are expressed as a percentage of the stimulation to 10 μM 2MeSATP.

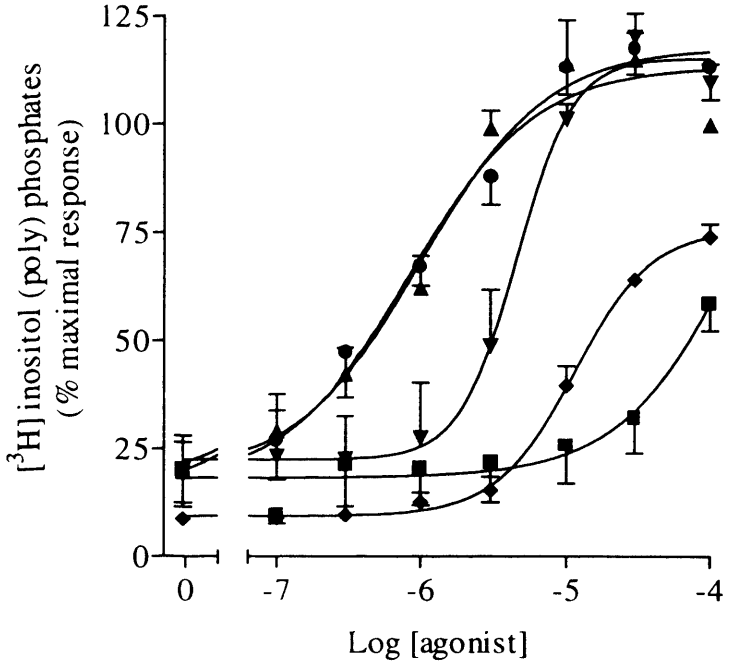


Figure 3.10

Agonist concentration response curves for [^{3}H]InsP$_X$ accumulation in human P2Y$_2$ 1321N1 cells.

Cells, prelabled overnight with [^{3}H] inositol were incubated for 10 minutes at 37 °C in 10 mM LiCl. Cells were then incubated for 15 minutes with increasing concentrations of 2MeSATP (■), UTP (▲), ADP (▼), ATP (●), and UDP (◆), before the aspiration of stimulating medium and addition of 0.5 ml TCA (0.5 M). Data are mean ± SEM of 3 separate experiment, each performed in triplicate, and are expressed as a percentage of the stimulation to 100 μM UTP.

were equipotent, with –log EC_{50} values of 6.02 (0.94 ± 0.23 μM) and 5.82 (1.50 ± 0.55 μM), respectively. ADP had a –log EC_{50} value of 5.08 (8.22 ± 3.0 μM), but had a steeper slope that reached the maximal response over approximately just one order of magnitude. The curve to UDP appeared to reach a plateau at approximately 60 % of the maximal response, and had a –log EC_{50} value of 4.80 (15.8 ± 0.51 μM). 2MeSATP was a very weak agonist, only significantly increasing the levels of [^{3}H]$InsP_x$ at the highest concentration used (100 μM).

3.5.4 Human $P2Y_4$

At the human $P2Y_4$ receptor, UTP was the most potent agonist, with a –log EC_{50} value of 5.93 (1.17 ± 0.19 μM) (Figure 3.11). None of the other agonists used achieved the same maximal response. The curve to UDP reached a plateau at 80.2 ± 1.0 % of the response to UTP and had –log EC_{50} of 5.24 (5.68 ± 1.19 μM). However, like ADP on the $P2Y_2$, the slope to UDP was much steeper than the other curves, moving from basal levels to maximal stimulation within one log unit. ATP was also a partial agonist, achieving a maximum of only 57.7 ± 2.12 % of that to UTP, and with a –log EC_{50} value of 4.79 (16.2 ± 4.76 μM). Both ADP and 2MeSATP were ineffective at the human $P2Y_4$ receptor.

3.6 Nucleotide interconversion

To date, characterisation of native P2 receptors has relied largely on differences in rank order of agonist potencies. As can be seen from the results shown in section 3.5, when the receptors are transfected into the same null host cell line, the agonist selectivity of each receptor can be determined individually, and this will aid the characterisation of native receptors. However, heterologous receptor populations and differences in tissue type are not the only factors effecting agonist potency orders. The interconversion of nucleotides by enzymes present on the surface of all cells presents a huge problem when interpreting the observed potencies of agonists. If a

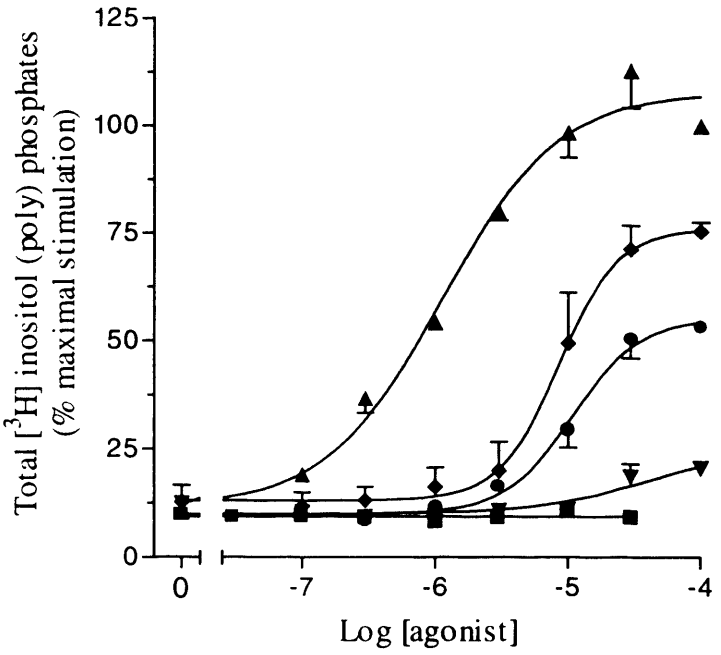


Figure 3.11

Agonist concentration response curves for [^{3}H]InsP$_X$ accumulation in human P2Y$_4$ 1321N1 cells.

Cells, prelabled overnight with [^{3}H] inositol were incubated for 10 minutes at 37 °C in 10 mM LiCl. Cells were then incubated for 15 minutes with increasing concentrations of 2MeSATP (■), UTP (▲), ADP (▼), ATP (●), and UDP (◆), before the aspiration of stimulating medium and addition of 0.5 ml TCA (0.5 M). Data are mean ± SEM of 3 separate experiment, each performed in triplicate, and are expressed as a percentage of the stimulation to 100 μM UTP.

potent agonist were more readily broken down than a less potent agonist, the observed potency order could be reversed, or dramatically altered.

Three separate approaches using shorter stimulation periods, an ectonucleotidase inhibitor, and hexokinase with glucose, were used to tackle this issue.

3.6.1 Effect of shorter stimulation periods

The [^{3}H]InsP$_x$ assay uses stimulation times of 15 minutes. This is a relatively long time in which nucleotides can be hydrolysed. Therefore, a simple comparison was made between UTP concentration response curves at the human P2Y$_2$ receptor using the [^{3}H]InsP$_x$ assay and the mass Ins(1,4,5)P$_3$ assay. The mass Ins(1,4,5)P$_3$ assay has a stimulation period of 15 seconds, a time in which much less hydrolysis would occur. If the observed potency of UTP were higher using the mass IP$_3$ assay, it would mean that between 15 seconds and 15 minutes there was an appreciable amount of UTP breakdown.

The results of this comparison are shown in Figure 3.12. UTP gave similar stimulations for both assays, in each case about 8 times basal counts. The curve with the [^{3}H]InsP$_x$ assay was slightly to the left of the curve with the mass Ins(1,4,5)P$_3$ assay, with -log EC$_{50}$ values of 6.15 (0.70 μM) and 5.75 (1.80 μM) respectively. Since UDP is less potent at this receptor than UTP, any breakdown that occurs has little effect when using the [^{3}H]InsP$_x$ assay.

3.6.2 ARL 67156

The nucleoside triphosphates ATP, 2MeSATP, and UTP are substrates of ectoATPase enzymes, which are present on the surface of all cells. If there were a high level of ectoATPase activity in these cells, the observed potency of these nucleotides would be lower than their true potency. In addition, the products of this degradation (ADP, 2MeADP and UDP) may act at the receptor, confusing issues further. To determine the level of ectoATPase activity in 1321N1 cells over the 15 minute stimulation period, the newly synthesised, potent ectoATPase inhibitor ARL

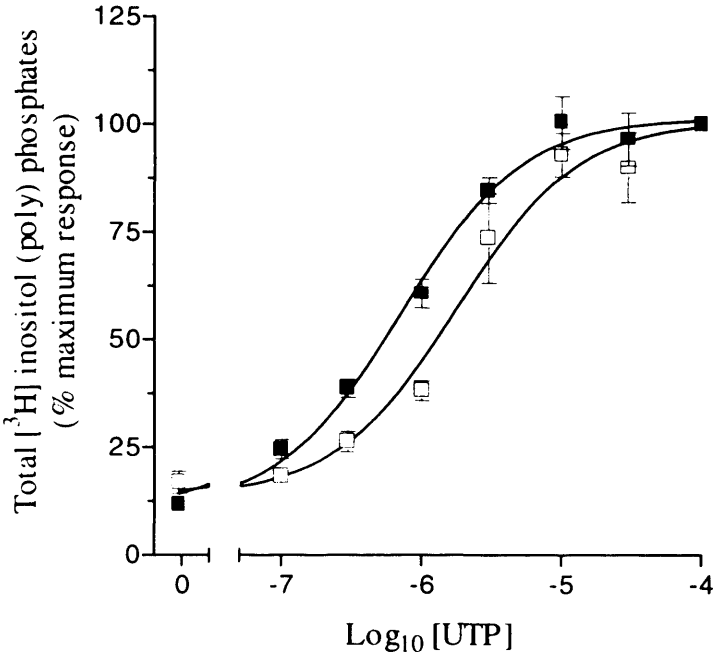


Figure 3.12

Agonist concentration response curve to UTP for [^{3}H]InsP$_X$ accumulation and Ins(1,4,5)P$_3$ production in human P2Y$_2$ 1321N1 cells.

Responses to increasing concentrations of UTP were measured in human P2Y$_2$ cells using method B for the total [^{3}H]InsP$_X$ assay (■) (see section 3.22), and the mass Ins(1,4,5)P$_3$ assay (⊿) (see section 3.31). Data are the mean ± SEM of 3 separate experiments for the [^{3}H]InsP$_X$ assay, and one experiment for the Ins(1,4,5)P$_3$ assay, each performed in triplicate. Data are expressed as a percentage of the stimulation to 100 μM UTP.

67156 (6-N,N-diethyl-β,γ-dibromomethylene-D-ATP) (formerly FPL 67156; Crack et al, 1995) was used.

Figure 3.13A shows the effect of ARL 67156 on the concentration response curve to 2MeSATP on the $P2Y_1$ cells. ARL 67156 was included with the 10 minute LiCl preincubation at a concentration of 100 μM, before the addition of 2MeSATP for 15 minutes. ARL 67156 produced an elevation of the concentration-response curve to 2MeSATP, and a small, though statistically insignificant shift of this curve to the left. The curves had -log EC_{50} values of 7.55 ± 0.17 (28.2 nM) in the absence, and 8.08 ± 0.19 (8.3 nM) in the presence of 100 μM ARL 67156.

Figure 3.13B shows the results obtained when the same experiment was performed with UTP on the $P2Y_2$ cells. Again ARL 67156 raised the top and bottom of the concentration-response curve to UTP, and gave a small, insignificant shift of the curve to the left. -log EC_{50} values were 5.85 ± 0.06 (1.4 μM) in the absence, and 6.18 ± 0.13 (0.66 μM) in the presence of 100 μM ARL 67156.

The small shift of the curves to the left with ARL 67156 indicates that ectoATPase activity is present in these cells to a small degree. The fact that curves to both 2MeSATP and UTP were shifted to the same degree indicates that the ectoATPase enzymes present on the surface of 1321N1 cells do not discriminate between 2MeSATP and UTP. The elevation of the curves are harder to explain, and will be approached in the discussion section of this chapter.

3.6.3 The effect of hexokinase and glucose

Recently, in a paper by Nicholas et al (1996b), the presence of ectonucleoside diphosphokinase activity on 1321N1 astrocytoma cells was reported. This enzyme catalyses the transfer of a terminal phosphate from a nucleoside triphosphate to a nucleoside diphosphate. Thus, if the cells release ATP due to sheer stress, it is possible that exogenously added UDP could be phosphorylated to UTP, and therefore display a different action at the receptor (refer to section 1.7 and Figure 1.8, this

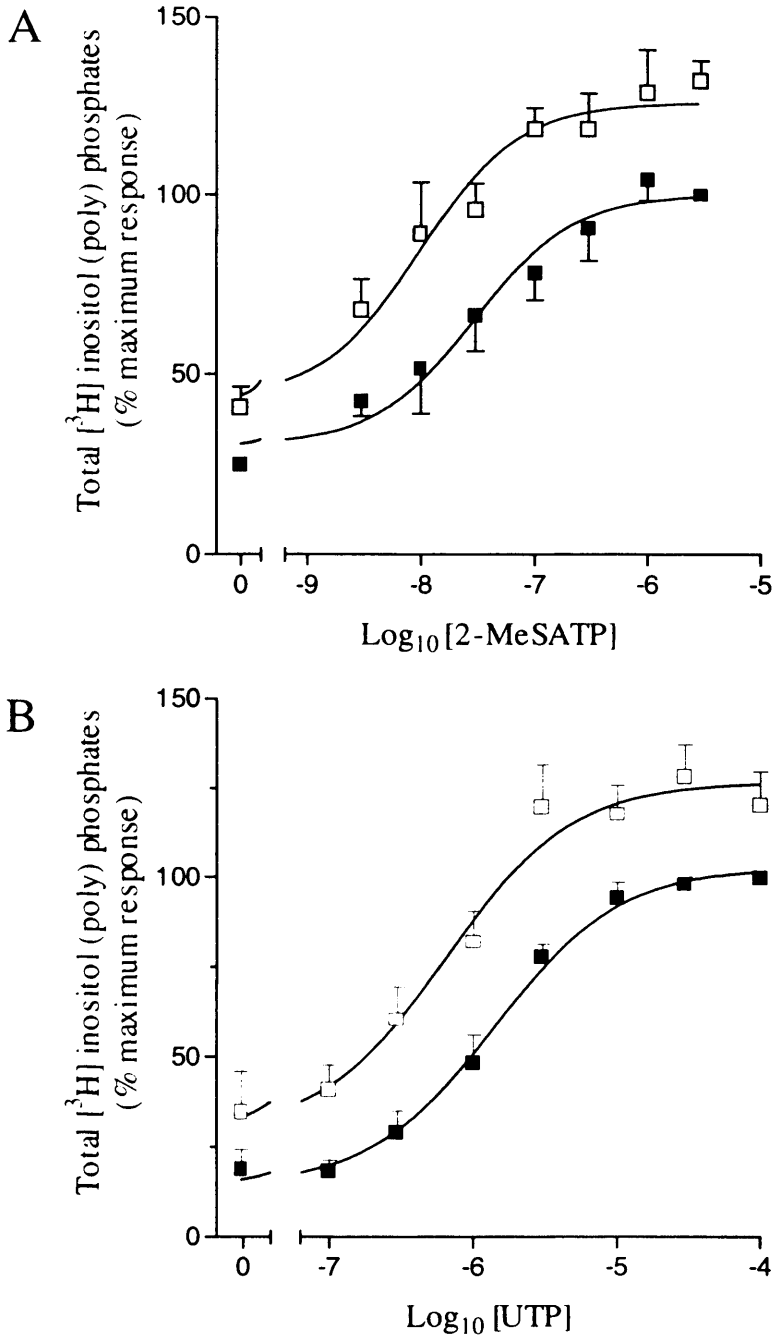


Figure 3.13

The effect of the ectonucleotidase inhibitor ARL 67156 on concentration response curves to 2MeSATP on turkey $P2Y_1$ 1321N1 cells and to UTP on the human $P2Y_2$ 1321N1 cells.

Concentration response curves were constructed (**A**) to 2MeSATP alone (■) or to 2MeSATP with ARL 67156 (100 μM) (❑), or (**B**) to UTP alone (■) or to UTP with ARL 56156 (100 μM) (❑). ARL 67156 was preincubated with the cells for 10 minutes with 10mM LiCl. Cells were then stimulated with agonist for 15 minutes. Data are pooled from three separate experiments each in triplicate and are expressed as mean ± SEM of the percentage of the response to 3 μM 2MeSATP alone. This data is also presented as part of figure 25, chapter 4.

thesis). In the same study it was also suggested that triphosphate impurities in stock solutions of dinucleotide agonists might contribute to misinterpretation of nucleoside diphosphate potencies. The enzyme hexokinase has been used to circumvent this problem. Hexokinase catalyses the transfer of a terminal phosphate from a nucleoside triphosphate to the C6 position of glucose, thus converting any triphosphates produced by nucleoside diphosphokinase or contaminating stock solutions back into diphosphates.

Concentration response curves to the nucleoside diphosphates were repeated on the $P2Y_1$, $P2Y_2$ and $P2Y_4$ cells, this time in the presence of hexokinase and glucose. Stock solutions of 5 μM UDP and ADP were preincubated with 50 units/ml hexokinase and 110 mM glucose for 1 hour before experimentation. Hexokinase (1 unit/ml) and glucose (22 mM) were added to the cells with the 10 minute LiCl pre-incubation, and stimulations with the treated agonists were performed as described previously (method B, section 3.31).

3.6.3.1 Turkey $P2Y_1$

Figure 3.14 shows the effect of hexokinase on the concentration response curve to ADP at the turkey P2Y1 receptor. Hexokinase shifted the curve to the right, but the maximal response was maintained. $-\log EC_{50}$ values were 6.67 (0.21 ± 0.11 μM) in the absence and 5.55 (2.80 ± 0.56 μM) in the presence of hexokinase. The two curves were significantly different by two-way analysis of variance ($P<0.001$); data were from 3 separate experiments, each performed in triplicate.

3.6.3.2 Human $P2Y_2$

The effect of hexokinase on the concentration response curve to ADP at the human $P2Y_2$ receptor is shown in Figure 3.15A. As was seen in Figure 3.10, ADP in the absence of hexokinase and glucose gave a response with a steeper slope that reached the maximal response within one order of magnitude, with a $-\log EC_{50}$ value of 5.18 (6.53 ± 0.92 μM). When hexokinase and glucose were included, the response fell away dramatically, leaving a curve that only achieved a significant response at

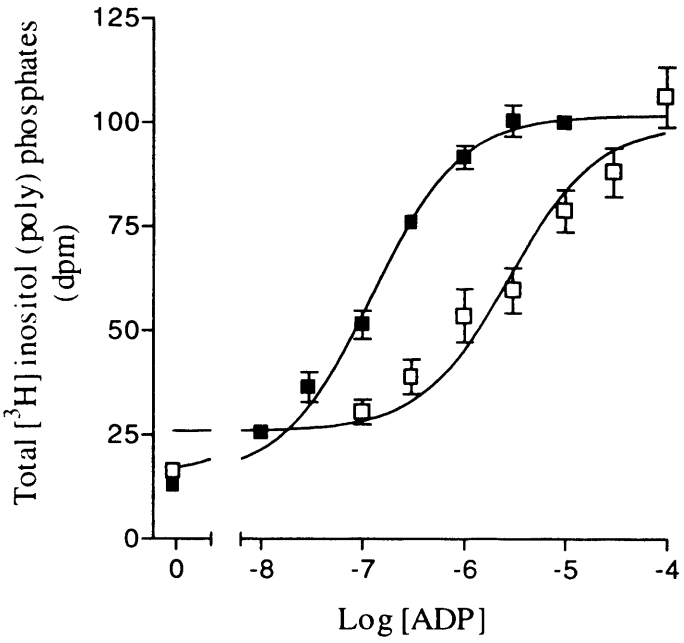


Figure 3.14

Concentration response curves to ADP on turkey $P2Y_1$ cells in the absence and presence of hexokinase.

ADP stock (5 mM) was preincubated with 50 units/ml hexokinase and 110 mM glucose for 1 hour prior to dilution of agonist. Cells were preincubated for 10 minutes with 10 mM LiCl with (❑) and without (■) 1 unit/ml hexokinase and 22 mM glucose. Cells were then incubated for 15 minutes with hexokinase treated ADP (❑) and untreated ADP (■), before termination of stimulations by aspiration and addition of 0.5 ml TCA (0.5 M). Data are pooled from three separate experiments each in triplicate and are expressed as mean ± SEM of the percentage of the response to 100 μM ADP alone.

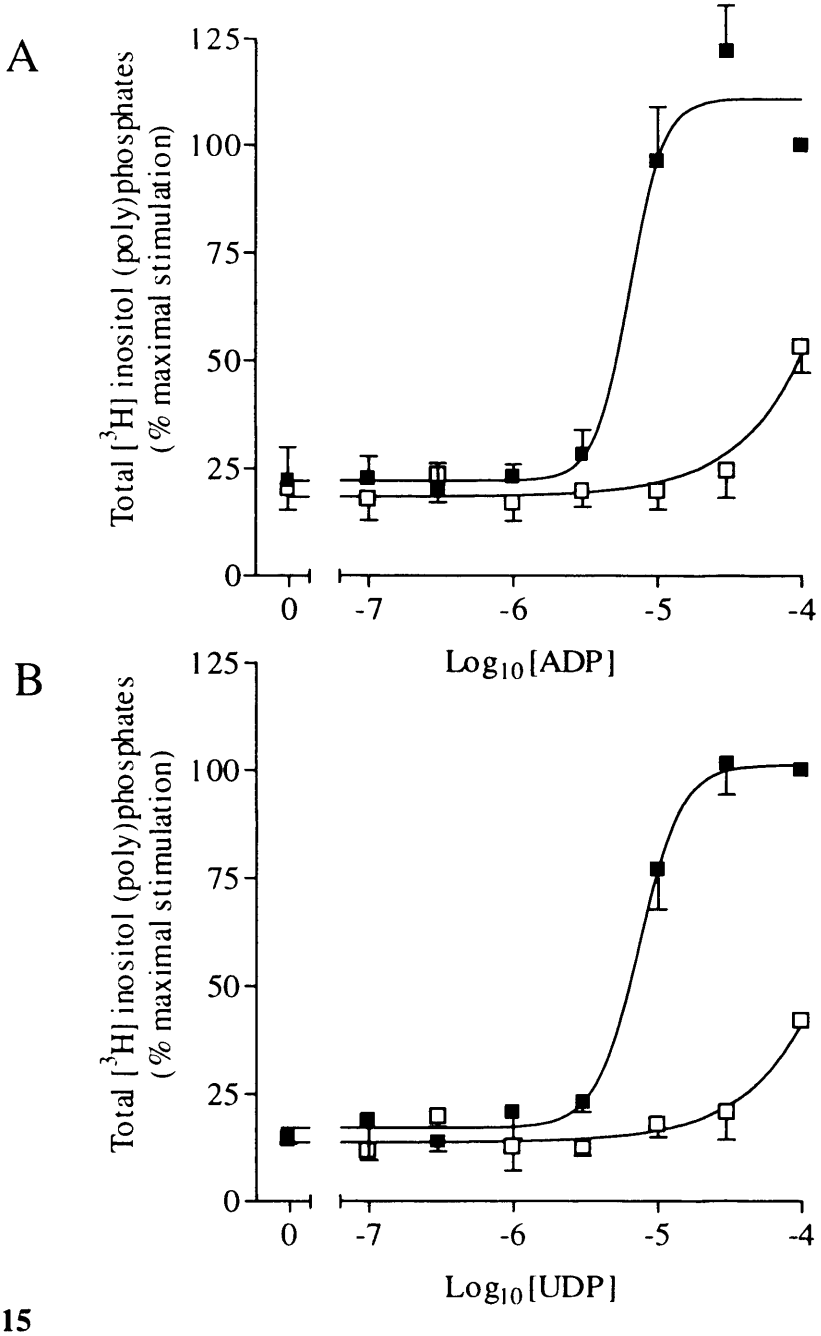


Figure 3.15

Concentration response curves to ADP and UDP on human $P2Y_2$ cells in the absence and presence of hexokinase.

ADP and UDP stocks (5 mM) were preincubated with 50 units/ml hexokinase and 110 mM glucose for 1 hour prior to dilution of agonist. Cells were preincubated for 10 minutes with 10 mM LiCl with (❑) and without (■) 1 unit/ml hexokinase and 22 mM glucose. Cells were then incubated for 15 minutes with (**A**): hexokinase treated ADP (❑) and untreated ADP (■), and (**B**): hexokinase treated UDP (❑) and untreated UDP (■), before termination of stimulations by aspiration and addition of 0.5 ml TCA (0.5 M). Data are pooled from three separate experiments each in triplicate and are expressed as mean ± SEM of the percentage of the response to 100 μM ADP (for **A**) or UDP (for **B**) alone.

100 μM ADP. The effect of hexokinase on the UDP concentration response curve was very similar, with a steep slope and a -log EC_{50} of 5.21 (6.09 ± 1.16 μM) (Figure 3.15B). Again, hexokinase all but abolished the response to UDP.

3.6.3.3 Human $P2Y_4$

Figure 3.16 shows the results when hexokinase and glucose were included in the UDP concentration response curve at the human $P2Y_4$ receptor. The curve to UDP without hexokinase had a –log EC_{50} of 5.55 (2.81 ± 0.27 μM), which was shifted to 4.35 (44.2 ± 7.21 μM) when hexokinase and glucose were included. The two curves were statistically significant at $P<0.001$ using two-way analysis of variance with Dunnet's post-hoc test.

3.7 Discussion

This chapter has described the preliminary investigation of several cloned P2Y receptors, transfected into 1321N1 astrocytoma cells. Transfection of the $P2Y_1$ and $P2Y_2$ receptors conferred an increase in PLC activity in the absence of added agonist to the 1321N1 cells. Moreover, this activity could be reduced to pre-transfection levels by the addition of an antagonist. One possible explanation for this observation is that the receptor displays constitutive activity, i.e. it can attain an active, high affinity state in the absence of agonist. An antagonist (in this case suramin) can occupy the receptor, stabilising it in an inactive state. Thus, the antagonist is said to act as an inverse agonist with negative efficacy. However, the results shown in Figure 3.3B show that the basal activity is dependent on the conditions used for stimulation, so is unlikely to be due to constitutive activity. A more likely explanation for this phenomenon is that 1321N1 cells exposed to sheer stress release ATP, which stimulates the transfected receptors, increasing PLC activation.

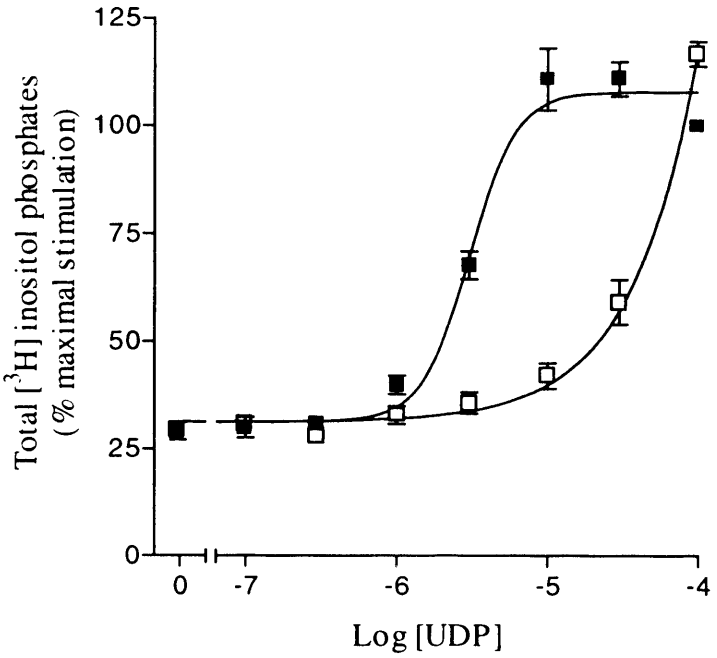


Figure 3.16

Concentration response curves to UDP on human P2Y4 cells in the absence and presence of hexokinase.

UDP stock (5 mM) was preincubated with 50 units/ml hexokinase and 110 mM glucose for 1 hour prior to dilution of agonist. Cells were preincubated for 10 minutes with 10 mM LiCl with (❑) and without (■) 1 unit/ml hexokinase and 22 mM glucose. Cells were then incubated for 15 minutes with hexokinase treated UDP (❑) and untreated UDP (■), before termination of stimulations by aspiration and addition of 0.5 ml TCA (0.5 M). Data are pooled from three separate experiments each in triplicate and are expressed as mean ± SEM of the percentage of the response to 100 μM UDP alone.

3.7.1 Nucleotide release from 1321N1 cells

The release of ATP from intact 1321N1 cells has been directly quantified using the luciferin-luciferase assay (Lazarowski et al, 1995). Up to 150 pmol ATP/10^6 cells (approximately 9 % of the cell content of ATP) was released upon replacing the incubation medium with fresh medium. This resulted in an extracellular ATP concentration of 200–300 nM for confluent cells in a well of a 12-well culture dish containing 0.5 ml medium. An estimate of the concentration of released ATP from the 1321N1 cells in response to sheer stress has been made from the results presented in this chapter. The equivalent concentration of 2MeSATP required to elicit the same response as that observed with unstimulated cells using the original method, was approximately 30–40 nM. It must be noted, however, that 2MeSATP is a more potent agonist than either ADP or ATP, and that approximately 6.5 times more ADP and 44 times more ATP is required to produce an equivalent stimulation. Thus, an estimated concentration of released nucleotide would be 230 nM ADP or 1.58 μM ATP.

Lazarowski et al (1995) also showed that the released ATP was degraded completely by 3 hours, but less than 10 % was hydrolysed in 20 minutes, so it would have a large impact on results obtained using the total inositol phosphate assay. Grierson & Meldolesi (1995) have provided evidence for the endogenous release of ATP in another cell line, mouse fibroblast cells, in response to sheer stress induced by puffs of saline.

3.7.1.1 Which is the predominant nucleotide released?

The differential success of method A and method B suggests that the $P2Y_1$ cells are more sensitive to medium changes than the $P2Y_2$ cells. Several factors may be responsible for this. For example, the transfection procedure may have isolated a $P2Y_1$ clonal cell line that is inherently more prone to ATP release upon sheer stress than the $P2Y_2$ clone used. Alternatively, the $P2Y_1$ cells may have a larger receptor reserve than the $P2Y_2$ cells, resulting in a higher sensitivity of the $P2Y_1$ cells to released nucleotides. However, the differences in basal levels of stimulation may reflect the receptor specificity of the nucleotide released. Lazarowski et al (1995) have shown that both ATP and ADP are present in the medium of 1321N1 cells, although their studies only investigated purine nucleotides and so do not rule out the

presence of UTP or UDP. However, the endogenous agonist activity has a relatively small effect on the $P2Y_2$ expressing cells, suggesting the endogenous agonist activity is not predominantly UTP. ATP is effective at both $P2Y_1$ and $P2Y_2$ receptors, so again is probably not the principle agonist. ADP, however, is selective for $P2Y_1$ receptors over $P2Y_2$ receptors (Nicholas et al, 1996a; this chapter) and is it therefore likely that this is the predominant endogenous nucleotide.

3.7.1.2 Is nucleotide release a physiological response?

The mechanism of release of nucleotides from 1321N1 cells has not been determined, and it is unclear whether it is a physiological response or not. The roles of astrocytes in the brain have not been clearly established, although 'buffering' of extracellular medium and release of neuroactive substances appear to be important (Walz, 1989; Martin, 1992). Primary cultures of astrocytes express P2 receptors (Bruner & Murphey, 1993; Kastritsis et al, 1992), and therefore, the release of ATP and activation of endogenous P2 receptors may be an autocrine response.

3.7.2 Nucleotide degradation and interconversion

To date, classification of P2 receptors has relied heavily on rank order of agonist potency. However, interconversion of nucleotides by enzymes on the surface of all cells can lead to the misinterpretation of agonist potencies. The issue of nucleotide interconversion was tackled in 3 different ways. Firstly, the use of two assays with different stimulation periods provided evidence against any appreciable nucleotide breakdown in 1321N1 cells. The effect of ARL 67156, an ectoATPase inhibitor, and hexokinase, were also examined.

3.7.3.1 ARL 67156

The ectoATPase inhibitor ARL 67156 has been used at concentrations of 100 μM to prevent approximately 70 - 80 % nucleoside triphosphate hydrolysis by human blood cells (Crack et al, 1995). In this thesis, the inhibitor was used to check whether

any appreciable amount of nucleotide breakdown occurred with 1321N1 cells. Although the positions of the curves to 2MeSATP and UTP were not altered by ARL 67156 to a statistically significant degree, there was a small shift to the right in both cases. This may represent a small inhibition of nucleotide breakdown by ARL 67156. More puzzling is the tendency for ARL 67156 to raise the concentration response curves to both agonists. ARL 67156 has been shown to have low potency agonist effects at $P2Y_2$ receptors, with a pA_{50} value of approximately 3.5 (Crack et al, 1995). However, if ARL 67156 was acting as an agonist, although the response in the absence of additional agonist would be increased, the maximal response attained by stimulation of the P2Y receptor would not. Therefore, if ARL 67156 acts as an agonist, it must be at a receptor not stimulated by UTP or 2MeSATP. If this phenomenon was due to the inhibition of ectonucleotidases, then ARL 67156 raises the level of an endogenous nucleotide that acts at a receptor not activated by either UTP in the $P2Y_2$ cells or 2MeSATP in the $P2Y_1$ cells.

One interesting hypothesis is that ARL 67156 can enter cells and inhibit the GTPase activity of G protein α subunits. This would increase the time that G proteins spend in their GTP bound, uncomplexed active state, and may increase the activation of phospholipase C. This would explain why ARL 67156 has an effect on basal levels of total inositol phosphates. This question could be further examined by performing the same experiment with ARL 67156 in untransfected cells, to determine whether it was a receptor-specific effect.

3.7.3.2 Hexokinase

Hexokinase has been used by Nicholas et al (1996b) to remove contaminating triphosphates in stock solutions and stimulating medium during the construction of concentration response curves to nucleoside diphosphates. They reported that previous responses observed to UDP and ADP at the $P2Y_2$ and $P2Y_4$ receptors were actually due to action of UTP and ATP, and that the $P2Y_2$ and $P2Y_4$ receptors were activated by nucleoside triphosphates and not nucleoside diphosphates.

The results obtained with the $P2Y_2$ and $P2Y_4$ receptor in this thesis agree with this observation. At the human $P2Y_2$ receptor, the inclusion of hexokinase dramatically reduced the potency of ADP and UDP, suggesting they are not agonists

at this receptor. Hexokinase also significantly reduced the response to UDP at the human $P2Y_4$ receptor, again suggesting that responses previously observed to this agonist were actually to UTP. The steeper concentration-response curves seen with the nucleoside diphosphates in the $P2Y_2$ and $P2Y_4$ cells in the absence of hexokinase may be because they exert their effects through conversion to nucleoside triphosphates.

However, results obtained with the turkey $P2Y_1$ receptor cast doubt on this assumption. The response to ADP at the turkey $P2Y_1$ receptor was used as a control for hexokinase, because ATP is less potent than ADP at this receptor. Thus, if ADP was converted to ATP by nucleoside diphosphokinase, the true concentration response curve to ADP would lie to the right, and not to the left of the original curve. However, when hexokinase was used with the $P2Y_1$ cells, the potency of ADP at the $P2Y_1$ receptor was significantly reduced.

There are two possible explanations for this phenomenon. Firstly, *hexokinase and glucose may exert non-specific effects on the cells or receptors, reducing the capability of the cells to respond.* The osmolarity of cell culture medium is approximately equivalent to 300 mM glucose, so an increment of 22 mM glucose would not significantly increase the osmotic pressure to the cells. In addition, evidence against the non-specific action of hexokinase on 1321N1 cells has been provided by Nicholas et al (1996b), (also Harden et al, 1997), who showed that hexokinase had no effect on the response to UDP on 1321N1 cells transfected with the human $P2Y_6$ receptor.

An alternative explanation is that *by removing ATP, hexokinase interferes with the recycling of AMP to ADP at the cell surface.* Typically, only small amounts of interconversion have been reported when bulk phase levels of nucleotides were measured (Harden et al 1997; Pearson et al, 1980), but at the surface of the cells, or 'biophase', the interconversion of nucleotides may occur at a greater rate, and may play an important role. A theoretical model explaining this hypothesis is presented in Figure 3.17, and is described below:

ADP is broken down by the enzyme ectoADPase to AMP, which is in turn hydrolysed to AMP by 5'-nucleotidase. 5'-nucleotidase has a high K_m value, resulting in the accumulation of AMP at the cell surface (Pearson et al, 1980). AMP can be

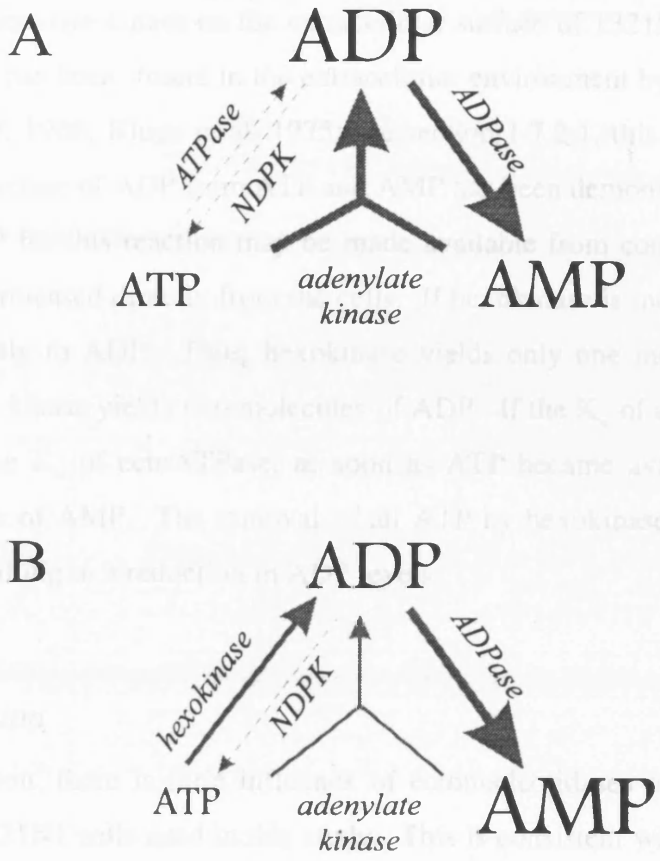


Figure 3.17

Cartoon showing a theoretical model for the reduction of ADP levels by hexokinase.

The model shows how inclusion of hexokinase reduces ATP levels, and may therefore reduce the recycling of AMP to ADP by adenylate kinase. **A:** In the normal situation, AMP formed by ADPase is combined with ATP (endogenously released) by adenylate kinase to form 2 molecules of ADP. **B:** The inclusion of hexokinase hydrolyses ATP to ADP, but prevents the recycling of AMP, resulting in reduced ADP resynthesis.

recycled to ADP by the action of adenylate kinase, which catalyses the transfer of the terminal phosphate group of ATP to AMP, yielding two molecules of ADP. Although the presence of adenylate kinase on the extracellular surface of 1321N1 cells has not been examined, it has been found in the extracellular environment by several groups (e.g. Lindena et al, 1986; Kluge et al, 1975; see section 1.7.2.1, this thesis), and the extracellular production of ADP from ATP and AMP has been demonstrated by Kluge et al (1975). ATP for this reaction may be made available from contaminated ADP stocks, or may be released directly from the cells. If hexokinase is included, the ATP is converted directly to ADP. Thus, hexokinase yields only one molecule of ADP whereas adenylate kinase yields two molecules of ADP. If the K_m of adenylate kinase was lower than the K_m of ectoATPase, as soon as ATP became available it would recycle a molecule of AMP. The removal of all ATP by hexokinase would prevent this recycling, resulting in a reduction in ADP levels.

3.7.3.3 Conclusion

In conclusion, there is little influence of ectonucleotidases on the [^{3}H]InsP_x response in the 1321N1 cells used in this study. This is consistent with other studies performed on 1321N1 cells which show that although ATP is completely hydrolysed by 3 hours, 90 % remains after 20 minutes (Lazarowski et al, 1995). The cells do appear to have considerable nucleoside diphosphokinase activity, although this is undoubtedly exaggerated by contamination of the ADP/UDP stocks with ATP/UTP. It is, however, possible that 1321N1 cells do possess a high level of ectonucleotidase activity, but that this is not observed because the nucleoside diphosphates are rapidly cycled back to their triphosphate forms by nucleoside diphosphokinase. The rapid cycling of nucleotides at the surface of cells may have huge implications on the study of P2 receptors. Table 3.2 shows a summary of the rank order of agonist potencies at the 4 cloned receptors, taking nucleotide interconversion into consideration.

3.7.4 Species variation

Many of the results with the $P2Y_1$ receptor presented in this thesis are on the turkey $P2Y_1$ clone, whilst the $P2Y_2$ and $P2Y_4$ receptors are both cloned from human

	Rank order of agonist potencies
Turkey $P2Y_1$	2MeSATP > ADP > ATPγS = ATP, not UTP
Bovine $P2Y_1$	2MeSATP > ADP > ATP, not UTP
Human $P2Y_2$	UTP = ATP, not UDP, ADP or 2MeSATP
Human $P2Y_4$	UTP >> ATP (partial) > UDP, not ADP, 2MeSATP

Table 3.2

Summary of the rank order of agonist potencies at the 4 cloned P2Y receptors, corrected for nucleotide interconversion.

The rank orders presented assume that hexokinase does not have non-specific effects.

sources. It must therefore be considered whether the differences seen between the receptor types might actually be due to the species of origin, rather than the intrinsic receptor subtype. Review of the amino acid sequence reveals that the receptor types are each highly conserved across species, while the receptor subtypes show only selective and limited homology with each other. For example, the human $P2Y_1$ receptor is 83 % homologous to the turkey $P2Y_1$ receptor, and ≥ 95 % homologous to the bovine $P2Y_1$ receptor, but has only about 35 % sequence identity to the human $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors (see section 1.5.1, this thesis). However, it must also be noted that all of the mammalian $P2Y_1$ receptors possess an 11 amino acid insert near the N-terminus that is absent in the avian sequences, with other differences occurring in TM4 and TM5 (Schachter et al, 1996).

3.7.4.1 Comparison between the turkey and bovine $P2Y_1$ receptors

To determine whether these differences in receptor structure result in different receptor characteristics, a mammalian $P2Y_1$ receptor, the bovine $P2Y_1$ (Henderson et al, 1995) was transfected into 1321N1 cells. A comparison of the $P2Y_1$ species homologous revealed that the agonist profile was identical, suggesting that the differences in sequence do not alter agonist specificity. However, although the position of the curves did not change in relation to each other, the potency of each of the agonists was reduced by approximately a factor of 10 in the bovine $P2Y_1$ 1321N1 cells. For example, the EC_{50} values for 2MeSATP were 28.12 ± 3.65 nM at the turkey $P2Y_1$ and 387 ± 22 nM at the bovine $P2Y_1$ receptor, and EC_{50} values for ADP were 0.18 ± 0.01 μM at the turkey $P2Y_1$, and 1.10 ± 0.52 μM at the bovine $P2Y_1$ receptor.

It is possible that the different potencies reflects differences in the intrinsic receptor structure, but it is more likely that it is due to a higher expression level of turkey $P2Y_1$ receptors compared to bovine $P2Y_1$ receptors. If the turkey $P2Y_1$ 1321N1 cells had a higher receptor reserve, the potency of agonists would appear higher (Kenakin et al, 1992). In the absence of reliable radioligand binding assays, there is no way of determining exact receptor number, so this question can not be adequately addressed.

3.7.5 Is ATP an agonist at the $P2Y_1$ receptor?

Several reports have shown that ATP acts as a partial agonist or even an antagonist at mammalian $P2Y_1$ receptors. For example, measuring increases in cytosolic Ca^{2+}, Dainty et al (1996) showed that ATP was a partial agonist with an estimated pK_A of 5.44 ± 0.09 at cloned bovine $P2Y_1$ receptors expressed in Jurkat cells. Also, Feolde et al (1995) have described a Ca^{2+} response following stimulation of a $P2Y_1$ receptor in rat brain microvascular endothelial cells by ADP, that is inhibited by ATP. The maximum response to ATP at this receptor was only 55 % of that to ADP. A recent report by Leon et al (1997) has shown the presence of the $P2Y_1$ receptor in human platelets, and has suggested that this receptor corresponds to the P_{2T} receptor. They show that ADP is the true agonist for the cloned human $P2Y_1$ receptor with ATP acting as an antagonist, and attribute previous observations of ATP agonism to contamination of commercial ATP with ADP, or breakdown of the trinucleotide to the dinucleotide. However, the platelet ADP antagonist ARL 67085 has been reported to have no effect on endothelial $P2Y_1$ receptors, which presumably correspond to the cloned $P2Y_1$ receptor (Humphries et al, 1995).

The results presented in this thesis are not consistent with the suggestion that ATP is an antagonist at mammalian $P2Y_1$ receptors. A possible explanation for these inconsistencies is differences in host cell. The cells that these ATP insensitive receptors were studied in may have less signal transduction capacity, or lower $P2Y_1$ receptor expression than in the 1321N1 cells. Thus, if ATP were a very low efficacy agonist in a system where there were not many receptors or other transduction components, it would be unable to achieve the maximal stimulations observed to other, more efficacious agonists. But, if there were a large receptor reserve, ATP would appear to be a full agonist. This is borne out by other studies on both avian (turkey) and mammalian (human) $P2Y_1$ receptors cloned into 1321N1 cells that have shown ATP to be a full agonist in both cases (Filtz et al, 1994; Schachter et al 1996). Thus, the same cloned receptor may show different agonist profiles depending upon the host cell it was transfected into e.g. the cloned bovine $P2Y_1$ transfected into Jurkat and 1321N1 cells.

Chapter 4: Antagonist Studies

4.1 Introduction

The characterisation of P2 receptors has to date relied largely on the use of rank orders of agonist potencies and differential effects of antagonists. There is, however, a severe lack of selective antagonists, and those antagonists available are poorly characterised with respect to the actions at the different types of G protein-coupled P2 receptors. The inconsistencies in the literature pertaining to the selectivity of antagonists have arisen through the use of different cell/tissue, the co-expression of two or more receptors in one preparation, and the effects of ectonucleotidases. In this chapter, the actions of 3 commonly utilised antagonists, suramin, PPADS and NF023, are investigated at 4 different P2Y receptors. The receptors were transfected into the same host cells, so the comparison between the receptors could not be contaminated by differences in the host cell. The question of nucleotide breakdown is also addressed in this chapter using the ectonucleotidase inhibitor ARL 67156.

4.2 Suramin

Suramin has been described in the past as a non-selective P2 receptor antagonist, although there have been instances where suramin insensitive responses have been observed to UTP (e.g. in bovine aortic endothelial cells; Wilkinson et al, 1994b). A detailed overview of the properties of suramin has been provided elsewhere in this thesis (section 1.4.3.1). In this section, the effect of suramin at 4 cloned and transfected P2Y receptors is thoroughly investigated to determine whether it could be used as a tool for selecting between P2Y receptor subtypes.

4.2.1 Turkey $P2Y_1$

Figure 4.1 shows the effect of suramin on concentration-response curves to

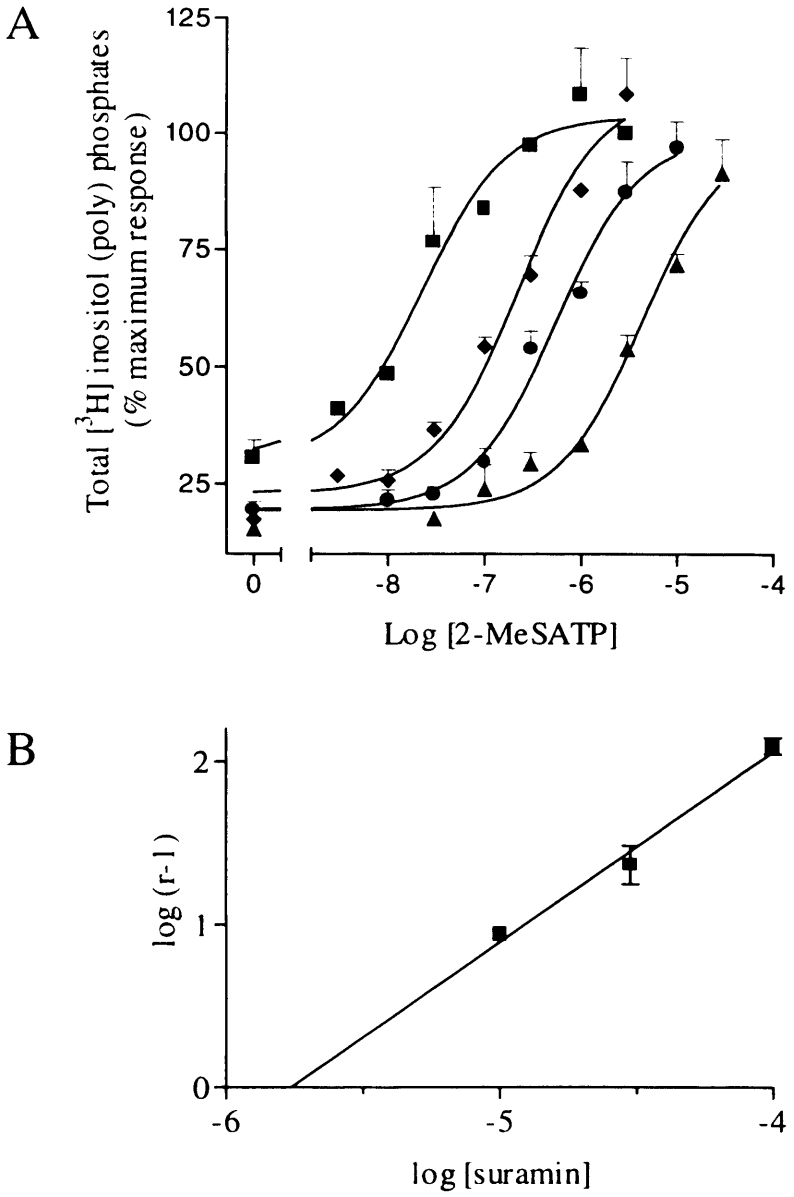


Figure 4.1

The effect of suramin on concentration-response curves to 2MeSATP on turkey $P2Y_1$ 1321N1 cells.

(**A**) Agonist concentration-response curves to 2MeSATP were constructed in either the absence (■) or presence of 10 μM (◆), 30 μM (●) and 100 μM (▲) suramin. Suramin was preincubated with the cells for 10 minutes prior to agonist stimulation. Data are the mean ± SEM of three triplicate experiments and are expressed as a percentage of the response to 3 μM 2MeSATP alone. (**B**) Schild analysis of the data presented in **A**. r, the dose ratio was calculated from the ratio of the EC_{50} value for each curve with suramin against the control curve, using the method of Arunlakshana and Schild (1959).

2MeSATP on the turkey $P2Y_1$ receptors. Suramin was included in the 10 minute BSS + LiCl (10mM final concentration) pre-incubation at 11× final concentration prior to the addition of 2MeSATP (0.003 – 30 μM) for 15 minutes. Suramin at concentrations of 10, 30 and 100 μM gave parallel rightward shifts of the concentration-response curve to 2MeSATP (Figure 4.1A). Schild analysis of these data generated a straight line with a slope of 1.16 ± 0.08, which was not significantly different from unity (Figure 4.1B). The data in figure 4.1 are pooled from 3 separate experiments; when each experiment was analysed separately the Schild plots gave pA_2 values of 5.77 ± 0.11.

4.2.2 Human $P2Y_2$

When the same analysis was undertaken for the human $P2Y_2$ receptor, the effect of suramin was much less marked (Figure 4.2A). Suramin at 30 μM had no effect on the position of the concentration response curve to UTP, and the rightward shift of the EC_{50} with 100 μM suramin was 4.6 ± 0.7 fold compared to 128.8 ± 15.5 for the turkey $P2Y_1$ cells (significantly different at $P < 0.001$). As expected from these data, the resulting Schild plot was less satisfactory (Figure 4.2B), giving an apparent pA_2 value of 4.32 ± 0.13 (from 3 separate experiments), but with a slope of 1.57 ± 0.19.

4.2.3 1 hour suramin incubations

The previous experiments were carried out with an antagonist preincubation time of 10 min. However, suramin is known to be a slowly equilibrating antagonist (Leff et al, 1991) and so these experiments were repeated using 1 hr incubations with suramin. The results obtained with this longer incubation time were essentially the same as the 10 min incubations. Figure 4.3A shows results obtained for the turkey $P2Y_1$ using 1 hour suramin preincubations. The concentration-response curve to 2MeSATP was shifted to the right in a concentration-dependant manner consistent with competitive antagonism. The Schild plot generated from these data gives a pA_2 value of 5.76 (n=1), compared to 5.77 ± 0.11 with 10 min preincubations (Figure

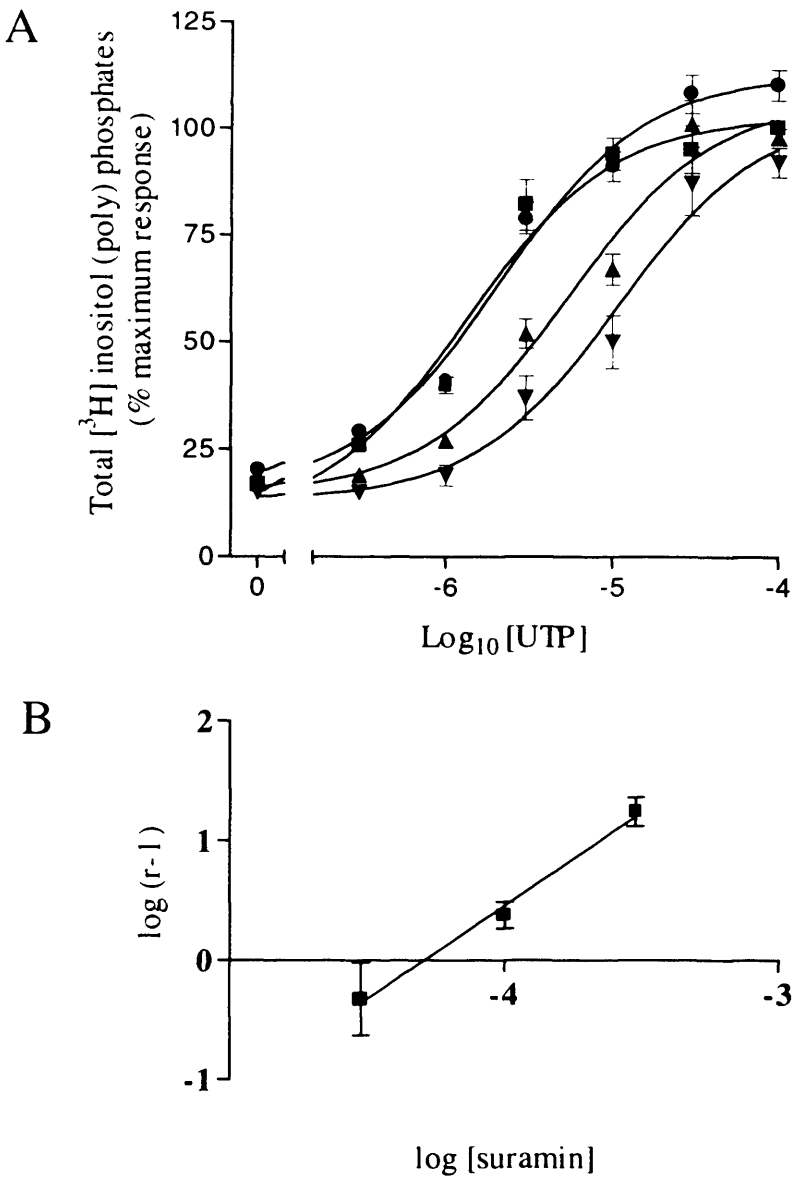


Figure 4.2

The effect of suramin on concentration-response curves to UTP on human $P2Y_2$ 1321N1 cells.

(**A**) Agonist concentration-response curves to UTP were constructed in either the absence (■) or presence of 30 μM (●), 100 μM (▲) and 300 μM (▼) suramin. Suramin was preincubated with the cells for 10 minutes prior to agonist stimulation. Each curve is the mean ± SEM of three triplicate experiments and are expressed as a percentage of the response to 100 μM UTP alone. (**B**) Schild analysis of the data presented in **A**. r, the dose ratio was calculated from the ratio of the EC_{50} value for each curve with suramin against the control curve, using the method of Arunlakshana and Schild (1959).

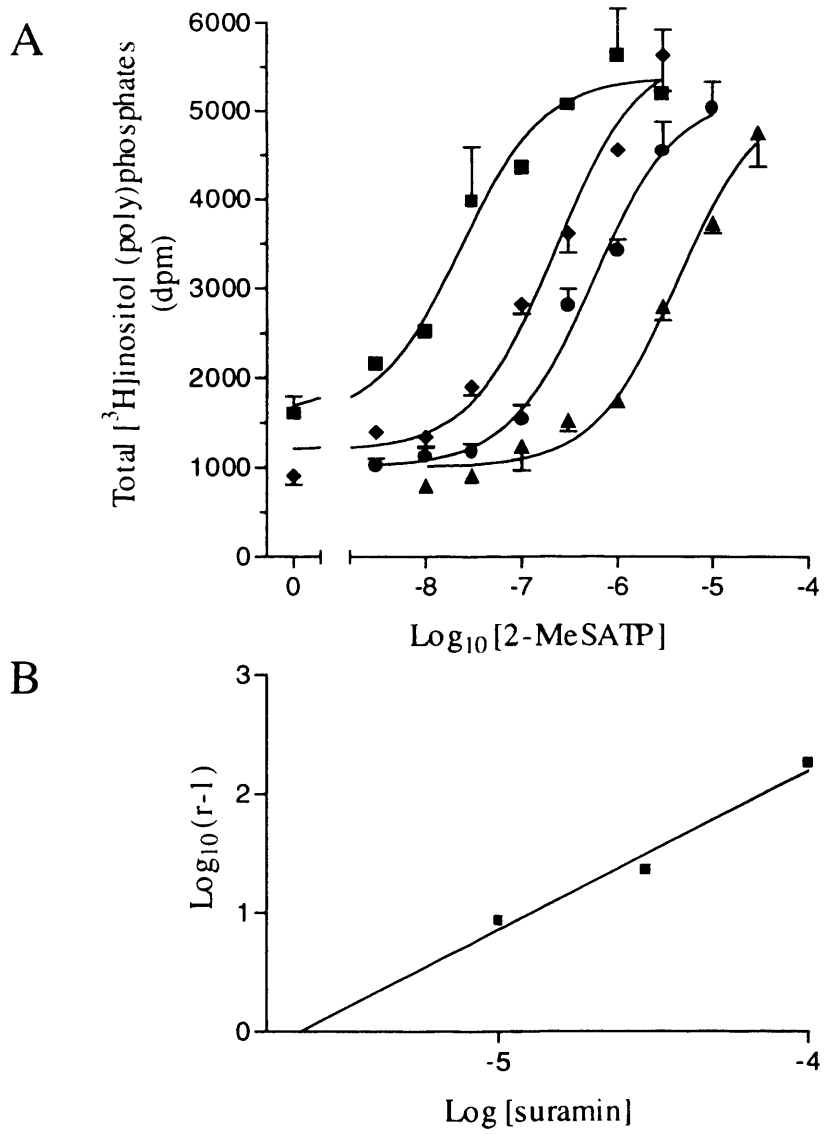


Figure 4.3

The effect of 1 hour preincubations with suramin on concentration-response curves to 2MeSATP on turkey $P2Y_1$ 1321N1 cells.

(**A**) Agonist concentration-response curves to 2MeSATP were constructed in either the absence (■) or presence of 10 μM (◆), 30 μM (●) and 100 μM (▲) suramin. Suramin was preincubated with the cells for 1 hour prior to agonist stimulation. Data are the mean ± SEM of one triplicate experiments and are expressed as a percentage of the response to 3 μM 2MeSATP alone. (**B**) Schild analysis of the data presented in **A.** r, the dose ratio was calculated from the ratio of the EC_{50} value for each curve with suramin against the control curve, using the method of Arunlakshana and Schild (1959).

4.3B). Similarly, results on the human $P2Y_2$ cells showed no significant difference between the two incubation times (figure 4.4A). However, the concentration-response curve to UTP in the presence of 100 μM suramin failed to reach the maximal response seen in the absence of the antagonist. The subsequent pA_2 of 4.20 was calculated (compared to 4.32 with a 10 minute incubation time) on the assumption that this curve would reach the same maximum as the others if higher concentrations of UTP were used (Figure 4.4B). As there was no significant difference in the effect of suramin with longer preincubations periods, subsequent experiments were performed using 10 minute preincubations.

4.2.4 ARL 67156

The previous results show that suramin has some selectivity for the turkey $P2Y_1$ over the human $P2Y_2$ receptor. However, as discussed before, nucleotide agonists such as 2MeSATP and UTP are subject to degradation by ectonucleotidases, and suramin is known to be an ectonucleotidase inhibitor (Hourani & Chown, 1989). It is therefore possible that apparent effects of suramin as an antagonist are influenced by its action as an inhibitor. Differential effects of ectonucleotidase breakdown on the two agonists used here could therefore lead to an erroneous estimate of the selectivity of suramin as an antagonist. The effects of ectonucleotidase breakdown on the concentration response curves in the absence and presence of suramin were investigated using the ectonucleotidase inhibitor ARL 67156 (Crack et al, 1995).

ARL 67156 was included with the LiCl incubation, 10 minutes prior to addition of agonist. Figure 4.5A shows that the presence of ARL 67156 produced an elevation of the concentration-response curve to 2MeSATP on the turkey $P2Y_1$ cells, and a small, insignificant shift of this curve to the left (-log EC_{50} of 7.55 ± 0.17 in the absence, and 8.08 ± 0.19 in the presence of 100 μM ARL 67156). In the presence of suramin, the concentration-response curves in the absence and presence of ARL 67156 were even closer (-log EC_{50} values of 5.53 ± 0.01 and 5.79 ± 0.17 respectively; not significantly different). Suramin generated the expected shift of the concentration-response curve to 2MeSATP to the right. In the absence of ARL 67156 the pA_2 values for suramin were 6.06 ± 0.40, and in the presence of ARL 67156 the apparent pA_2

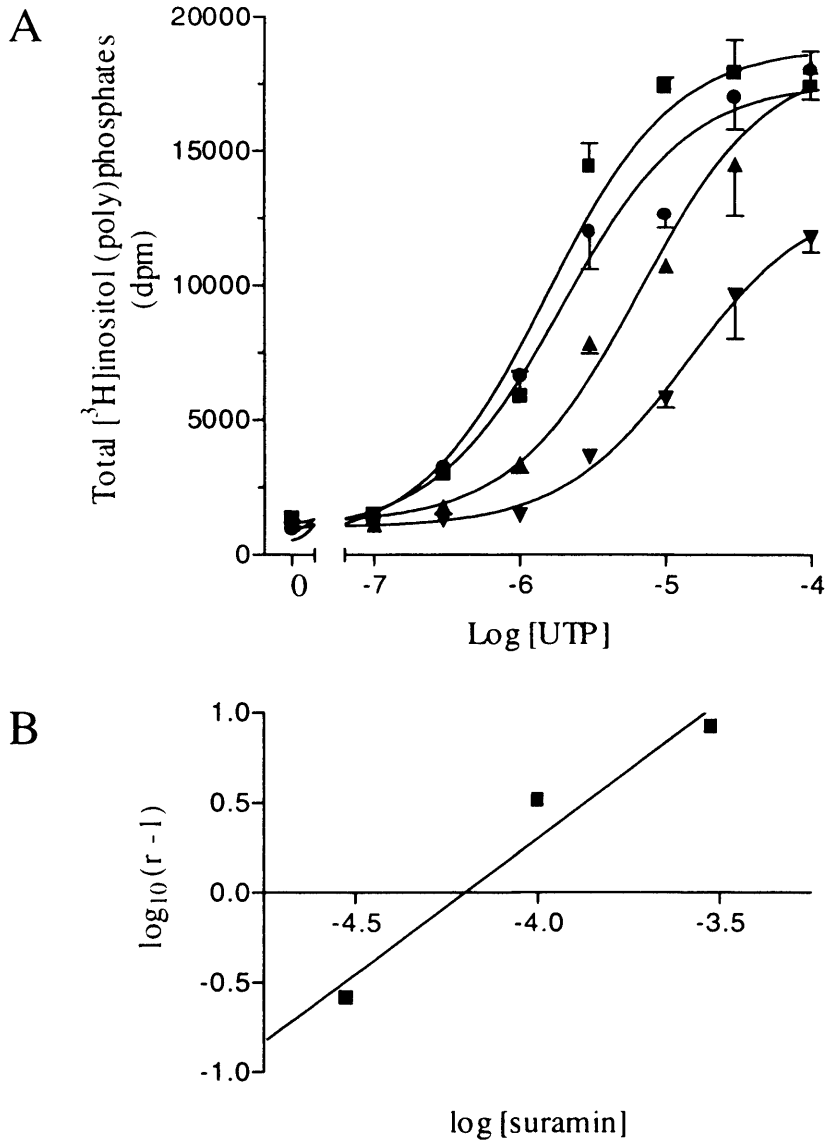


Figure 4.4

The effect of 1 hour preincubations with suramin on concentration-response curves to UTP on human P2Y2 1321N1 cells.

(**A**) Agonist concentration-response curves to UTP were constructed in either the absence (■) or presence of 30 μM (●), 100 μM (▲) and 300 μM (▼) suramin. Suramin was preincubated with the cells for 1 hour prior to agonist stimulation. Each curve is the mean ± SEM of three triplicate experiments and are expressed as a percentage of the response to 100 μM UTP alone. (**B**) Schild analysis of the data presented in **A**. r, the dose ratio was calculated from the ratio of the EC_{50} value for each curve with suramin against the control curve, using the method of Arunlakshana and Schild (1959).

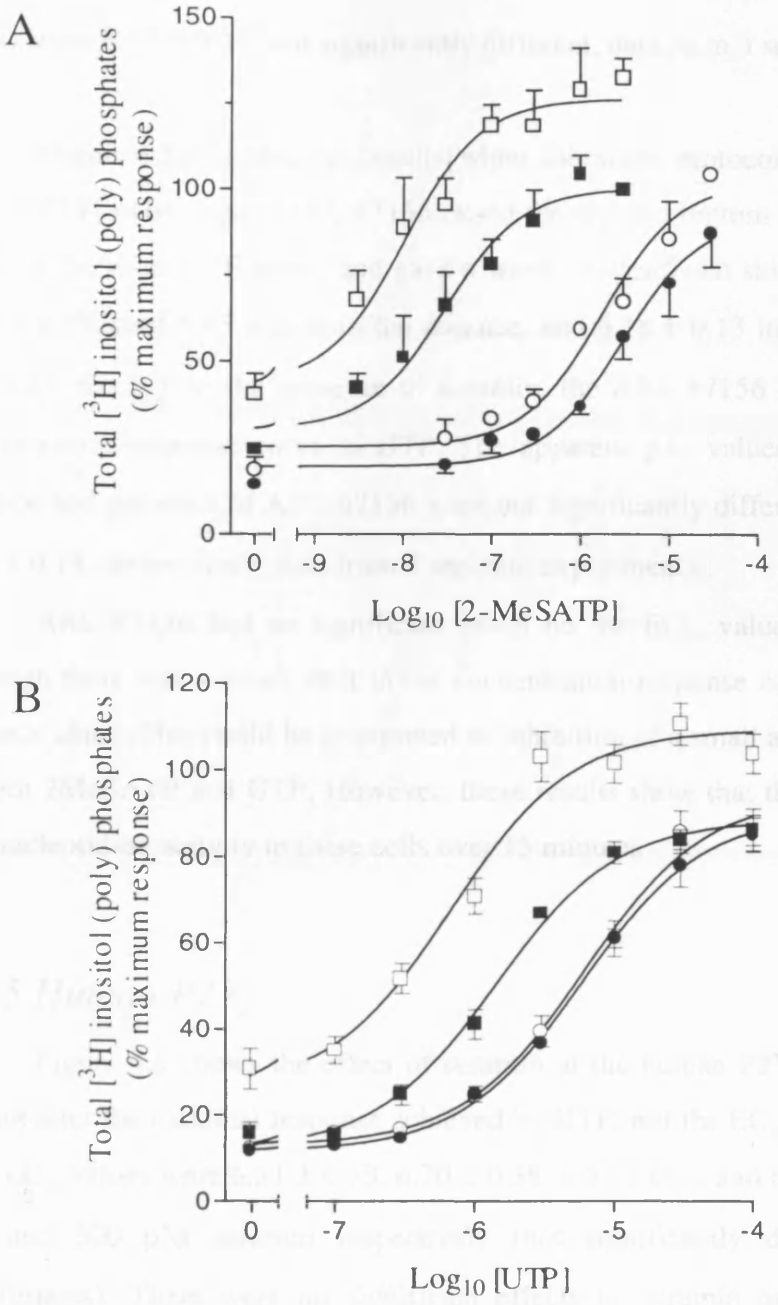


Figure 4.5

The effect of ARL 67156 on concentration response curves to 2MeSATP in the absence and presence of suramin on turkey $P2Y_1$ and human $P2Y_2$ 1321N1 cells.

Concentration response curves were constructed to 2MeSATP alone (■), 2MeSATP with ARL 67156 (❑), 2MeSATP with suramin (●) and 2MeSATP with both ARL 67156 and suramin (○). Suramin (100 μM) and ectonucleotidase inhibitor ARL 67156 (100 μM) were preincubated with the cells for 10 minutes prior to agonist stimulation. Data are pooled from three separate experiments each in triplicate and are expressed as mean ± SEM of the percentage of the response to 3 μM 2MeSATP alone.

values were 6.53 ± 0.27 (not significantly different, data from 3 separate experiments).

Figure 4.5B shows the results when the same protocol was applied to the human $P2Y_2$ cells. Again ARL 67156 raised the top and bottom of the concentration-response curve to UTP alone, and gave a small, insignificant shift of the curve to the left (-log EC_{50} of 5.85 ± 0.06 in the absence, and 6.18 ± 0.13 in the presence of 100 μM ARL 67156). In the presence of suramin, the ARL 67156 had no effect on the concentration-response curve to UTP. The apparent pA_2 values for suramin in the absence and presence of ARL 67156 were not significantly different (4.91 ± 0.16 and 4.72 ± 0.14, respectively; data from 3 separate experiments).

ARL 67156 had no significant effect on the EC_{50} values at either receptor, although there was a small shift in the concentration-response curves to the left with agonists alone. This could be interpreted as inhibition of a small amount of breakdown of both 2MeSATP and UTP. However, these results show that there is no significant ectonucleotidase activity in these cells over 15 minutes.

4.2.5 Human $P2Y_4$

Figure 4.6 shows the effect of suramin at the human $P2Y_4$ receptor. Suramin did not alter the maximal response achieved by UTP, nor the EC_{50} values of the curve. -log EC_{50} values were 6.21 ± 0.13, 6.70 ± 0.38, 6.63 ± 0.16 and 6.58 ± 0.06 for 0, 30, 100 and 300 μM suramin respectively (not significantly different, 3 separate experiments). There were no significant effects of suramin on the concentration-response curve to UTP, as determined by analysis of variance and Dunnett's multiple range post hoc test.

4.2.6 Bovine $P2Y_1$

Figure 4.7A shows the effect of suramin (10–100 μM) at 1321N1 cells transfected with the bovine $P2Y_1$ receptor. Suramin at concentrations of 10 and 30 μM caused rightward shifts of the curves to 2MeSATP, which maintained near maximal responses in each case. EC_{50} values were 80.5 nM and 306.2 nM for 10 and 30 μM

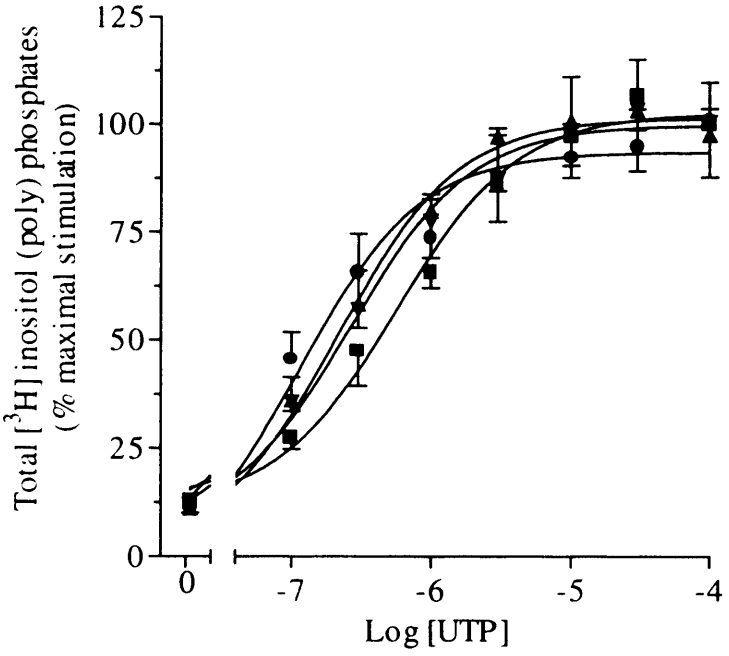


Figure 4.6

The effect of suramin on concentration-response curves to UTP on human $P2Y_4$ 1321N1 cells.

Agonist concentration-response curves to UTP were constructed in either the absence (■) or presence of 30 μM (●), 100 μM (▲) and 300 μM (▼) suramin. Suramin was preincubated with the cells for 10 minutes prior to agonist stimulation. Each curve is the mean ± SEM of three triplicate experiments and are expressed as a percentage of the response to 100 μM UTP alone.

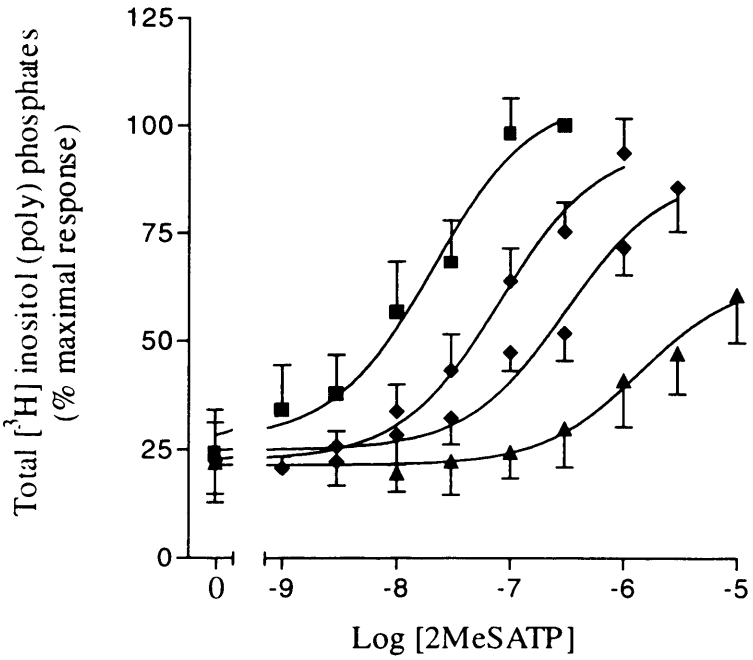


Figure 4.7

The effect of suramin on concentration-response curves to 2MeSATP on bovine $P2Y_1$ 1321N1 cells.

Agonist concentration-response curves to 2MeSATP were constructed in either the absence (■) or presence of 10 μM (◆), 30 μM (●) and 100 μM (▲) suramin. Suramin was preincubated with the cells for 10 minutes prior to agonist stimulation. Each curve is the mean ± SEM of three triplicate experiments and are expressed as a percentage of the response to 3 μM 2MeSATP alone.

respectively, compared to 20.8 nM for control. However, preincubation with 100 μM suramin resulted in a flatter curve that attained only 64.7 % of the maximal response to 2MeSATP in control conditions. Thus, a Schild plot could not be constructed, so estimated pA_2 values of -5.48 and -5.91 were derived from the curves with 10 and 30 μM suramin, respectively (Furchgott, 1972).

4.3 Pyridoxal-phosphate-6-azophenyl-2', 4'-disulphonic acid (PPADS)

PPADS was originally described as a P2X selective antagonist (Lambrecht et al, 1992; Ziganshin et al, 1993; Windscheif et al, 1994), but has since been reported as an effective antagonist at bovine aortic endothelial $P2Y_1$, but not $P2Y_2$ receptors (Brown *et al.*, 1995). The effect of this antagonist was investigated in each of the 4 receptor systems.

4.3.1 Turkey $P2Y_1$

Figure 4.8 shows the influence of 3–30 μM PPADS on the response of the turkey $P2Y_1$ cells to 2MeSATP. PPADS shifted the curves to the right in a concentration-dependant manner. For example, in one typical experiment the EC_{50} in the absence of PPADS was 24 nM, which shifted to 31, 85 and 260 nM with 3, 10 and 100 μM PPADS respectively. Schild analysis gave data consistent with a straight line, with apparent pA_2 values of 5.98 ± 0.65, but with a slope of 0.55 ± 0.15 (Figure 4.8B). Examination of the data shows that there was a tendency for the presence of PPADS to cause a reduction in both the basal levels of $[^3H]IP_X$, and those achieved by 2MeSATP. This reduction in maximal response contributed to the shift in EC_{50} values, reflected by the Schild plot with a slope of less than unity. These observations indicate that the effect of PPADS was not due to classical competitive antagonism.

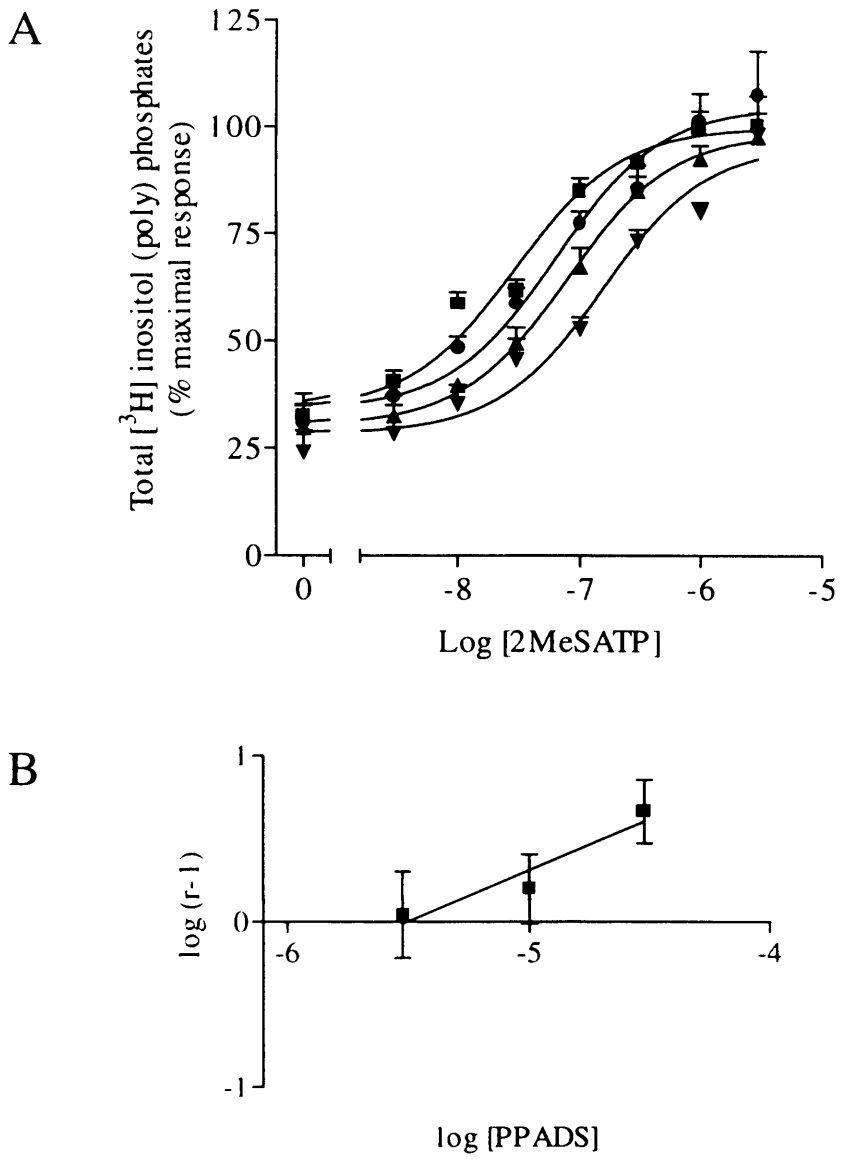


Figure 4.8

The effect of PPADS on concentration-response curves to 2MeSATP on turkey $P2Y_1$ 1321N1 cells.

(**A**) Agonist concentration-response curves to 2MeSATP were constructed in either the absence (■) or presence of 3 μM (●), 10 μM (▲) and 30 μM (▼) PPADS. PPADS was preincubated with the cells for 10 minutes prior to agonist stimulation. Each curve is the mean ± SEM of three triplicate experiments and are expressed as a percentage of the response to 3 μM 2MeSATP alone. (**B**) Schild analysis of the data presented in **A**. r, the dose ratio was calculated from the ratio of the EC_{50} value for each curve with PPADS against the control curve, using the method of Arunlakshana and Schild (1959).

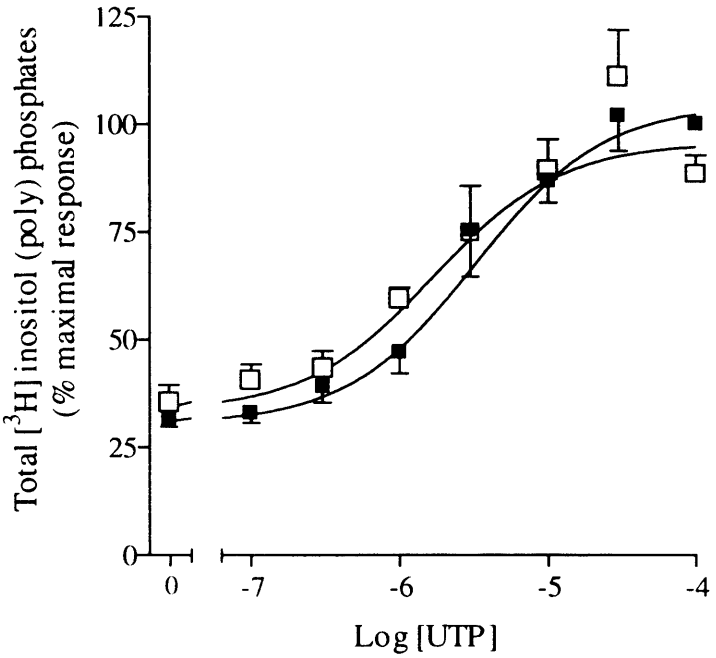


Figure 4.9

The effect of PPADS on concentration-response curves to UTP on human $P2Y_2$ 1321N1 cells.

Agonist concentration-response curves to UTP were constructed in either the absence (■) or presence of 30 μM PPADS (❑). PPADS was preincubated with the cells for 10 minutes prior to agonist stimulation. Data are the mean ± SEM of three triplicate experiments and are expressed as a percentage of the response to 100 μM UTP alone.

4.3.2 Human $P2Y_2$

In contrast with the turkey $P2Y_1$ receptor, there was no antagonistic effect of PPADS at the human $P2Y_2$ receptor (Figure 4.9). The concentration-response curves to UTP in the absence and presence of PPADS (30 μM) were not significantly different by analysis of variance.

4.3.3 Human $P2Y_4$

Figure 4.10 examines the effect of PPADS on the human $P2Y_4$ receptor. Pooled across 3 separate experiments the -log EC_{50} values for UTP were 6.15 ± 0.005 in the absence, and 6.82 ± 0.17 in the presence of 30 μM PPADS ($P < 0.05$; Student's paired *t* test), showing that PPADS produced a slight shift of the curve to the left (Figure 4.10A). There was also a small effect on the maximal response (to 100 μM UTP), which was reduced to 82.5 % by 30 μM PPADS ($P < 0.025$; Student's paired *t* test). This reduction in maximal response is consistent with the effect of increasing concentrations of PPADS (0.3 – 100 μM) on the response to 10 μM UTP (Figure 4.10B).

4.3.4 Bovine $P2Y_1$

Figure 4.11 shows the effect of PPADS on the bovine $P2Y_1$ receptor. PPADS was used at the higher concentration range of 10–100 μM in an attempt to achieve a more satisfactory Schild plot than that obtained for the turkey $P2Y_1$ receptor. However, the results obtained were less satisfactory, with none of the curves with PPADS reaching the maximal response achieved by 2MeSATP alone. Although the curve with 10 μM PPADS appeared to be parallel with the control, the slope of the curves with 30 and 100 μM PPADS were flattened. In addition, the concentration-response to 2MeSATP with 100 μM PPADS appeared to be reaching a plateau at approximately 70 % of the response to 2MeSATP alone.

These results clearly dismiss the notion that PPADS is a competitive antagonist at $P2Y_1$ receptors (see Boyer et al, 1994; Ralevic & Burnstock 1996). Several possible explanations are considered in the discussion section of this chapter.

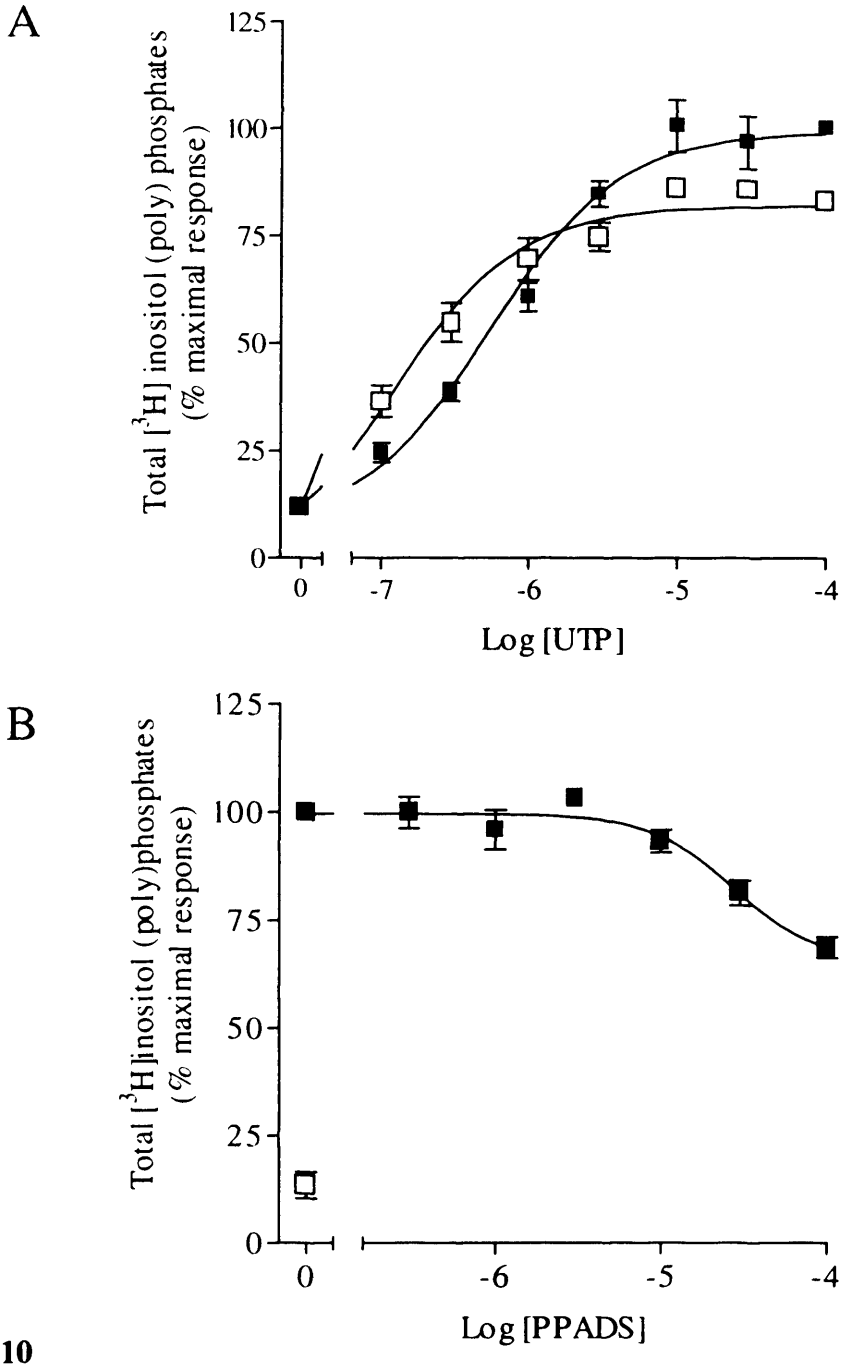


Figure 4.10

The effect of PPADS on concentration-response curves to UTP on human $P2Y_4$ 1321N1 cells.

(**A**) Agonist concentration-response curves to 2MeSATP were constructed in either the absence (■) or presence of 100 μM PPADS (❑). PPADS was preincubated with the cells for 10 minutes prior to agonist stimulation. Data presented are the mean ± SEM of three triplicate experiments and are expressed as a percentage of the response to 100 μM UTP alone. (**B**) The response to 10 μM UTP in the presence of increasing concentrations of PPADS (■). Data from no UTP and no PPADS control are also shown (❑). Data are pooled from three separate experiments and expressed as a percentage of the response to 10 μM UTP.

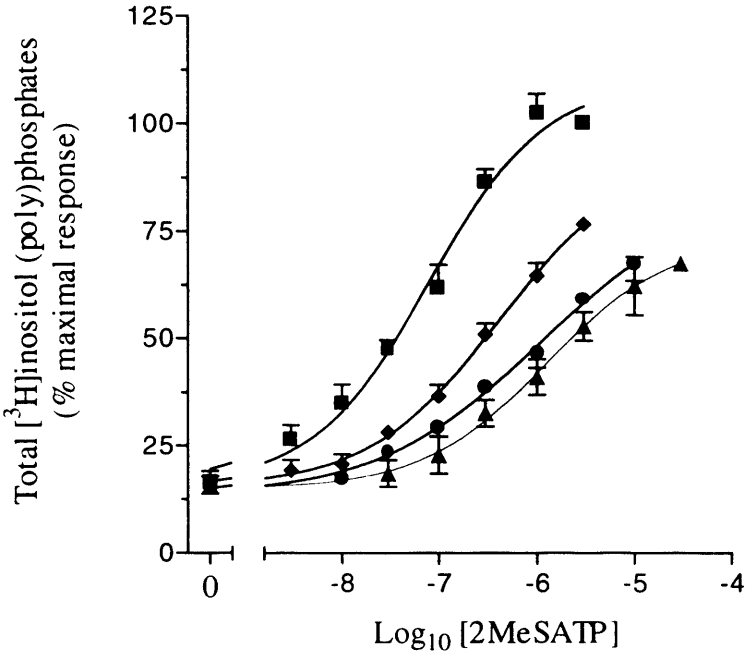


Figure 4.11

The effect of PPADS on concentration-response curves to 2MeSATP on bovine $P2Y_1$ 1321N1 cells.

Agonist concentration-response curves to 2MeSATP were constructed in either the absence (■) or presence of 10 μM (◆), 30 μM (●) and 100 μM (▲) PPADS. PPADS was preincubated with the cells for 10 minutes prior to agonist stimulation. Data are the mean ± SEM of three triplicate experiments and are expressed as a percentage of the response to 3 μM 2MeSATP alone.

4.4 NF023

NF023 (8,8' – (carboxylbis (imino-3,1-phenylene)) bis – (1,3,5-napthalentrisulfonic acid), a novel suramin analogue, is the symmetrical 3'-urea of 8-(benzamido) napthalene-1,3,5-trisulphonic acid. It was introduced as a specific, directly interacting, competitive P2 receptor antagonist, being highly selective for P2X subtypes (pA_2 value of 5.96 ± 0.04) over $P2Y_1$ and $P2Y_2$ receptors (Ziyal et al, 1994, 1995; Lambrecht et al, 1996). The effect of NF023 on responses at the cloned turkey $P2Y_1$, human $P2Y_2$ and human $P2Y_4$ receptors was examined to determine whether NF023 is an antagonist at P2Y receptors.

4.4.1 Turkey $P2Y_1$

NF023 at concentrations of 10, 30 and 100 μM produced rightward shifts in the concentration-response curve to 2MeSATP, although the curve with 100 μM NF023 did not achieve the same maximum response as to 2MeSATP alone (Figure 4.12A). EC_{50} values were 0.054 μM for 2MeSATP alone, 0.15 μM for 10 μM NF023 and 0.30 μM for 30 μM NF023. When the curve with 100 μM NF023 was projected to reach the same maximal response as the control curve, the EC_{50} value was 0.59 μM. A Schild plot using this projected value generated a straight line with a slope of 0.78 ± 0.02, and an estimated pA_2 value of 5.33 (Figure 4.12B).

4.4.2 Human $P2Y_2$

Figure 4.13 shows concentration-response curves to UTP (0.1–100 μM) in the absence and presence of NF023 (10 – 100 μM). NF023 caused a small, dose-independent shift of the curve to the left, which was not significant by analysis of variance. EC_{50} values with 10, 30 and 100 μM NF023 were 0.70 ± 0.06, 0.44 ± 0.09

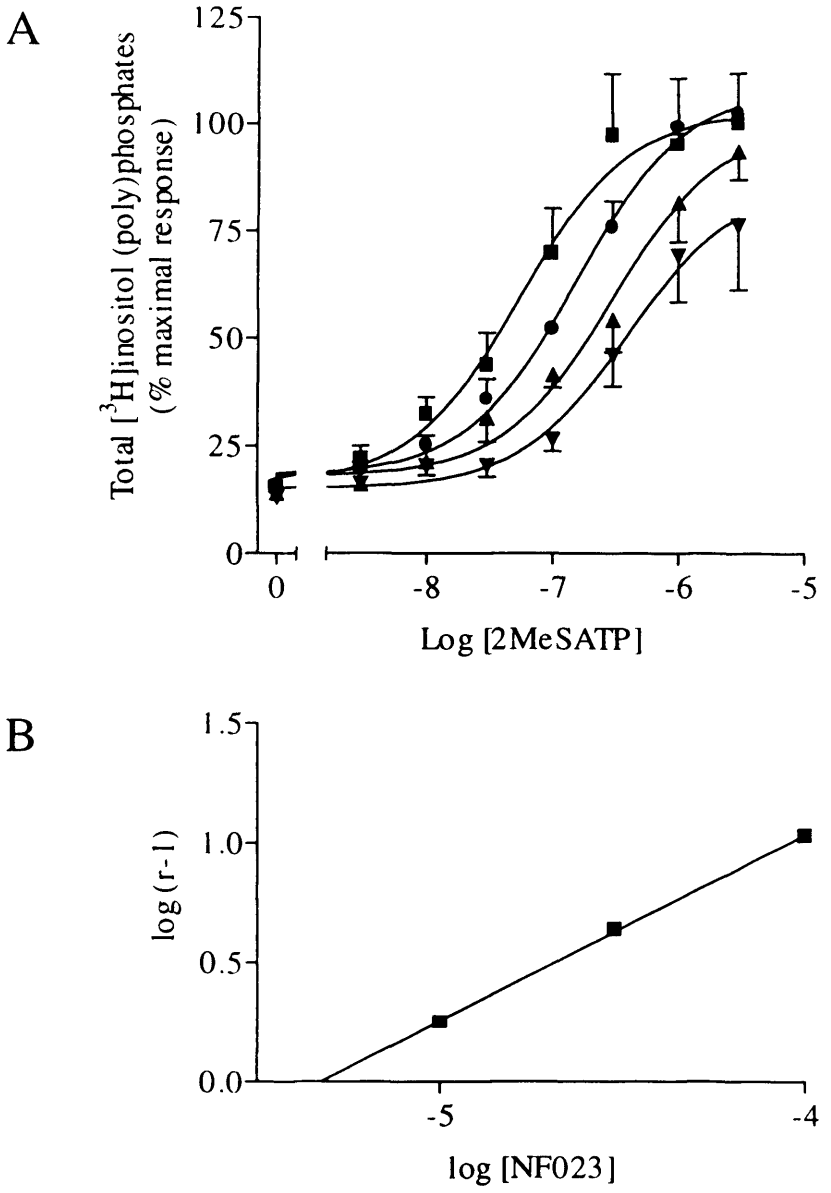


Figure 4.12

The effect of NF023 on concentration-response curves to 2MeSATP on turkey $P2Y_1$ 1321N1 cells.

Agonist concentration-response curves to 2MeSATP were constructed in either the absence (■) or presence of 10 μM (●), 30 μM (▲) and 100 μM (▼) NF023. NF023 was preincubated with the cells for 10 minutes prior to agonist stimulation. Data are the mean ± SEM of three triplicate experiments and are expressed as a percentage of the response to 3 μM 2MeSATP alone. (**B**) Schild analysis of the data presented in **A**. r, the dose ratio was calculated from the ratio of the EC_{50} value for each curve with NF023 against the control curve, using the method of Arunlakshana and Schild (1959).

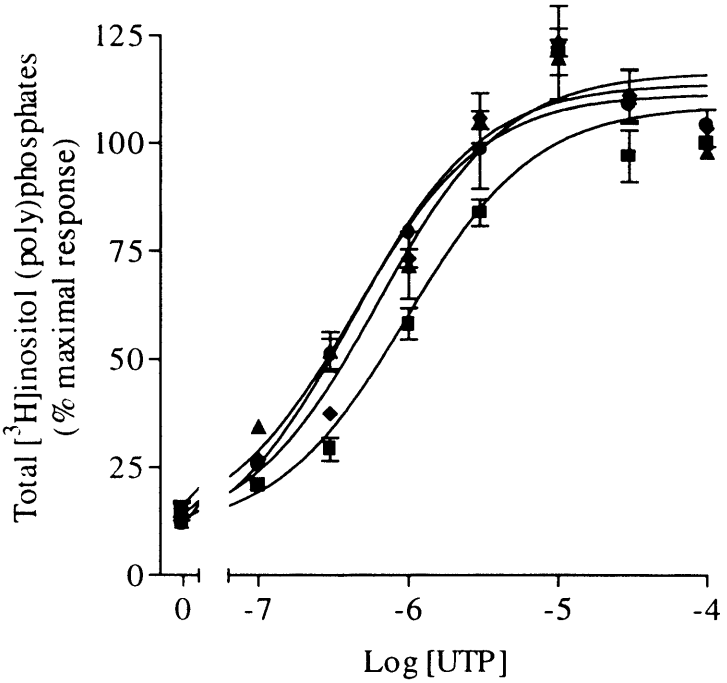


Figure 4.13

The effect of NF023 on concentration-response curves to UTP on human $P2Y_2$ 1321N1 cells.

Agonist concentration-response curves to UTP were constructed in either the absence (■) or presence of 10 μM (◆), 30 μM (●) and 100 μM (▲) NF023. NF023 was preincubated with the cells for 10 minutes prior to agonist stimulation. Data are the mean ± SEM of three triplicate experiments and are expressed as a percentage of the response to 100 μM UTP alone.

and 0.50 ± 0.19 μM, which were not significantly different from 1.32 ± 0.08 μM with UTP alone.

3.4.3 Human $P2Y_4$

Figure 4.14 shows the effect of NF023 (100 μM) on the concentration-response curve to UTP on the human $P2Y_4$ cells. NF023 caused a leftward shift in the curve to UTP, with EC_{50} values of 1.42 ± 0.46 μM in the absence and 0.315 ± 0.15 μM in the presence of NF023, which were statistically significant to the $P<0.001$ level (Student's paired t test; GraphPad Prism).

4.5 ATP as an antagonist at the human $P2Y_4$ receptor

As discussed previously (section 3.54), ATP acts as a partial agonist at the human $P2Y_4$ receptor and should therefore antagonise responses seen to a full agonist. Figure 4.15 shows the effect of increasing concentrations of ATP (30–1000 μM) on the response to 10 μM UTP. In this experiment, ATP was not pre-incubated with the cells prior to the addition of UTP, in order to avoid any possible desensitisation of the receptor. Although this experimental design did not allow ATP to reach equilibrium with the receptor before addition of agonist, there was a clear reduction in the response to UTP at higher ATP concentrations. ATP at concentrations of 100, 300 and 1000 μM caused 16.8, 25.1 and 34.7 % reductions in response to 10 μM UTP respectively ($P<0.01$ by analysis of variance and Dunnett's post test; 3 separate experiments).

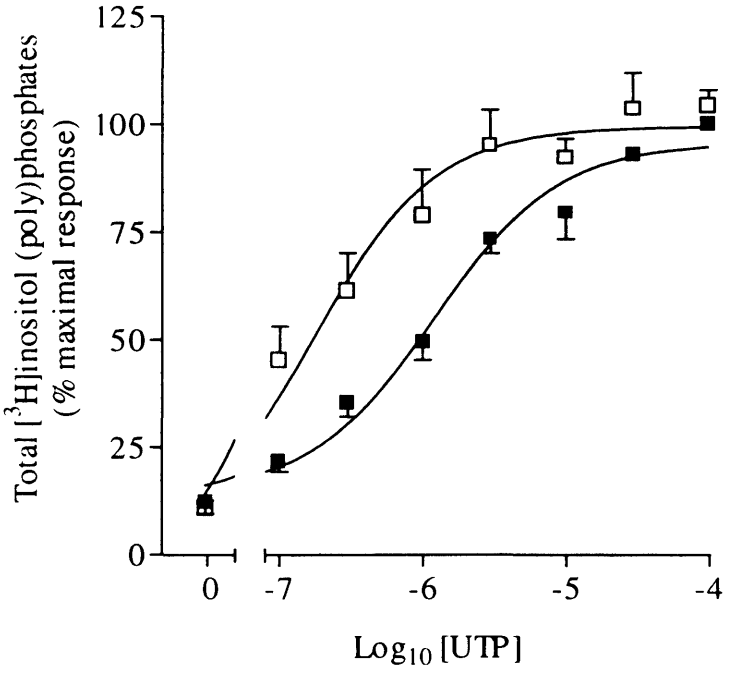


Figure 4.14

The effect of NF023 on concentration-response curves to UTP on human $P2Y_4$ 1321N1 cells.

Agonist concentration-response curves to UTP were constructed in either the absence (■) or presence of 100 μM NF023 (❑). NF023 was preincubated with the cells for 10 minutes prior to agonist stimulation. Data are the mean ± SEM of three triplicate experiments and are expressed as a percentage of the response to 100 μM UTP alone.

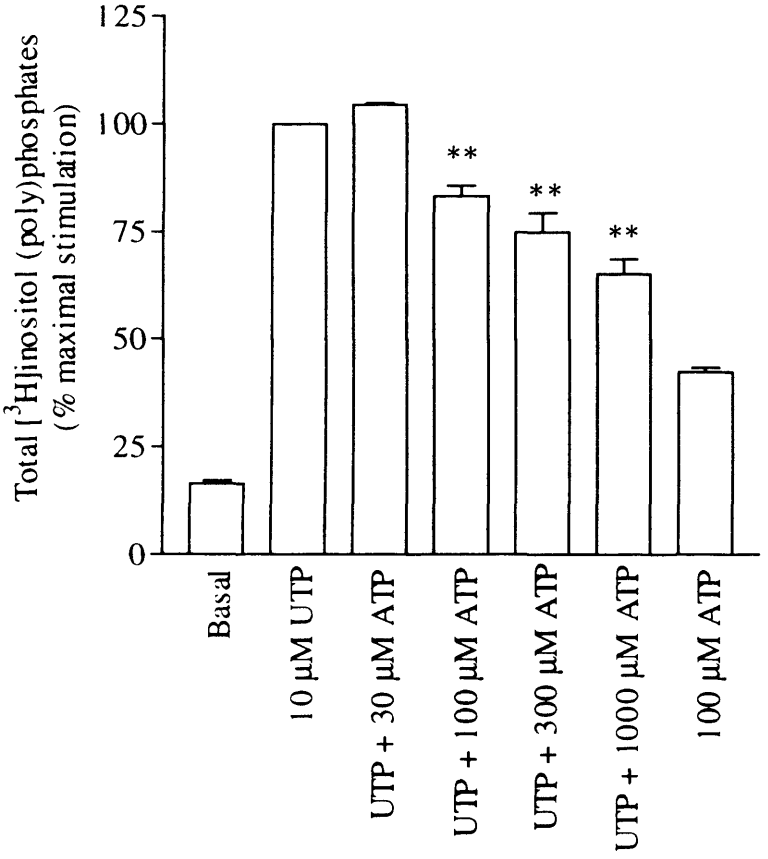


Figure 4.15

Antagonism of the response to UTP on human $P2Y_4$ 1321N1 cells by ATP.

Cells were stimulated for 15 minutes with 10 μM UTP in the absence or presence of increasing concentrations of ATP (1-1000 μM). A stimulation to 100 μM ATP (maximal concentration) was included as a reference. Data are the mean ± SEM of three separate experiments, each in triplicate. Where indicated, values were significantly different from 10 μM UTP control at the $P<0.01$ level, as determined by analysis of variance with Dunnet's post test.

4.6 Discussion

4.6.1 Suramin

The trypanocidyl drug suramin has long been used as a P2 antagonist. There are, however, many inconsistencies in the literature pertaining to the effect of suramin at the different P2 receptor subtypes. For example, the existence of $P2Y_2$ subtypes has been suggested on the basis of differential sensitivity to suramin.

4.6.1.1 Multiple $P2Y_2$ receptors?

Stimulation of the $P2Y_2$ receptor on PC12 rat phaechromocytoma cells is competitively inhibited by suramin (Murrin & Boarder, 1992), whilst in bovine aortic endothelial cells, responses to UTP at $P2Y_2$ receptors are not (Wilkinson et al, 1993). Also, Dainty et al (1994) have reported suramin sensitive $P2Y_2$ receptors on rat aortic rings, and suramin insensitive $P2Y_2$ receptors in canine tracheal epithelium.

The results presented in this chapter show that the cloned human $P2Y_2$ receptor is mildly sensitive to suramin, and it is possible that the differential effects of suramin observed in previous studies may have been due to different ectonucleotidase activity in the different tissues. For instance, in a tissue where ectonucleotidase activity is low, observed responses at a suramin sensitive receptor would be antagonised by suramin. However, if the tissue had high ectonucleotidase activity, then the antagonism by suramin would be 'cancelled out' by the inhibition of nucleotide breakdown.

Also, a suramin insensitive response to UTP in rabbit isolated aorta lead Chinellato et al (1992) to conclude that a P2 receptor was not involved, but that a novel class of receptors (which they named P_3 purinoceptors) was mediating the response. However, the results obtained in this thesis show that suramin insensitive responses to UTP can be observed at $P2Y_4$ receptors. It is probable that many more of the suramin insensitive responses to UTP which were originally attributed to action at $P2Y_2$ receptors, will turn out to be at $P2Y_4$ receptors.

4.6.1.2 G protein uncoupling?

There are, however, several important issues that must be addressed when assessing the effect of suramin at these receptors. Suramin has been shown to suppress [^{35}S]GTPγS binding to purified, recombinant G protein α subunits (selective for $G_{\alpha s}$), an effect that is due to inhibition of GDP release (Freissmuth et al, 1996). In their study, it was found that suramin did not interfere with the interaction between α subunits and G protein βγ dimers but competed with binding of the G protein heterotrimer to the receptor in a competitive manner. It is therefore possible that the antagonism to suramin observed in this chapter was actually due to the non-specific effects at the G protein level.

The results presented in this chapter will be carefully considered with respect to the following hypothesis; *in this study, suramin acted at the G protein level to affect the responses to agonists*. There are several arguements both for and against this statement.

Firstly, suramin was shown to specifically inhibit G_s coupling, and none of the receptors have been reported to couple to G_s. Secondly, the study described above (Freissmuth et al, 1996) was performed in isolated plasma membranes, where suramin would have full access to the G proteins. However, suramin is a large, highly charged molecule, and would be unlikely to cross the plasma membrane when used with whole cells. Thirdly, suramin acts as a competitive antagonist at P2X receptors with a similar pA_2 as that observed at $P2Y_1$ receptors. P2X receptors are not coupled to G proteins, demonstrating the ability of suramin to directly compete with ATP binding to a receptor. These points suggest that in the context of this thesis, suramin is likely to be acting as an antagonist of nucleotide binding.

However, if suramin were acting in a non-specific manner at the G protein level, it would be expected that the maximal response to agonists would be reduced, whilst similar EC_{50} values would be observed. While this did not occur for the turkey $P2Y_1$ or the human $P2Y_2$ receptors, 100 μM suramin reduced the maximal response observed to 2MeSATP at the bovine $P2Y_1$ receptor. This indicates that antagonism of the bovine $P2Y_1$ receptor was uncompetitive.

In a situation where receptor levels were higher in the turkey $P2Y_1$ cells than the bovine $P2Y_1$ cells (discused in Chapter 3), the action of suramin at the G protein level could explain the different modes of antagonism observed. If suramin were acting to "knock-out" receptor-G protein complexes, the response at cells with a high receptor reserve would shift to the right in a parallel fashion. This observeration would be consistent with competitive antagonism observed with the $P2Y_1$ turkey cells.

However, in cells with a low receptor reserve, the concentration response curves to agonist in the presence of increasing suramin concentrations would be expected to behave differently. The curves would shift to the right as the 'spare' receptors were 'knocked out', but at the point where the agonist concentration response curve was superimposable upon the agonist occupation curve, the concentration response curve would stop shifting and there would be no further change in the EC_{50} value. Instead, increasing concentrations of suramin would cause a reduction in the maximal response. This rightward shift and then collapse of the concentration response curve was observed with the bovine $P2Y_1$ cells.

While the implications of this differential mode of antagonism are concerning, a more detailed examination of the data show that this explanation for suramin antagonism is not adequate. It is apparent that in the presence of 100 μM suramin, 10 μM 2MeSATP is capable of eliciting the maximal response in the turkey $P2Y_1$ cells, whilst in the bovine $P2Y_1$ cells, only 50 % of the maximal response is achieved in the same conditions. This would only be expected if the occupation curves for 2MeSATP were different for the turkey $P2Y_1$ and the bovine $P2Y_1$ receptors, and it therefore seems that differences in receptor number were not responsible for the differential effect of suramin.

Thus, it appears that suramin acts as a competitive antagonist at the turkey $P2Y_1$ receptor, but not at the bovine $P2Y_1$ receptor, and is thus able to discriminate between the two receptors. While at first this seems unlikely, results obtained with PPADS suggest that this might be the case. Although PPADS did not behave as a purely competitive antagonist at the turkey $P2Y_1$ cells, the maximum response to 2MeSATP was maintained at 30 μM PPADS. However, the bovine $P2Y_1$ cells were

more sensitive, with 30 μM PPADS reducing the maximal response to approximately 60 % of that achieved by 2MeSATP alone.

Whether the cells or receptors themselves are responsible for this differential sensitivity to antagonists will only be determined when a reliable binding assay with a high affinity ligand is made available. Until that time, the use of suramin and PPADS as competitive antagonists at P2Y receptors should be undertaken with caution.

4.6.2 ARL 67156

The effect of the ectoATPase inhibitor ARL 67156 on agonist concentration response curves has been described in the previous chapter. However, in the current chapter, it was shown that when suramin was included, ARL 67156 had no effect on the position of the agonist concentration-response curve. This suggests that the putative receptor at which ARL 67156 acts is also antagonised by suramin. Again, a good control experiment would be to examine the effect of ARL 67156 on wild type 1321N1 cells in the absence and presence of suramin. This would show whether ARL 67156 caused a P2Y receptor-independant response, and would establish whether surmin could inhibit this effect.

4.6.4 NF023

NF023 was introduced as a specific, directly interacting, competitive P2 receptor antagonist, being highly selective for P2X subtypes over $P2Y_1$ and $P2Y_2$ receptors (Ziyal et al, 1994, 1995; Lambrecht et al, 1996). However, at the turkey $P2Y_1$ receptor, NF023 shifted the concentration response curve to 2MeSATP to the right in a dose-dependant manner, consistent with competitive antagonism. The estimated pA_2 value for NF023 at the turkey $P2Y_1$ receptor was 5.3, compared to 6.0 at the P2X receptors in rabbit saphenous artery (Lambrecht et al, 1996). Thus, NF023 can no longer be described as a selective antagonist at P2X receptors.

Like suramin, NF023 uncouples G_α subunits from their effector enzymes (Freissmuth et al, 1996). However, unlike suramin, which is selective for recombinant $G_{\alpha s}$ subunits, NF023 is selective for recombinant $G_{\alpha i\text{-}1}$ subunits. Work detailed in Chapter 5 shows that the $P2Y_2$ receptor shows a certain degree of coupling to pertussis

toxin sensitive G proteins, whilst responses to $P2Y_1$ receptor activation are pertussis toxin insensitive. Thus, if NF023 acted by uncoupling G proteins, it would be more likely to reduce responses at the $P2Y_2$ receptor than at the $P2Y_1$ receptor. However, the effect of NF023 at the human $P2Y_2$ receptor was to shift the concentration response curve to UTP to the left, not the right. This effect was even more apparent at the $P2Y_4$ receptor, where the leftward shift of the EC_{50} values were significantly different to the $P<0.001$ level.

Although NF023 has been shown to inhibit ectonucleotidase enzymes with an activity similar to suramin (Beukers et al, 1995), the leftward shift observed at the $P2Y_4$ receptor was unlikely to be due to prevention of UTP hydrolysis. This is because all assessment of ectonucleotidase activity in 1321N1 cells presented so far in this thesis has shown that very little breakdown occurs. However, ectonucleotidase activity has not been determined in the $P2Y_4$ 1321N1 cells, although it seems unlikely that they would differ significantly from the 1321N1 cells used as hosts to the other receptors.

4.6.5 ATP is a partial antagonist at the $P2Y_4$ receptor

The experiment involving the co-addition of UTP and ATP to the human $P2Y_4$ receptor showed that ATP was capable of displace binding of UTP to the receptor, and thus reduce the response observed to 10 μM UTP. Although ATP has been described as a partial agonist at the human $P2Y_4$ receptor (see Communi & Boeynams, 1997), the direct antagonism by ATP of the response to UTP has never before been directly demonstrated.

4.6.6 Conclusions

In conclusion, the results presented in this chapter show that suramin can be used to discriminate between responses at $P2Y_1$ or $P2Y_2$ receptors and those at $P2Y_4$, and that PPADS can select for $P2Y_1$ receptors over $P2Y_2$ or $P2Y_4$ receptors. Used together, these tools may be useful in the characterisation of native receptors. Table 4.1 summarises the effects of the antagonists at the different P2Y receptors.

	Suramin	PPADS	NF023
Turkey $P2Y_1$	5.77 ± 0.11	5.98 ± 0.65 (estimated)	5.33 (estimated)
Bovine $P2Y_1$	5.34 (estimated)	non-competitive	not done
Human $P2Y_2$	no effect	no effect	non-competitive
Human $P2Y_4$	no effect	augmentation	augmentation

Table 4.1

Summary of the effects of suramin, PPADS, and NF023 on four cloned P2Y receptors.

Numbers represent pA_2 values, calculated using the method of Arunlakshana and Schild (1959). Where the values are labelled as estimated, the slope of the Schild plot was significantly different from unity, or the curves with antagonist failed to achieve the maximal response to agonist alone.

However, these data also demonstrate that all of the P2 antagonists tested show non-competitive inhibition and other non-specific effects. Thus, the results in this chapter highlight perhaps the single most important obstacle faced in the P2 receptor field; the complete lack of high affinity, highly selective ligands.

Chapter 5: Signal transduction

5.1 Introduction

The cloned P2Y receptors used in this study are all linked to phospholipase C through G proteins. On binding of agonist to the receptor, a conformational change is transmitted to the α subunit of the G protein, reducing its affinity for GDP. GDP is released and GTP, which is at much higher concentrations in the cell, binds at the active site. GTP binding activates the α subunit, which then dissociates from the receptor and $\beta\gamma$ subunits. Both α_q and $\beta\gamma$ subunits are capable of activating PLC, catalysing the hydrolysis of phosphatidyl inositol(4,5) bisphosphate (PtdIns(4,5)P_2). The two products of this reaction are inositol 1,4,5-triphosphate (Ins(1,4,5)P_3) and *sn* 1,2-diacylglycerol (DAG), both of which are second messengers. Ins(1,4,5)P_3 is soluble and diffuses across the cytosol where it releases sequestered Ca^{2+} by action at the Ins(1,4,5)P_3 receptor. DAG is lipophilic and remains within the plasma membrane where it is able to stimulate most isoforms of protein kinase C (PKC) (see Section 1.6.3 for detail).

The two previous chapters have established a rank order of agonist potencies and the sensitivity to antagonists for each of the cloned receptors. In this chapter, several aspects of the signal transduction mechanisms of each receptor are explored, in particular with reference to previous work in this laboratory on bovine aortic endothelial cells (BAECs). BAECs possess two subtypes of P2Y receptors, the $P2Y_1$ and $P2Y_2$ subtypes, which are differentially coupled to PLC. Pre-treatment of BAECs with pertussis toxin reduces the response to stimulation of the $P2Y_2$ receptors, but not to stimulation of $P2Y_1$ receptors, suggesting some coupling of the $P2Y_1$ receptor to the G_q family and of the $P2Y_2$ receptor to $G_{i/0}$ (Purkiss et al, 1994). The regulation of this coupling also varies. Responses to $P2Y_1$ stimulation are inhibited by activation of PKC (using phorbol ester), and enhanced by inhibition of PKC (using Ro 31-8220). In contrast, responses to the $P2Y_2$ receptor are unaffected by both compounds, showing that PKC provides inhibitory feedback for $P2Y_1$ receptors, but not for $P2Y_2$ receptors (Purkiss et al, 1994).

Thus, the work presented in this chapter was undertaken to determine whether the differences in $P2Y_1$ and $P2Y_2$ receptor signal transduction described above were due to inherent differences in the receptor subtype, or were a property of the different host cells examined. This study was also expanded to examine the transduction characteristics of the cloned human $P2Y_4$ receptor, and finally to assess the suitability of cloned systems for the study of transduction pathways of different receptors.

5.2 Time course of Ins(1,4,5)P_3 accumulation

The characteristics of Ins(1,4,5)P_3 production by each receptor was assessed by performing a time course of receptor stimulation. The mass Ins(1,4,5)P_3 assay, described in section 3.3.1, was used to measure Ins(1,4,5)P_3 production during stimulation of the $P2Y_1$ 1321N1 cells with 30 μM 2MeSATP and the $P2Y_2$ 1321N1 cells with 100 μM UTP (Figure 5.1). Activation of both receptors resulted in a rapid rise in Ins(1,4,5)P_3 levels, which reached maximal levels within 5 seconds (the shortest time point measured). However, the period of time that these levels were sustained varied between the receptor types. In the $P2Y_1$ cells (Figure 5.1A), the increase in Ins(1,4,5)P_3 was transient, with a reduction in peak levels at 15 seconds. In contrast, Ins(1,4,5)P_3 levels in the $P2Y_2$ cells were more sustained (Figure 5.1B), remaining maximal up to 20 seconds. Ins(1,4,5)P_3 levels had returned to basal by 60 seconds in both cell types.

5.3 The effect of Pertussis toxin

The bacterial product, pertussis toxin, has been utilised in many studies to establish which family of G proteins a receptor couples to. Pertussis toxin catalyses the ADP-ribosylation of a cysteine residue in the $G_{\alpha i}$ family (Bokcoh et al, 1984), except $G_{\alpha z}$ (Fong et al, 1988), which results in the inhibition of receptor-G protein coupling.

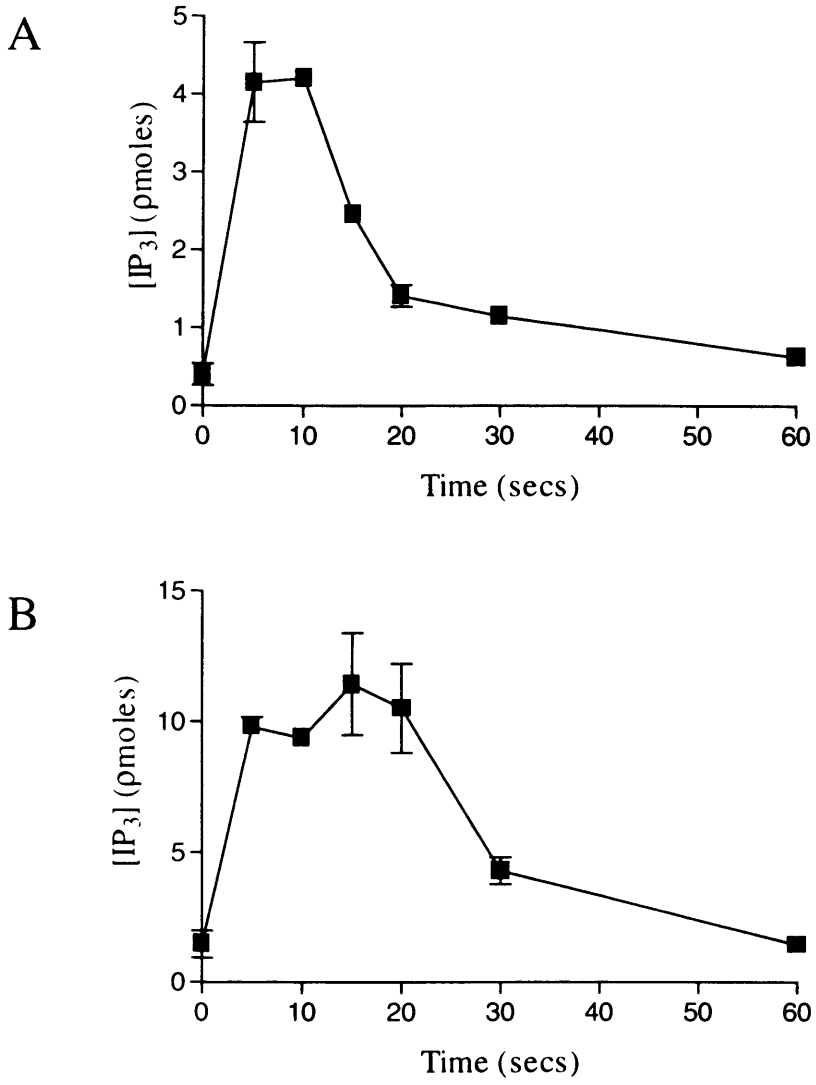


Figure 5.1

Mass inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) time courses on turkey $P2Y_1$ and human $P2Y_2$ 1321N1 cells.

60 second time course of Ins(1,4,5)P_3 production in turkey $P2Y_1$ (**A**) and human $P2Y_2$ (**B**) 1321N1 cells in response to 2MeATP (30 μM) and UTP (100 μM), respectively. Data are mean ± SEM from one representative experiment of 2, each performed in triplicate.

As has already been described, the $P2Y_1$ and $P2Y_2$ receptors in BAECs activate PLC through distinct G proteins, the $P2Y_1$ receptor coupling to pertussis toxin insensitive G proteins, and the $P2Y_2$ receptor coupling to pertussis toxin sensitive G proteins (Purkiss et al, 1994). This has also been shown in other tissues. For example, in human skin fibroblasts and CF/T43 human airway epithelial cells, pertussis toxin reduces the $P2Y_2$ induced stimulation of $[^3H]InsP_x$ by around 40 %, whereas responses at $P2Y_1$ receptors in porcine coronary artery and rat liver are unaffected (Fine et al, 1989; Brown et al, 1991; Flavahan et al, 1989; Siddiqui & Exton, 1992).

The cloned $P2Y_2$ receptor has also been shown to couple to a G protein of the G_i family, as pertussis toxin inhibits 40 % of the $P2Y_2$ mediated Ca^{2+} increase (Erb et al, 1993). Communi et al, (1995) showed that $[^3H]InsP_x$ accumulation in 1321N1 cells transfected with the human $P2Y_4$ receptor was modulated by preincubation with pertussis toxin at the 30 seconds time point, but not at 15 minutes. They suggested that the $P2Y_4$ receptor is capable of coupling to different G proteins depending on the time course of activation.

5.3.1 Turkey $P2Y_1$

Figure 5.2 shows the time course of $[^3H]InsP_x$ accumulation in turkey $P2Y_1$ 1321N1 cells, in the absence and presence of pertussis toxin. Pertussis toxin (100 ng/ml) was incubated with the cells for 16 hours prior to stimulations with 3 μM 2MeSATP. There was an initial rapid rise in levels of $[^3H]InsP_x$ between 0 and 30 seconds. The rate of $[^3H]InsP_x$ accumulation then slowed, becoming linear between 1 and 20 minutes. The time course of $[^3H]InsP_x$ accumulation with pertussis toxin was not significantly different at any point (two-way analysis of variance).

5.3.2 Human $P2Y_2$

Figure 5.3A shows the time course of $[^3H]InsP_x$ accumulation in human $P2Y_2$ 1321N1 cells, in the absence and presence of pertussis toxin. Pertussis toxin (100 ng/ml) was incubated with the cells for 16 hours prior to stimulations with 10 μM UTP. As was observed with the turkey $P2Y_1$ cells, there was an initial rapid rise in $[^3H]InsP_x$ accumulation, although this initial rate was linear up to 1 minute. At the 2

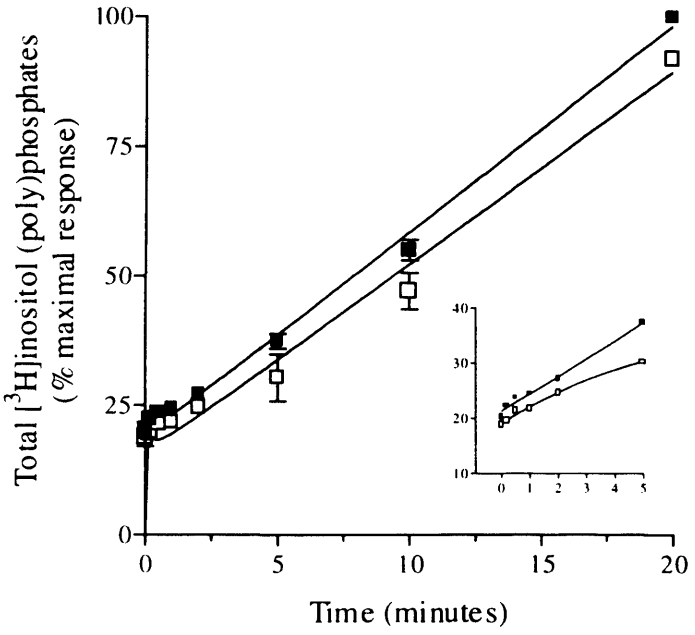


Figure 5.2

The effect of pertussis toxin on the time course of [^{3}H]InsP$_X$ accumulation in turkey P2Y$_1$ 1321N1 cells.

Time course of [^{3}H]InsP$_X$ accumulation in turkey P2Y$_1$ 1321N1 cells in response to 3 μM 2MeSATP with (❑), or without (■) pre-treatment with pertussis toxin. Pertussis toxin was incubated with the cells for 16 hours prior to stimulations. Data are mean ± SEM of 3 experiments, each performed in triplicate.

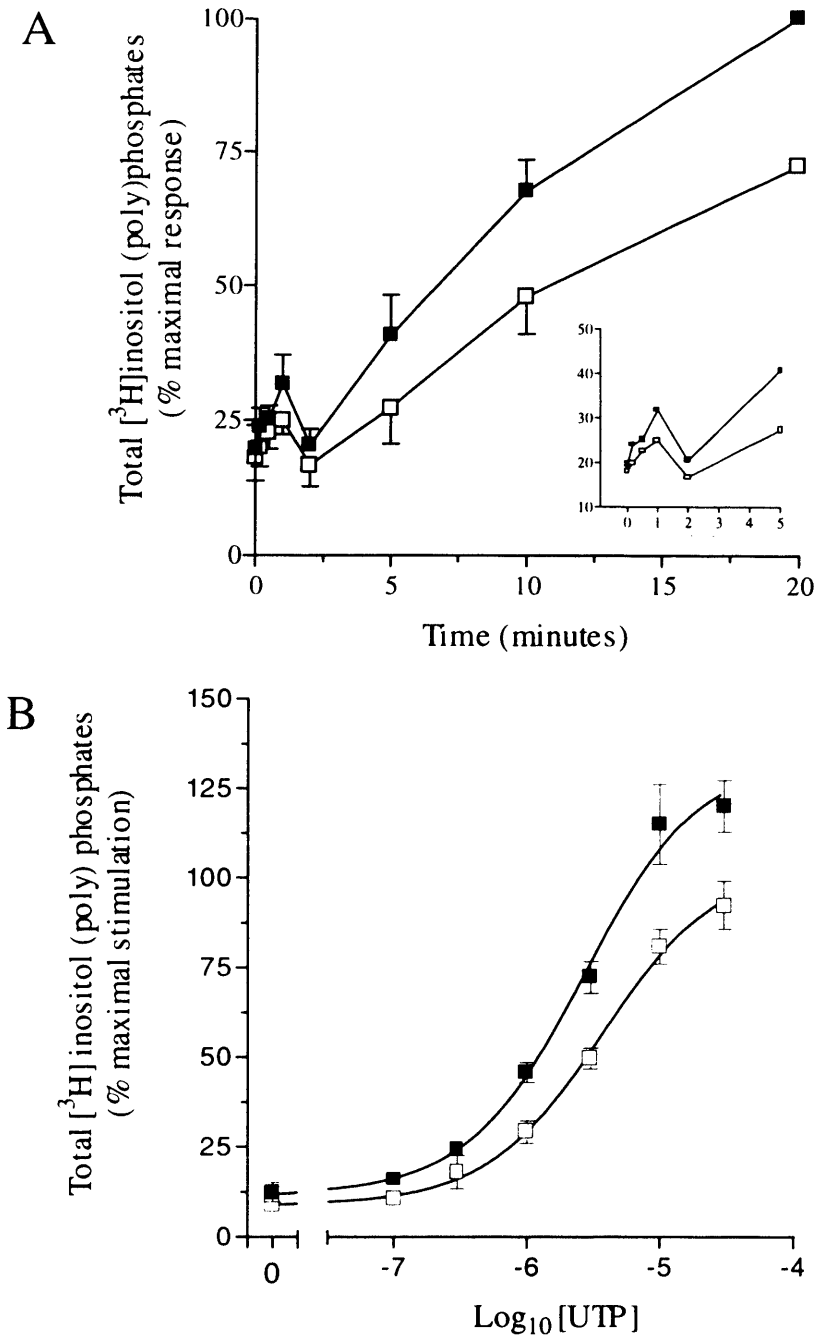


Figure 5.3

The effect of pertussis toxin on the human $P2Y_2$ 1321N1 cells.

Time course of [^{3}H]$InsP_X$ accumulation in response to 10 μM UTP (**A**) and concentration-response curves to UTP (**B**) in human $P2Y_2$ 1321N1 cells with (❑), or without (■) pre-treatment with pertussis toxin. Pertussis toxin was incubated with the cells for 16 hours prior to stimulations. Data are mean ± SEM of 3 experiments, each performed in triplicate. Data in **B** are normalised to the response to 100 μM UTP.

minute time point, levels of [^{3}H]InsP$_x$ fell almost to basal levels, after which there was a second phase of [^{3}H]InsP$_x$ accumulation. It should not be possible to observe a reduction in [^{3}H]InsP$_x$ levels using this assay, because its metabolism is inhibited by LiCl. This result therefore shows two things. Firstly, at 2 minutes there is a reduction in [^{3}H]InsP$_x$ production and/or an increase in it's metabolism, and second, there is an incomplete LiCl block in these cells which is only observed at these short time points.

Pertussis toxin reduced the levels of [^{3}H]InsP$_x$ accumulation at every time point, becoming significantly different at 10 and 20 minutes, where it reduced levels by 20.6 ± 3.8 % and 27.4 ± 0.6 %, respectively (P<0.05, Students t-test). However, the curves were not significantly different by two-way analysis of variance.

The effect of pertussis toxin on the human P2Y$_2$ 1321N1 cells was examined in more detail. Figure 5.3B shows the effect of pertussis toxin on the concentration response curve to UTP. Again, pertussis toxin reduced the levels of [^{3}H]InsP$_x$ accumulation at each UTP concentration, resulting in a 20.8 ± 4.0 % reduction in the maximal response achieved. The response to 1-30 μM UTP was significantly inhibited by pertussis toxin (Student's t-test, P<0.05), but the curves were not significantly different by two-way anova. The EC$_{50}$ values were 1.82 ± 0.10 μM in the absence and 2.72 ± 0.74 μM in the presence of pertussis toxin (not significantly different by Student's t test).

5.3.3 Human P2Y$_4$

The effect of pertussis toxin on the human P2Y$_4$ 1321N1 cells is shown in Figure 5.4. The time course in the absence of pertussis toxin had a rapid phase up to 30 seconds, which then gradually slowed up to 20 minutes. Pertussis toxin had no significant effect on the time course.

5.4 PKC involvement

Protein kinase C (PKC) is a family of serine/throenine protein kinases. It is activated by *sn* 1-2 diacylglycerol (DAG), generated from phosphatidylinositol 4,5-

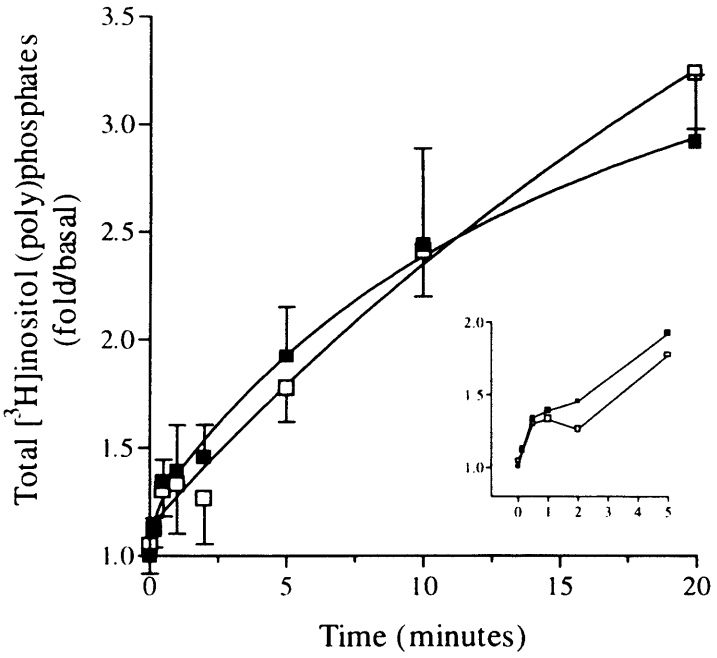


Figure 5.4

The effect of pertussis toxin on the time course of [^{3}H]InsP$_X$ accumulation in human P2Y$_4$ 1321N1 cells.

Time course of [^{3}H]InsP$_X$ accumulation in human P2Y$_4$ 1321N1 cells in response to 10 μM UTP with (❑), or without (■) pre-treatment with pertussis toxin. Pertussis toxin was incubated with the cells for 16 hours prior to stimulations. Data are mean ± SEM of 3 experiments, each performed in triplicate, and are normalised to fold over control basal.

phosphate (PtdIns(4,5)P_2) hydrolysis by phospholipase C. The activity of PKC can be regulated experimentally using certain compounds. PKC can be potently activated by the use of tumor promoting phorbol esters such as 12-O-tetradecanoyl phorbol 13-acetate (TPA) (Nishizuka, 1992). The first PKC inhibitors to be used included staurosporin and H7 (Tamaoki et al, 1986), but these compounds also inhibited other protein kinases. A newer compound based on the structure of staurosporin, Ro 31-8220, is 100 to 1000 times more selective for PKC over other protein kinases (Davis et al, 1989). This compound, however, is not selective for PKC isoforms (see Harris et al, 1996).

Short-term (5–10 minute) preincubation of phorbol esters with cells has been shown to attenuate agonist induced PLC activation (Boarder & Challiss, 1992; Challiss et al, 1993; Helper et al, 1988; Purkiss et al, 1994), by activation of a short feedback loop involving PKC. However, long term exposure to phorbol esters can result in potentiation of agonist induced PLC activation, e.g. with angiotensin II in vascular smooth muscle cells (Pfeilschifter et al, 1989), attributed to down regulation of PKC.

As has been described earlier, responses to stimulation of $P2Y_1$ receptors in BAECs are inhibited by PKC, whereas $P2Y_2$ receptors are unaffected (Purkiss et al, 1994). Contrastingly, in C6-2B rat glioma cells, $P2Y_2$ receptor stimulation of PLC is inhibited by over 80 % by PKC activation (Munshi et al, 1993). Although this indirect evidence appears to show that P2Y receptor activation results in an inhibitory feedback loop involving PKC, there is no direct evidence for the activation of PKC by P2Y receptors (see Boarder et al, 1994). The following experiments were performed to test whether the differences in PKC sensitivity of the receptors observed in BAECs were evident in this cloned system.

5.4.1 Turkey $P2Y_1$

[^{3}H]$InsP_x$ were measured in the turkey $P2Y_1$ cells in response to 3 μM 2MeSATP in the absence and presence of TPA (100 nM) and Ro 31-8220 (Ro) (10 μM). TPA and Ro 31-8220 were included with the LiCl incubation for 10 minutes prior to addition of agonist. Stimulations were terminated after 15 minutes. 3 μM

2MeSATP produced a 5 fold over basal stimulation (Figure 5.5). The inclusion of TPA reduced this stimulation to 40.8 ± 7.9 % (significant at $P<0.05$), but did not significantly affect basal accumulation (77.3 ± 11.5 % of control values). In the presence of Ro 31-8220, 3 μM 2MeSATP produced a response that was 144.5 ± 18.4 % of control values. Basal values with Ro 31-8220 were 145.0 ± 30.7 % of the basal control. However, these values were not significantly different by Student's t-test.

5.4.2 Bovine $P2Y_1$

When the same protocol was applied to the bovine $P2Y_1$ cells, the effects of TPA and Ro 31-8220 were even more marked (Figure 5.6). TPA reduced stimulated levels of [^{3}H]$InsP_x$ to 17.6 ± 0.5 % (significant at $P<0.001$) of control values, whilst the basal accumulation remained unchanged at 96.1 ± 4.2 % of control values. Ro 31-8220 enhanced [^{3}H]$InsP_x$ basal and stimulated levels to 297.3 ± 60.0 and 135.1 ± 3.8 % of control values (both significant at $P<0.05$, Student's t-test), respectively.

5.4.3 Human $P2Y_2$

TPA and Ro 31-8220 produced the same pattern when used with human $P2Y_2$ 1321N1 cells stimulated with 100 μM UTP (Figure 5.7). TPA significantly reduced accumulation to 10 μM UTP 37.0 ± 3.1 % of the control values ($P<0.05$). However, Ro 31-8220 had no significant effect on the response to 10 μM UTP, with [^{3}H]$InsP_x$ levels of 118.0 ± 21.7 % of the control stimulated values. Basal levels were unchanged in the presence of both compounds.

5.4.3 Human $P2Y_4$

Figure 5.8 shows the results obtained when TPA and Ro 31-8220 were used with the human $P2Y_4$ 1321N1 cells. TPA reduced the stimulation of accumulation by 10 μM UTP to 45.9 ± 2.0 % of the control value (significant to $P<0.05$), and stimulations in the presence of Ro 31-8220 125.3 ± 11.7 % of the control value (not

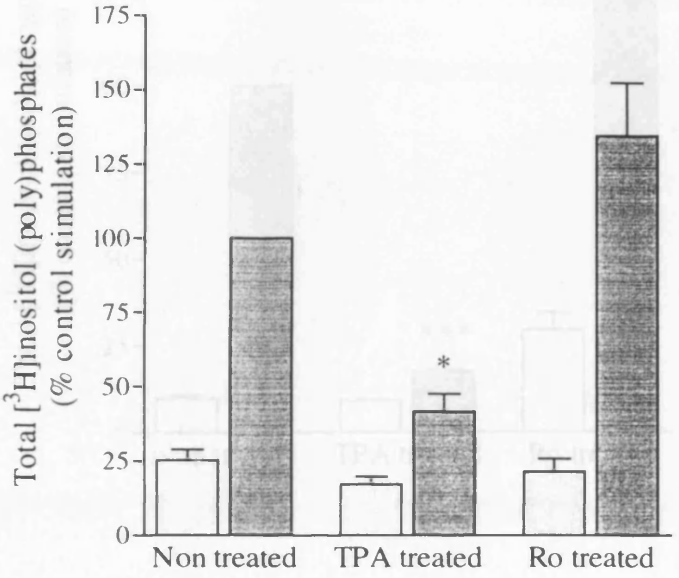


Figure 5.5

The effect of TPA and Ro 31-8220 on [^{3}H]InsP$_X$ accumulation to 3 μM 2MeSATP in turkey P2Y$_1$ 1321N1 cells.

The [^{3}H]InsP$_X$ assay was performed on turkey P2Y$_1$ 1321N1 cells treated with 3 μM 2MeSATP (dark bars) or vehicle control (light bars) in the absence and presence of TPA (100 nM) and Ro 31-8220 (10 μM). Data are mean ± SEM of 3 separate experiments, each performed in triplicate. Where indicated, values were significantly different from control (P<0.05), determined by Student's t test.

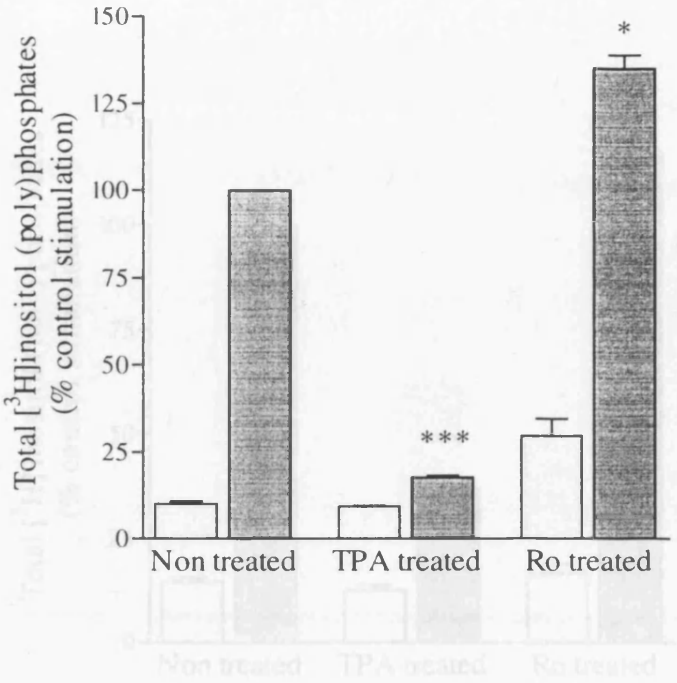


Figure 5.6

The effect of TPA and Ro 31-8220 on [^{3}H]InsP$_X$ accumulation to 3 μM 2MeSATP in bovine P2Y$_1$ 1321N1 cells.

The [^{3}H]InsP$_X$ assay was performed on bovine P2Y$_1$ 1321N1 cells treated with 3 μM 2MeSATP (dark bars) or vehicle control (light bars) in the absence and presence of TPA (100 nM) and Ro 31-8220 (10 μM). Data are mean ± SEM of 3 separate experiments, each performed in triplicate. Where indicated, values were significantly different from control (*, $P<0.05$; **, $P<0.001$), determined by Student's t test.

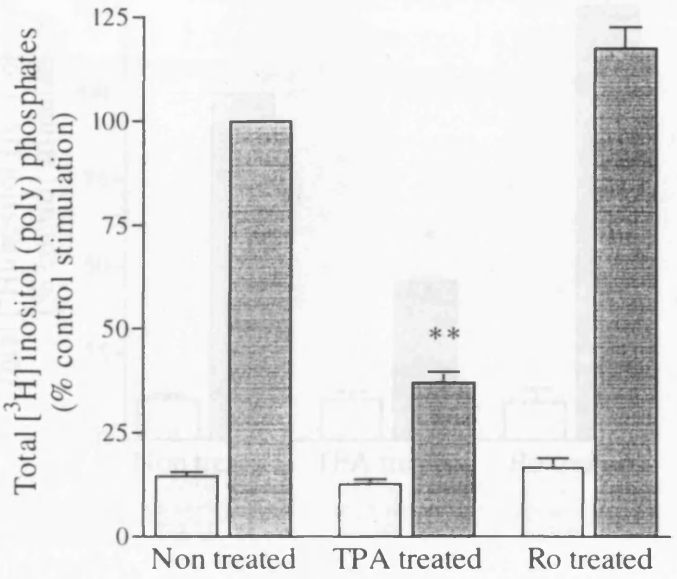


Figure 5.7

The effect of TPA and Ro 31-8220 on [^{3}H]InsP$_X$ accumulation to 10 μM UTP in human P2Y$_2$ 1321N1 cells.

The [^{3}H]InsP$_X$ assay was performed on human P2Y$_2$ 1321N1 cells treated with 10 μM UTP (dark bars) or vehicle control (light bars) in the absence and presence of TPA (100 nM) and Ro 31-8220 (10 μM). Data are mean ± SEM of 3 separate experiments, each performed in triplicate. Where indicated, values were significantly different from control (P<0.001), determined by Student's t test.

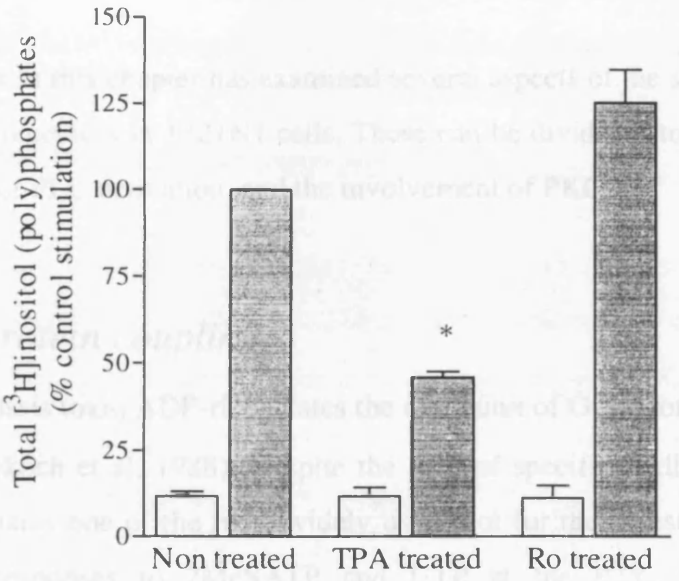


Figure 5.8

The effect of TPA and Ro 31-8220 on [^{3}H]InsP$_X$ accumulation to 10 μM UTP in human P2Y$_4$ 1321N1 cells.

The [^{3}H]InsP$_X$ assay was performed on human P2Y$_4$ 1321N1 cells treated with 10 μM UTP (dark bars) or vehicle control (light bars) in the absence and presence of TPA (100 nM) and Ro 31-8220 (10 μM). Data are mean ± SEM of 3 separate experiments, each performed in triplicate. Where indicated, values were significantly different from control (P<0.05), determined by Student's t test.

significantly different by Students t-test). The two compounds had no significant effect on basal levels of [^{3}H]InsP$_x$.

5.5 Discussion

Work in this chapter has examined several aspects of the signal transduction of cloned P2Y receptors in 1321N1 cells. These can be divided into G protein coupling, the kinetics of PLC activation, and the involvement of PKC.

5.5.1 G protein coupling

Pertussis toxin ADP-ribosylates the α subunit of $G_{i/0}$ proteins, rendering them inactive (Bokoch et al, 1988). Despite the lack of specificity displayed by pertussis toxin, it remains one of the most widely used tool for the investigation of G protein coupling. Responses to 2MeSATP and UTP at the $P2Y_1$ and $P2Y_4$ receptors respectively were unaffected by pretreatment with pertussis toxin. However, the effect of pertussis toxin on the human $P2Y_2$ 1321N1 cells was to reduce the level of [^{3}H]InsP$_x$ accumulation in response to UTP. Levels were reduced by approximately 25 %, whereas in other systems, pertussis toxin has been shown to reduce $P2Y_2$ stimulated [^{3}H]InsP$_x$ accumulation by 50-70 % (Motte et al, 1993; Fine et al, 1989; Purkiss et al, 1994). Assuming that the batch of PTX used was fully active, there are two possible explanations for this disparity in results. Firstly, pertussis toxin may not be as efficient in 1321N1 cells as in other cell types, providing less of an inhibition of $G_{i/0}$ α subunits, or secondly, when the $P2Y_2$ receptor is transfected into 1321N1 cells, it may couple to other G proteins in combination with the G_i family.

There have been many studies showing the relative promiscuity of receptor G protein interactions, initially performed using reconstruction studies (e.g Cerione et al, 1986; Senogles et al, 1990; Munshi et al, 1991). More recently, Gudermann et al (1996) have shown that α_2 adrenergic and M_4 muscarinic receptors, which couple primarily to $G_{i/0}$ can couple to G_S at high density. It is possible that transfection of the $P2Y_2$ receptor results in a larger number of receptors at the cell surface than is

present in native systems. Indirect evidence for this comes from the different EC_{50} values observed in native and transfected systems. For example, in bovine aortic endothelial cells, the EC_{50} for UTP was 32.8 ± 9.4 μM, compared to 0.94 ± 0.22 μM in the $P2Y_2$ transfected 1321N1 cells. The $P2Y_2$ receptor in the transfected system may therefore be capable of coupling to additional G proteins than those it couples to in native systems. Thus, increased receptor density would not only alter the observed potency of agonists (as discussed in section 3.7.4.1), but may also alter the nature of coupling to transduction pathways.

In these circumstances, differences in the abundance of specific G proteins (i.e. variations between different host cell types) would be expected to determine the nature of the agonist responses. Most cells appear to contain one or more of the members of the G_q and G_i families. For example, Nakahata et al (1995) have shown by western blotting with specific antisera that $G_{\alpha s}$, $G_{\alpha i/0}$, and $G_{\alpha q/11}$ are all present in the membranes of 1321N1 cells. However, although in native systems G proteins are generally in great excess relative to receptors (Neubig, 1994; Exton), they could become the limiting factor in transfected cells expressing large number of receptors.

In conclusion, the results presented in this chapter with pertussis toxin suggest that the $P2Y_1$ and $P2Y_4$ receptors couple to $G_{q/11}$, but that the $P2Y_2$ receptor shows a degree of coupling to $G_{i/0}$. This is consistent with most other studies performed on the $P2Y_1$ and $P2Y_2$ receptors. However, in a recent study by Communi et al (1996), it was shown that responses in 1321N1 cells mediated by the cloned $P2Y_4$ receptor were inhibited by pertussis toxin at a 30 second time point, but not at any other time. They suggest that the $P2Y_4$ receptor is capable of coupling to different G proteins, depending upon the time after addition of agonist. This effect was not observed in the present study, and although a small effect of pertussis toxin was observed at 2 minutes (Figure 5.4), it was not significant using Students t-test.

5.5.2 Kinetics of PLC activation

In this chapter, the kinetics of PLC activation by the turkey $P2Y_1$ and the human $P2Y_2$ receptors was examined to assess whether the differences observed between the receptors coexpressed in bovine aortic endothelial cells was retained

when the receptors were transfected into 1321N1 cells. The issue of species difference of the two receptors is important, but has been adddressed previously (Chapter 3). The kinetics of PLC activation was examined using two separate methods, measuring $Ins(1,4,5)P_3$ production and $[^3H]InsP_x$ accumulation. $Ins(1,4,5)P_3$ levels are dependent upon both generation and metabolism, and in some instances metabolism may be influenced by agonist and/or $Ins(1,4,5)P_3$ concentration. However, $[^3H]InsP_x$ accumulation is independent of metabolism and therefore gives a better index of PLC activation.

There are several possible reasons for the differences in time courses of $Ins(1,4,5)P_3$ production between the turkey $P2Y_1$ and human $P2Y_2$ 1321N1 cells. Firstly, *activation of $P2Y_1$ receptors may result in more rapid desensitisation than activation of the $P2Y_2$ receptors*. Rapid desensitisation (i.e. within a few seconds) has been described with the cloned m3 muscarinic receptor transfected into chinese hamster ovary cells (Tobin et al, 1993). This is consistent with the early time points of $[^3H]InsP_x$ accumulation in section 5.3. The rapid phase of $[^3H]InsP_x$ accumulation in the human $P2Y_2$ cells continues up to 1 minute after the initial challenge with agonist, whereas with the turkey $P2Y_1$ cells, desensitisation appears to occur after 30 seconds. The issue of desensitisation is discussed in more depth later in this chapter.

Secondly, *the GTPase activity of the G proteins associated with each receptor may differ*. Activation of PLC by a G protein is terminated by the intrinsic GTPase activity of the G protein, converting it from the active, GTP-bound form to the inactive, GDP-bound form. Different G protein subtypes have different rates of GTP hydrolysis (for summary see Fields and Casey, 1997). For example, Carty et al (1990) showed that GTP hydrolysis was several-fold faster for $G_{\alpha i2}$ than for $G\alpha_{i-1}$ or $G_{\alpha i-3}$. Thus, the differential G protein coupling of the two P2Y receptors may be responible for the different PLC activation kinetics.

Thirdly, *the receptors may activate different PLC isoforms, which have different kinetics*. $G_{\alpha q}$ subunits activate the PLCβ isoforms in a rank order of responsiveness of β1> β3 > β4 > β2, while the activation order for βγ subunits is β3 > β2 > β1 (Lee & Rhee, 1995), with the β4 isoform being unaffected (Jiang et al, 1994). Thus, the $P2Y_1$ receptor (coupled via $G_{q/11}$), may preferentially activate the β1

isoform, and the $P2Y_2$ receptor (partially coupled via the βγ subunits of $G_{i/0}$) may preferentially activate the β2 isoform. Interestingly, PLC acts as a GTPase-activating protein (GAP), i.e. it stimulates the hydrolysis of GTP by the G protein, and recently this activity has been attributed to the C-terminal tail of PLC (Paulsson et al, 1996). An example of the effect of PLC on GTPase activity was provided by Bernstein et al (1992), when they showed that the presence of PLCβ1 increased the hydrolysis of $G_{q/11}$-bound GTP by at least 50-fold. It is possible that the rate of GTPase activity varies according to PLC subtype, and that this may also play a part in producing differential activation kinetics.

In addition to the suggestions outlined above, another possible explanation for the rapid reduction in rate of $Ins(1,4,5)P_3$ that is often overlooked is that of *substrate depletion*. The inositol phospholipids are relatively minor components of most eukaryotic cells, and $PtdIns(4,5)P_2$, the primary substrate for PLC activity, constitutes less than 5 % of the cellular inositol lipid pool (see Fisher and Slowiejko, 1996). For example, it has been calculated that in SH-SY5Y cells, PLC activity can increase by 150-fold within 5 seconds of muscarinic agonist addition (Fisher et al, 1994). This meant that even if the entire $PtdIns(4,5)P_2$ pool were available following receptor activation, it would need to have been completely replenished every 13-19 seconds during the initial rapid phase of phosphoinositide hydrolysis. However, Batty & Downes (1994) have shown that production of inositol phosphates in 1321N1 cells declines as a direct consequence of substrate depletion over a longer time period of 30-60 minutes (see also Chapter 3). Also, in studies where the rate of activation of the enzymes responsible for the synthesis of $PtdIns(4,5)P_2$ has been examined, it has been observed that re-synthesis usually begins within seconds (e.g. Stephens et al, 1993). It therefore seems that another mechanism is responsible for the reduced rate of $PtdIns(4,5)P_2$ hydrolysis at these early time points.

Finally, *the rate of $Ins(1,4,5)P_3$ metabolism may vary between receptor subtypes*. Metabolism of $Ins(1,4,5)P_3$ occurs by two pathways, phosphorylation to inositol (1,3,4,5) tetrakisphosphate by 3-kinase, and dephosphorylation to inositol (1,4) bisphosphate by 5-phosphatase (see Figure 1.6). Both these enzymes have been shown to be regulated by PKC (King & Rittenhouse, 1989; Sim et al, 1990), and

$Ins(1,4,5)P_3$ 3-kinase activity is increased by raised Ca^{2+} levels (Shears, 1991). It is therefore possible that the different receptors may differentially affect the activities of these enzymes, altering the time courses of $Ins(1,4,5)P_3$ levels.

The rate of stimulated phosphoinositide hydrolysis was not constant for any of the receptors, but rapidly declined within the first 30-60 seconds of exposure of the cells to agonist. Similar results have been obtained by Martin & Harden (1989) with the PLC linked $P2Y_1$ receptor on turkey erythrocytes, and for a number of other PLC linked receptors e.g. bradykinin (Francel et al, 1987), substance P (Menniti et al, 1991), and angiotensin II (Stauderman & Pruss, 1990). This indicates a general susceptibility of stimulated PLC activity to a rapid desensitisation. However, the rate of desensitisation is not necessarily a characteristic of a given receptor. For example, angiotensin receptors present on chromaffin cells desensitise (Stauderman & Pruss, 1990), whereas those on adrenal glomerulosa cells do not (Balla et al, 1988).

The potential mechanisms by which this desensitisation occurs are limited by the rapid nature of the effect. For example, although agonist-mediated downregulation of cell surface receptors and G proteins have both been shown to reduce $PtdIns(4,5)P_2$ hydrolysis, these events do not occur within the defined time frame (see Milligan, 1996; Lameh, 1996). However, many studies have focused on the rapid phosphorylation of receptors, which can occur within seconds of addition of agonist. For example, the rapid desensitisation of the cAMP response mediated by the β_2-adrenergic receptor (β_2-AR) is achieved by the β-adrenergic receptor kinase (β-ARK) (Benovic et al, 1989). β-ARK is a member of the newly discovered family of G protein-coupled receptor kinases (GRKs) which recognise and phosphorylate the G protein-coupled receptors only in their agonist-occupied, active conformations (Inglese et al, 1993). The subsequent uncoupling of the receptor and G protein is then mediated by arrestin proteins which specifically bind to the phosphorylated receptor (Lohse et al, 1990). There are to date only 7 members of the GRK family and it has been suggested that different GRKs can recognise several receptors. This is strengthened by the observation that β-ARK not only recognises β-ARs, but that it can also phosphorylate other receptors, including the α_{2A}-AR (Benovic et al, 1987), and the m2-muscarinic receptor (Richardson et al, 1993). Most G protein-coupled

receptors have a C-terminus that is rich in serine and threonine residues, and it seems that GRKs target this region of the receptor. A variety of truncated receptors without these residues have been constructed, and have shown varying degrees of resistance to agonist-induced desensitisation e.g. the receptors for platelet activating factor (Takano et al, 1994) and substance P (Sasakawa et al, 1994).

Recently, the m3-muscarinic receptor was shown to be phosphorylated in an agonist-dependant manner by caesine kinase (Tobin et al, 1997). The consensus sequence for caesin kinase is: [S T]-x x-[D E], and all of the cloned receptors used in this study have at least one such site in their C-terminal tail.

The fall in the level of accumulated [^{3}H]InsP$_x$ in the P2Y$_2$ cells at 2 minutes is puzzling, as the inhibition of [^{3}H]InsP$_x$ metabolism by 10 mM LiCl should be complete. This would mean that even if [^{3}H]InsP$_x$ production ceased, levels of accumulated [^{3}H]InsP$_x$ would remain constant, and certainly not fall. However, as there was a fall in [^{3}H]InsP$_x$, the lithium block must have been incomplete. A possible situation where an incomplete LiCl block may occur is under conditions of slow [^{3}H]InsP$_x$ production. Lithium uncompetitively inhibits inositol monophosphatase, and therefore, in conditions of slow substrate turnover, there would be less lithium-enzyme interaction. However, at higher levels of activity, the enzyme would be more rapidly inhibited. This phenomenon has been described previously (Nahorski et al, 1991). Indeed, the second phase of [^{3}H]InsP$_x$ accumulation that was observed with the P2Y$_2$ cells may possibly be because at this time point, the enzyme was completely inhibited, so an increased rate of accumulation was observed.

Assuming there was an incomplete lithium block, the reduction in [^{3}H]InsP$_x$ levels observed with the P2Y$_2$ cells may be explained by several other possibilities.

1. Substrate depletion has been discussed earlier in this chapter, and it was concluded that this was probably not responsible for the reduction in Ins(1,4,5)P$_3$ levels observed. However, the initial rate of PtdIns(4,5)P$_2$ hydrolysis may have exhausted the [^{3}H]inositol-labelled PtdIns(4,5)P$_2$ pool (see section 3.4), resulting in an apparent reduction in the rate of [^{3}H]InsP$_x$ accumulation. Others have shown that 1321N1 cells have a large intracellular pool of inositol (Batty et al, 1993), and are capable of sustained PtdIns(4,5)P$_2$ hydrolysis for at least 30 minutes (Batty & Downes, 1994).

However, data presented in this thesis were collected from non-equilibrium labelled cells (see Chapter 3). This means that not all PtdIns(4,5)P_2 was labelled with [^{3}H]inositol when the experiments were performed. It is likely that the most accessable pool of PtdIns(4,5)P_2 is the first to be labelled, as this pool is probably involved in basal turnover of PtdIns(4,5)P_2.

So, if the most accessable pool of PtdIns(4,5)P_2 was fully labelled, a burst of [^{3}H]InsP_x accumulation would be observed on activation of PLC. Then, assuming subsequent PtdIns(4,5)P_2 pools were not labelled to the same degree, the observed [^{3}H]InsP_x accumulation would decline, even if the rate of PtdIns(4,5)P_2 hydrolysis remained constant. However, resynthesis of the PtdIns(4,5)P_2 pools would result in the incorporation of more [^{3}H]inositol (from the large intracellular pool), and a second phase of accumulation would be observed.

However, if this explanation were true, it would imply that the labelling of PtdIns(4,5)P_2 in P2Y_2 transfected 1321N1 cells was different than in cells transfected with the other receptors, and there is no evidence that this is true. It therefore seems unlikely that depletion of the labelled PtdIns(4,5)P_2 was responsible for the fall in [^{3}H]InsP_x accumulation, as it would be expected to be dependant upon the host cell and not the different receptors.

2. The two phases may be the result of action of different PLC isoforms. PLCβ is stimulated very rapidly, and may be responsible for the early phase, whilst other isoforms may be activated more slowly by downstream events, e.g. PLCδ is regulated only by Ca^{2+}. However, although the presence of PLCδ has been demonstrated in primary cultures of astrocytes, no studies have determined whether PLCδ is present in 1321N1 cells. In addition, if increased Ca^{2+} levels and PLCδ activation were the cause of this phenomenon, then it would be expected in all cells, and not just those transfected with the P2Y_2 receptor.

3. Differential coupling to G proteins may result in differences in [^{3}H]InsP_x accumulation. For example, this effect is most prominent at the P2Y_2 receptor, which appears to couple to both G_q and $G_{i/0}$. $G\alpha_q$ subunits activate PLC rapidly, whereas βγ subunits (from $G_{i/0}$) have a slower rate of activation. This is because the

concentrations of βγ subunits required to stimulate PLC are are two orders of magnitide greater than those of Gαq (see Exton, 1994).

Taken together, the time courses of $Ins(1,4,5)P_3$ production and $[^3H]InsP_x$ accumulation give important information of the kinetics of PLC activation by both the turkey $P2Y_1$ and human $P2Y_2$ receptors. There are, however, several anomalies that should be discussed. For instance, *why did levels of $[^3H]InsP_x$ continually rise, when $Ins(1,4,5)P_3$ levels were reduced to basal levels by 60 seconds?* This question is best approached by considering the receptor activation of PLC.

On initial activation of PLC by the receptor, there is a peak in $Ins(1,4,5)P_3$ production, which lasts only 10-20 seconds. This peak corresponds to the initial rapid phase of $[^3H]InsP_x$ accumulation. Interestingly, the initial phase of $[^3H]InsP_x$ accumulation is more prolonged in the $P2Y_2$ cells than the $P2Y_1$ cells, and this may reflect the more sustained peak of $Ins(1,4,5)P_3$ in the $P2Y_2$ cells. After 0.5-1 minute, $Ins(1,4,5)P_3$ levels return to basal levels in both receptors, perhaps due to desensitisation of the receptor, PLC, or the G protein. However, levels of $[^3H]InsP_x$ steadily rise throughout this period, albeit at a slower rate than the initial phase. This is because the rate of $Ins(1,4,5)P_3$ production slows down after the initial peak to a rate that is matched by it's degradation (by now, the degradation pathways are fully stimulated), so there is no net increase in production. Thus, with the mass $Ins(1,4,5)P_3$ assay, the levels of $Ins(1,4,5)P_3$ appear to return to basal, whereas with the $[^3H]InsP_x$ assay (which involves the inhibition of $[^3H]InsP_x$ degradation) there is a steady increase in $[^3H]InsP_x$ levels.

5.5.3 Effects of PKC

The results presented in this chapter show that activation of PKC by phorbyl esters inhibits agonist-induced $PtdIns(4,5)P_2$ hydrolysis. This has been shown in many other studies, e.g. $P2Y_2$ receptor stimulated sheep chondrocytes (Kaplan et al, 1996), angiotensin II stimulated rat aortic smooth muscle cells (Brock et al, 1985), histamine stimulated adrenal chromaffin cells (Boarder & Challiss, 1992), and the $P2Y_2$ stimulated Ca^{2+} response in CHO-K1 cells (Iredale & Hill, 1993). Thus, PKC activation may result in feedback inhibition of the phosphoinositide turnover.

The expression and functional roles of protein kinase C (PKC) isoforms in P2 receptor signalling have been examined in rat renal mesangial cells (Pfeilschifter & Huwiler, 1996). It was observed that rat mesangial cells express four PKC isoforms, PKC-α, -δ, -ε and ζ, as determined by Western blot analysis. By using specific PKC inhibitors and down-regulation experiments they provided evidence that PKCα acts as a negative feedback regulator of ATP- and UTP-stimulated phosphoinositide turnover, whereas PKCε activates PLA_2 and PLD. Trilivas et al (1991) have demonstrated the translocation of PKCα from the cytosol to the membrane of 1321N1 cells during muscarinic receptor activation. This is consistent with PLCα playing a role in the desensitisation of the response to P2Y receptors in 1321N1 cells, and it would be interesting to examine the contribution of each PKC isoform to this phenomenon.

PKC has been shown to directly phosphorylate several G protein-coupled receptors, including the α_{1B}-AR (Leeb-Lundberg et al, 1985), and the m3 muscarinic receptor (Tobin & Nahorski, 1993) and it must be noted that the cloned $P2Y_1$, $P2Y_2$, and $P2Y_4$ receptors all have potential PKC phosphorylation sites in the C-terminal tail. The consensus pattern for PKC is:

[S T]-x-[R K]

where S or T is the phosphorylation site and x is any amino acid. The consensus sites for each of the receptors are as follows:

Human $P2Y_2$ receptor: LGLRR ***S***DR TDMQR and GSSED ***S***RR TESTP
Turkey $P2Y_1$ receptor: RLSRA ***T***RK SSRRS and ATRKS ***S***RR SEPNV
Bovine $P2Y_1$ receptor: RLSRA ***T***RK ASRRS and ATRKA ***S***RR SEANL
Human $P2Y_4$ receptor: DSSCS ***T***PR ADRL

It is therefore possible that desensitisation may occur through the direct phosphorylation of the receptors by PKC. However, only very few studies provide evidence that PKC-mediated desensitisation might result from receptor

phosphorylation. For example, truncated mutants of the 5-HT_{1A} and NK2 receptors lacking potential phosphorylation sites for PKC become resistant to phorbol ester-induced desensitisation (Lembo & Albert, 1995; Alblas et al, 1995).

In fact, there is little evidence in the literature to implicate PKC in the rapid regulation of PLC that was observed following agonist addition. Most experiments using downregulation or inhibition of PKC, have indicated that the initial rapid phase of agonist-mediated PtdIns(4,5)P_2 hydrolysis is relatively unaffected, e.g. with P2Y receptors in C6 glioma cells (Lin et al, 1993), and muscarinic receptors in SH-SY5Y cells (Wojcikiewicz et al, 1994). Also, distinction must be drawn between agonist-mediated desensitisation and that mediated by phorbol ester-activation of PKC. For example, Dickenson & Hill (1993) found that although activation of PKC caused a reduction in the Ca^{2+} response in DDT1MF-2 smooth muscle cells, inhibition of PKC did not effect the homologous and heterologous desensitisation of histamine and P2 receptors.

Many more studies have provided clear evidence that PKC exerts its action downstream of the receptor, and PKC has been shown to phosphorylate G proteins (Strassheim & Malbon, 1994), PLC (Ryu et al, 1990), and phosphatases involved in inositol phosphate metabolism (Majerus et al, 1986). Overexpression of $PKC\beta_1$ in rat fibroblasts inhibited the production of Ins(1,4,5)P_3 when the cells were permeabilised and stimulated with the non-hydrolysable GTP analogue, GTPγS, whereas direct stimulation of PLC with Ca^{2+} was not affected (Pachter et al, 1992). GTPγS acts by locking the α subunit into its active state, so it appears that in this case, the target of PKC action is located upstream of PLC and may involve regulation of the interaction between the G protein subunits and PLC.

Despite the enormous effort expended, the mechanism by which PKC modulates PtdIns(4,5)P_2 hydrolysis and levels of inositol phosphates remains ill defined.

5.5.4 Conclusions

It appears from the results presented in this chapter, and those from other studies, that certain transduction characteristics are determined by the specific receptor involved, whilst others are more dependant upon the host cell. Specific receptor G protein coupling, even in systems with high receptor density, appears to be unchanged in different host cells. However, inhibitory feedback by PKC appears to be more dependent upon cell type than receptor type. This is exemplified by the $P2Y_2$ receptor. In BAECs, responses at this receptor are unaffected by PKC (Purkiss et al, 1994; see Pirroton et al, 1996), whereas in CHO-K1 cells (Iredale & Hill, 1993) and cloned 1321N1 cells (this thesis), PKC activation reduces $P2Y_2$ receptor mediated stimulation.

Purkiss et al (1994) suggested that the differential sensitivity of the BAEC $P2Y_1$ and $P2Y_2$ receptors to PKC activation lay in the involvement of different G proteins. This study shows that differential G protein linkage does not result in differential sensitivity to PKC, although the effect of overexpressing the receptors may result in promiscuous coupling with G proteins.

Chapter 6: Vascular smooth muscle cells

6.1 Introduction

It has long been recognised that ATP, co-released with noradrenaline from sympathetic nerves, causes vasoconstriction via activation of P2X receptors on vascular smooth muscle (Sneddon & Burnstock, 1984; Kennedy, 1996). ATP and its analogues are also known to mediate vasodilation. Nucleotides exert their vasodilatory actions at P2Y receptors on endothelial cells, activation of which leads to the release of nitric oxide (NO) and prostacyclin (PGI_2). These local mediators diffuse to the smooth muscle, causing relaxation. However, in addition to those found on endothelial cells, P2Y receptors coupled to PLC have recently been shown to exist on vascular smooth muscle. For example, UTP and ATP have been shown to cause contractions in a variety of tissues, e.g. rabbit ear artery (Von Kugelgen et al, 1987), and rat mesenteric vascular bed (Ralevic & Burnstock, 1991); responses that have been attributed to $P2Y_2$ receptors. There are also reports of responses to UTP alone, e.g. in rat aorta (Garcia-Velasco et al, 1995), and the dog maxillary internal vein (Saiag et al, 1992).

It also appears that P2 receptors on VSMCs mediate several responses apparently unconnected to contraction. For example, nucleotides have been shown to stimulate mitogensis in VSMCs (e.g. Miyagi et al, 1996). Yu et al (1996) showed that ATP triggered mitogenesis of VSMCs by activation of a $P2Y_1$ receptor, leading to the formation of inositol trisphosphate (see section 1.8.5), and Erlinge et al (1995) demonstrated rat aorta VSMCs proliferation stimulated via a $P2Y_2$ receptor. A recent report by James et al (1996) has demonstrated the release of ATP and UTP from contracted vascular smooth muscle cells, suggesting an autocrine role for both purine and pyrimidine nucleotides.

In order to provide more information on the possible roles of P2 receptors on VSMCs, the effect of purine and pyrimidine nucleotides were examined on two smooth muscle types. These were cells derived from the human saphenous vein, and from the aorta of spontaneously hypertensive rats.

6.2 Human saphenous vein smooth muscle cells

6.2.1 Introduction

The saphenous vein (SV) is one of the major blood vessels of the lower leg. It is used to replace diseased coronary arteries in coronary bypass procedures. However, in this procedure, re-stenosis is a common problem, involving the proliferation of SV smooth muscle after surgery, possibly as a result of damaged SV endothelium (see section 1.9.2). It would be of great clinical importance to determine the mechanisms by which this smooth muscle proliferation occurs, so that steps can be taken to prevent it. Several reports in the literature describe P2Y receptor-mediated stimulation of the mitogenic pathway in animal smooth muscle cells (Erlinge et al, 1993, 1995; Yu et al, 1996), and the aim of this section was to determine whether P2Y receptors exist on human saphenous vein smooth muscle cells (hSV-SMCs), and whether the action of nucleotides stimulates proliferation. The total [^{3}H]InsP$_x$ assay was used to examine possible PLC-coupled P2Y receptors, and the first section of this chapter is concerned with evaluating the optimal conditions for measurement of [^{3}H]InsP$_x$ accumulation.

6.2.2 [^{3}H]inositol incorporation

Cells were subcultured into 24 well multiplates in 1 ml of complete medium and grown to confluence. The growth medium was removed and the cells were labelled with 1 μCi/ml (0.074 MBq/ml) of D-myo-[2-^{3}H] inositol in three different conditions. (**1**) 0.5 ml of medium 199 (Gibco) supplemented with 25 iu/ml penicillin, 25 μg/ml streptomycin and 2 mM Glutamine solution, (**2**) As (1), but with 0.4 % foetal calf serum, and (**3**) As (1), but with 1 mg/ml bovine serum albumin. Cells were left to incubate with these labelling media at 37 °C for 0, 12, 24, 36, 48, 60 and 72 hours. Incorporation of [^{3}H]inositol into the lipid fraction of the 1321N1 cells was determined using the method described in section 2.3.1. Figure 6.1 shows the results of this experiment.

Incorporation of [^{3}H]inositol was slow up to 24 hours in each labelling condition, after which the rate increased linearly. The inclusion of 0.4 % FCS resulted

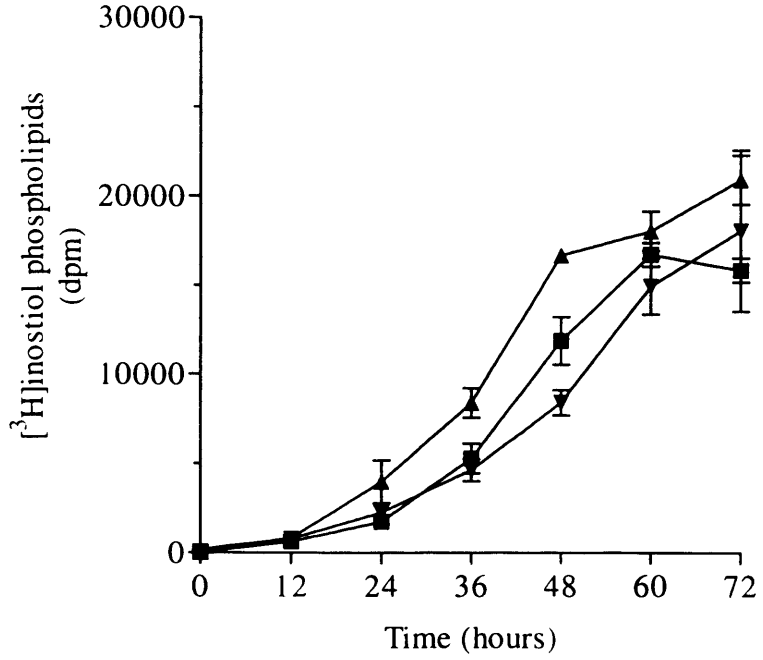


Figure 6.1

Time course of [^{3}H]inositol incorporation into the lipid fraction of human saphenous vein smooth muscle cells (hSVSMC).

Cells were incubated with [^{3}H]inositol for the specified times in three different media: (■) normal serum free medium (see section 2.1.3), (▲) supplemented with 0.4 % FCS, (▼) or supplemented with 1 mg/ml BSA. Incorporation into the lipid fraction was measured as described in section 2.3.1. Data are the mean ± SEM of one experiment.

in the largest incorporation, so this condition was used in the following experiments. Cells were labelled for both 48 and 60 hours; the labelling periods are stated for each experiment.

6.2.3 Time course of [^{3}H]InsP$_x$ accumulation

A time course of [^{3}H]InsP$_x$ accumulation was performed with the hSV-SMCs to determine the optimal time for terminating stimulations. Cells were labelled for 48 hours prior to experimentation. A preliminary time course was performed using ATP (300 μM) to stimulate the cells (Figure 6.2). Levels of [^{3}H]InsP$_x$ did not start to increase until after 2 minutes, after which a rapid accumulation was observed. At 15 minutes the accumulation reached a plateau which continued up to 30 minutes. A stimulation period of 15 minutes was used in all further experiments.

6.2.4 60 hours labelling with 2 μCi/ml [^{3}H]inositol

In an effort to increase fold over basal stimulations, cells were labelled for 60 hours with 2 μCi/ml [^{3}H]inositol. Cells were then challenged with 300 μM ATP, UTP, and ATPγS for 15 minutes. Table 6.1 shows the results of this experiment, and compares them with fold over basal stimulations achieved after labelling with 1 μCi/ml [^{3}H]inositol for 48 hours. Labelling with 2 μCi/ml for 60 hours did not significantly increase the fold over basal stimulations achieved with any of the agonists, so was not adopted for future experiments.

6.2.5 Higher LiCl concentrations

The effect of higher LiCl concentrations on nucleotide-stimulated [^{3}H]InsP$_x$ accumulation was examined to try to achieve higher fold over basal stimulations, as it was possible that a full block of [^{3}H]InsP$_x$ breakdown was not achieved with 10 mM LiCl in the 1321N1 cells (see section 5.3.2). Cells were labelled for 48 hours with 1 μCi/ml [^{3}H]inositol, and were incubated for 10 minutes with 10, 20, and 30 mM LiCl in BSS before being stimulated with 300 μM ATP. Figure 6.3 shows the results of this

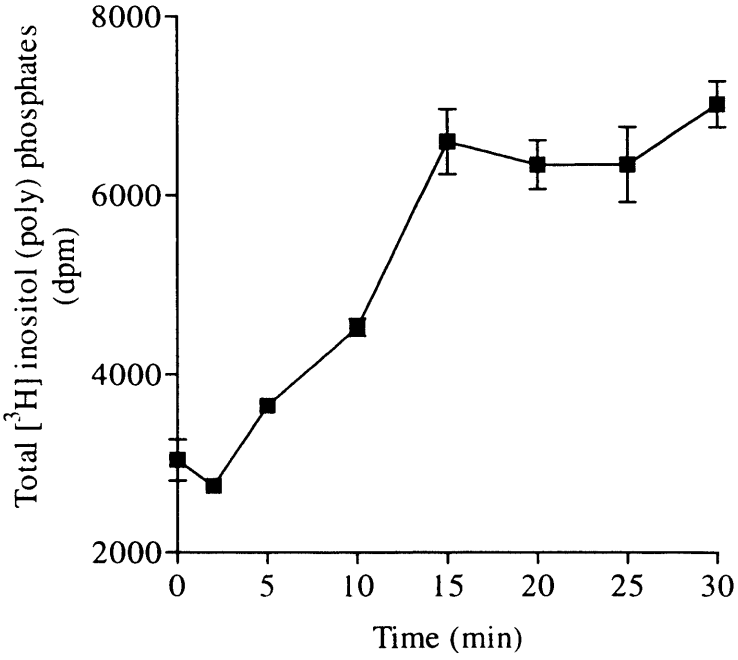


Figure 6.2

Time course of [^{3}H]InsP$_x$ accumulation in hSV-SMCs.

Time course of [^{3}H]InsP$_x$ accumulation in hSV-SMCs in response to 300 μM ATP. Cells were preincubated with 10 mM LiCl for 10 minutes before addition of agonist for 15 minutes. Incubations were terminated by aspiration of medium and addition of 0.5 ml TCA (0.5 M). Data are mean ± SEM from one experiment performed in triplicate.

	2 μCi/ml [^{3}H]inositol for 60 hours (fold over basal)	1 μCi/ml [^{3}H]inositol for 48 hours (fold over basal)
ATP (300 μM)	1.42 ± 0.23	1.6. ± 0.004
UTP (300 μM)	1.75 ± 0.11	1.75 ± 0.34
ATPγS (300 μM)	1.60 ± 0.10	1.58 ± 0.03

Table 6.1

Fold over basal stimulations using two different [^{3}H]inositol labelling conditions with the human saphenous vein smooth muscle cells. Data are from single experiments, each performed in triplicate.

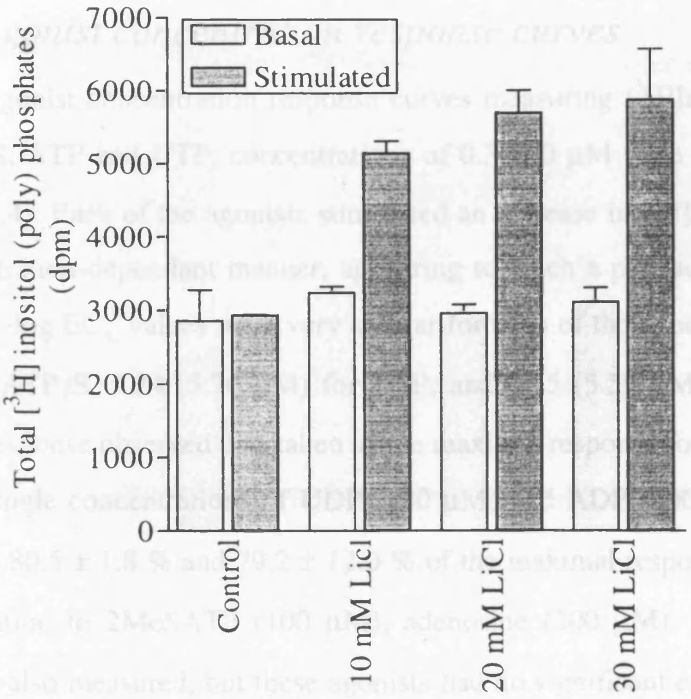


Figure 6.3

The effect of higher LiCl concentrations on [^{3}H]InsP$_x$ accumulation in hSV-SMCs.

Cells were labelled for 48 hours with [^{3}H]inositol, and were incubated for 10 minutes with LiCl in BSS at final concentrations of 10, 20, and 30 mM. Cells were stimulated with 300 μM ATP for 15 minutes. Data are mean ± SEM from one experiment performed in triplicate.

experiment. The responses observed to 300 μM ATP increased slightly with increased LiCl concentration, but were not significantly different from each other. Fold over basal stimulations were 1.6 ± 0.05 with 10 mM LiCl, 1.9 ± 0.1 with 20 mM LiCl, and 1.9 ± 0.24 with 30 mM LiCl.

6.2.6 Agonist concentration response curves

Agonist concentration response curves measuring [^{3}H]InsP$_x$ were constructed to ATPγS, ATP and UTP; concentrations of 0.3-300 μM were used for each agonist (Figure 6.4). Each of the agonists stimulated an increase in [^{3}H]InsP$_x$ accumulation in a concentration-dependant manner, appearing to reach a plateau at approximately 30 μM. The -log EC_{50} values were very similar for each of the agonists, being 5.57 (2.66 μM) for ATPγS, 5.24 (5.76 μM) for UTP, and 5.25 (5.59 μM) for ATP (when the highest response observed was taken as the maximal response for that receptor).

Single concentrations of UDP (300 μM) and ADP (300 μM) gave responses that were 80.5 ± 1.8 % and 79.2 ± 12.0 % of the maximal response to UTP. [^{3}H]InsP$_x$ accumulation to 2MeSATP (100 μM), adenosine (300 μM), and α,βMeATP (300 μM) was also measured, but these agonists had no significant effect over basal levels (data not shown). Angiotensin II (AII) at 100 nM has been shown to stimulate [^{3}H]InsP$_x$ accumulation in other vascular smooth muscle cells (Morton et al, 1995), so was tested on the hSV-SMCs. 100 nM AII failed to stimulate [^{3}H]InsP$_x$ accumulation (3 separate experiments, each in triplicate).

6.2.7 [^{3}H]thymidine incorporation

Previous studies have used [^{3}H]thymidine incorporation to measure vascular smooth muscle cell (VSMC) division in response to nucleotides (Erlinge et al, 1993, 1995; Yu et al, 1996). A preliminary investigation was undertaken to ascertain whether nucleotides were capable of stimulating mitogenesis in hSV-SMCs. AII was used as a reference, as it has been shown to stimulate proliferation of smooth muscle cells from a variety of vascular tissue (Morton et al, 1995). Initial experiments using

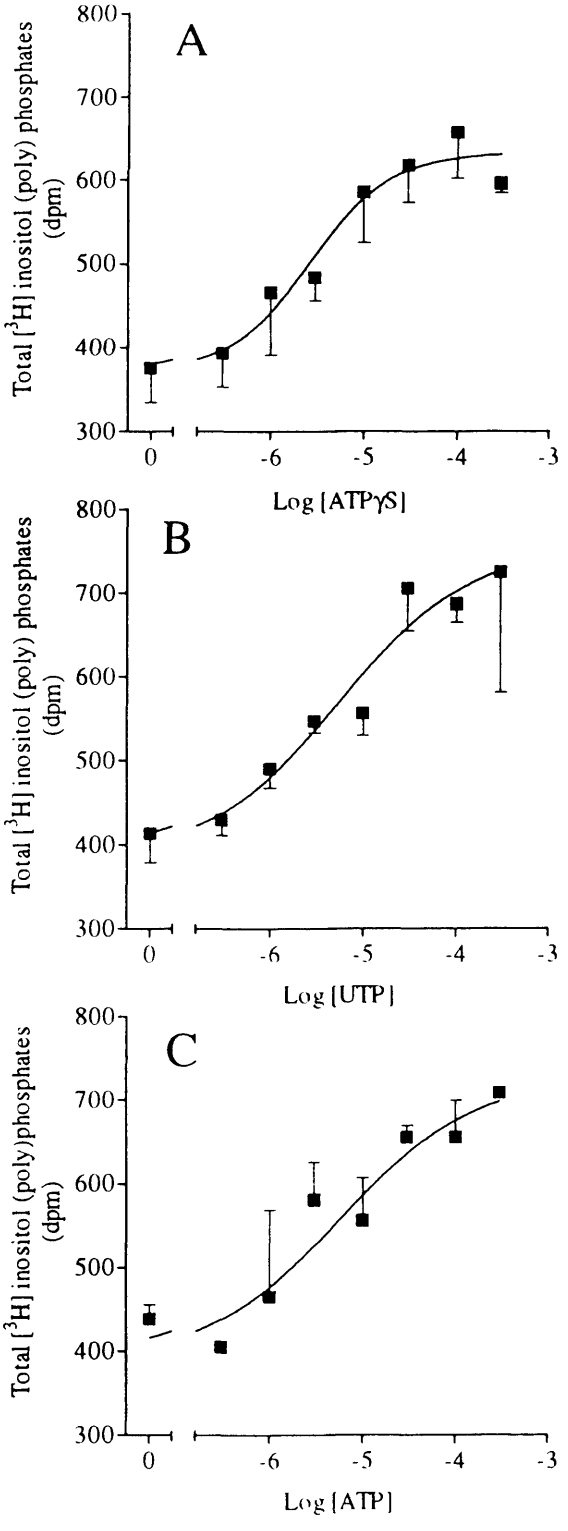


Figure 6.4

Agonist concentration response curves for [^{3}H]InsP$_x$ accumulation in hSV-SMCs.

Cells, prelabelled for 48 hours with [^{3}H]inositol, were incubated for 10 minutes in 10 mM LiCl. Cells were then incubated for a further 15 minutes with increasing concentrations of (**A**) ATPγS, (**B**) UTP, and (**C**) ATP. Data are mean ± SEM from one experiment performed in triplicate.

ATP, UTP and AII to induce proliferation showed that these agonists had little effect on [^{3}H]thymidine incorporation with hSV-SMCs. Counts for [^{3}H]thymidine incorporation were 14194 ± 1084 dpm for basal, 9940 ± 1339 with 300 μM UTP, 22496 ± 524 with 300 μM ATP, and 13607 ± 1462 with AII.

6.2.7.1 Platelet derived growth factor (PDGF)

In some studies it has been shown that nucleotides are unable to stimulated mitogenesis alone, but can greatly enhance a proliferative response to growth factors e.g. platelet derived growth factor (PDGF) (Miyagi et al 1996). PDGF stimulated the movement of quiescent serum-starved VSMCs from G_0 to G_1, but no further. ATP and UTP did not stimulate cells in G_0, but did stimulate progression of PDGF treated cells in G_1 into the completion of the cell cycle through S and M phases.

An experiment was therefore undertaken to examine whether PDGF stimulated proliferation in these cells, and to determine an optimal concentration to use with the nucleotides. Figure 6.5 shows [^{3}H]thymidine incorporation in hSV-SMCs stimulated with increasing concentrations of PDGF. PDGF stimulated [^{3}H]thymidine incorporation, reaching a plateau at 3 nM (2.25 ± 0.55 fold over control).

6.2.7.2 PDGF with nucleotides

Figure 6.6 shows the results when ATP and UTP were used in the presence of 3 nM PDGF. Both nucleotides had no effect alone, but with PDGF, they both gave large increases in [^{3}H]thymidine incorporation. UTP gave a 11.0 ± 0.8 fold over control stimulation with PDGF (compared to 0.7 ± 0.09 alone), and ATP gave a 14.7 ± 0.7 fold over control stimulation of proliferation with PDGF (compared to 1.6 ± 0.03 alone).

Despite obtaining some interesting results, the use of the [^{3}H]InsP$_x$ assay with hSV-SMCs resulted in a great deal of experimental variation. The fold over basal

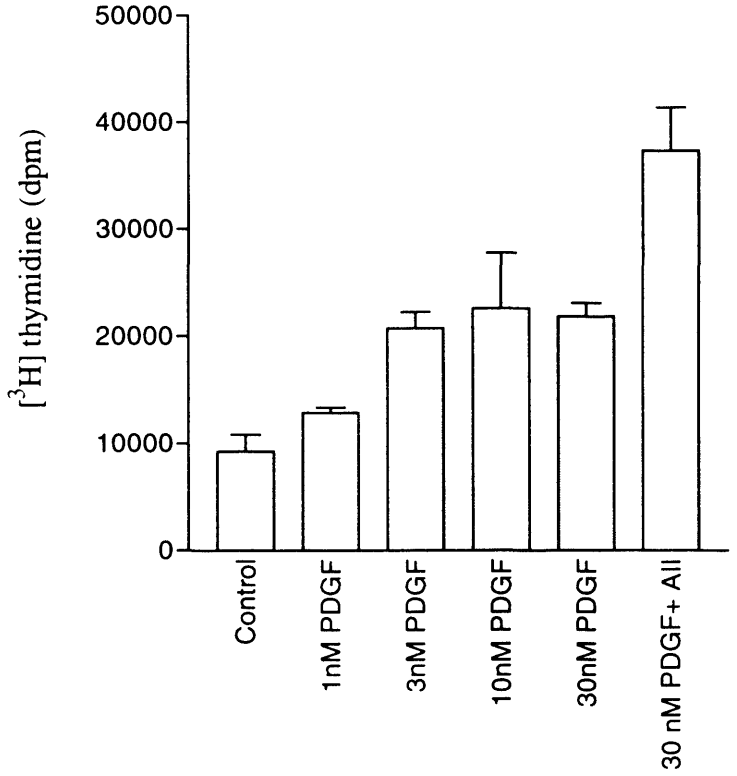


Figure 6.5

[^{3}H]thymidine incorporation into hSV-SMCs stimulated with PDGF and angiotensin II (AII).

Cells were incubated for 24 hours with increasing concentrations of PDGF and with 30 nM PDGF plus 100 nM AII. Data are mean ± SEM from one experiment performed in triplicate.

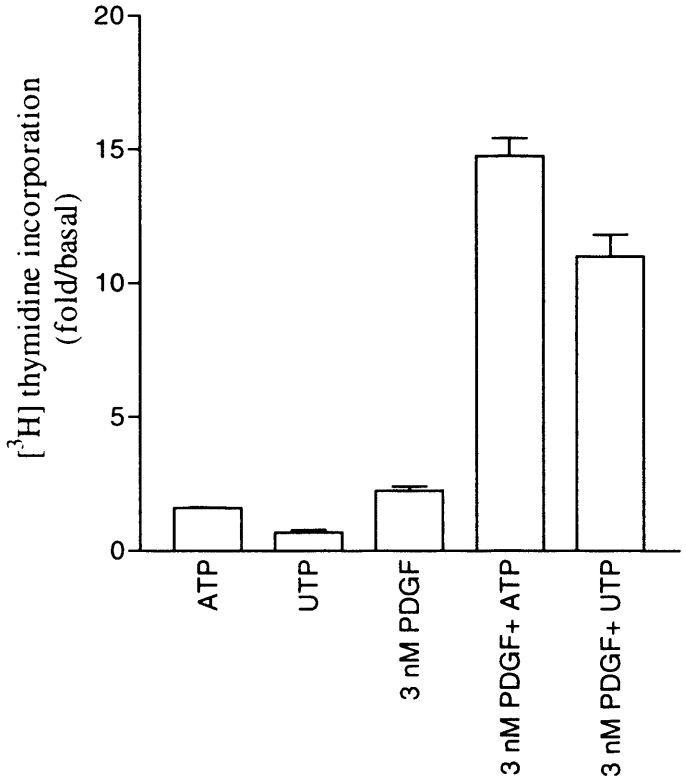


Figure 6.6

[^{3}H]thymidine incorporation into hSV-SMCs stimulated with PDGF plus ATP and UTP.

Cells were incubated for 24 hours with UTP (300 μM), ATP (300 μM) or PDGF (3 nM) alone, and with 3 nM PDGF plus ATP (300 μM) or UTP (300 μM). Data are mean ± SEM from one experiment performed in triplicate.

stimulations were less than 2 in every case, and the data were variable within triplicates. An example of experimental variation is provided by Figures 6.2 and 6.4. Although the assay procedures were identical, a maximal concentration of ATP elicited responses that were approximately 10 fold different. It is hard to explain this variation, although variations in cell number may contribute. Growth of smooth muscle cells does not cease once cell-cell contact has occured, unlike endothelial cells. It is therefore more difficult to bring each batch of cells to the same degree of confluency. Future experiments must include a protein determination with which to normalise data.

6.3 Spontaneously hypertensive rat smooth muscle cells

6.3.1 Introduction

The spontaneously hypertensive rat (SHR) is a strain of Wistar rats that was genetically bred to provide an experimental model of inherited hypertension (Okamoto & Aoki, 1963). Smooth muscle cells derived from the aorta of SHR cells (SHR-VSMCs) differ from their Wistar Kyoto normotensive control cells (WKY-VSMCs) in a variety of ways. Although receptor number and affinity have been shown not to differ (Bolger et al, 1991), agonists such as AII, endothelin-I and serotonin stimulate significantly higher levels of PIP_2 hydrolysis, intracellular Ca^{2+}, and mitogensis in SHR-VSMCs over WKY-VSMCs (Osanai & Dunn, 1992; Morton et al, 1995; Turla & Webb, 1990). This is in the absence of basal increases in cytosolic $Ins(1,4,5)P_3$ or Ca^{2+} (Morton et al, 1995). A growing body of work has tried to identify the possible causes of these differences. For example, mastoparan, a G-protein activator, has been used to demonstrate enhanced G_q sensitivity in SHR-VSMCs (Kanagy & Webb, 1994).

The SHR-VSMCs have been shown to give large stimulations in response to G protein-coupled receptor agonists, e.g. angiotensin II (Morton et al, 1995), so the presence of P2Y receptors was examined in these cells.

6.3.2 Concentration response curves

Experiments measuring [^{3}H]InsP$_x$ accumulation to various nucleotides were performed in SHR-VSMCs. As shown in Figure 6.7, UTP gave the largest response (16.2 ± 2.94 fold over basal) and had a -log EC_{50} of 5.40 ± 0.42. ATP gave a response which only reached 44.3 ± 5.3 % of that to UTP, but had a similar -log EC_{50} of 5.08 ± 0.14. The maximal stimulations and dose relationships of UTP and ATP were significantly different by Student's t test ($P<0.05$) and analysis of variance ($P<0.05$), respectively. The other agonists tested, 2MeSATP, α,βMeATP, and adenosine (data not shown) failed to significantly increase [^{3}H]InsP$_x$ accumulation above control levels.

6.3.2.1 Nucleoside diphosphates

The concentration response curves to the nucleoside diphosphates ADP and UDP were constructed in the absence and presence of hexokinase. For a detailed description of the protocol, see section 3.6.3. Briefly, stock solutions of 5 μM UDP and ADP were preincubated with 50 units/ml hexokinase and 110 mM glucose for 1 hour prior to experimentation. Hexokinase was also added to the cells during the stimulations at 1 unit/ml with 22 mM glucose.

Figure 6.8 shows that UDP elicits a response with a -log EC_{50} value of 5.15 ± 0.05. ADP at concentrations up to 300 μM did not significantly increase levels of [^{3}H]InsP$_x$ (data not shown). The maximal response achieved by 300 μM UDP was only 63.6 ± 26.4 % of that observed to 300 μM UTP. However, inclusion of hexokinase markedly reduced the potency of UDP to a -log EC_{50} of approximately 4, and the curve was significantly different to that in the absence of hexokinase (two-way analysis of variance; $P<0.05$). ADP did not significantly increase the accumulation of [^{3}H]InsP$_x$ above control values, and so no significant effect of hexokinase was observed (analysis of variance).

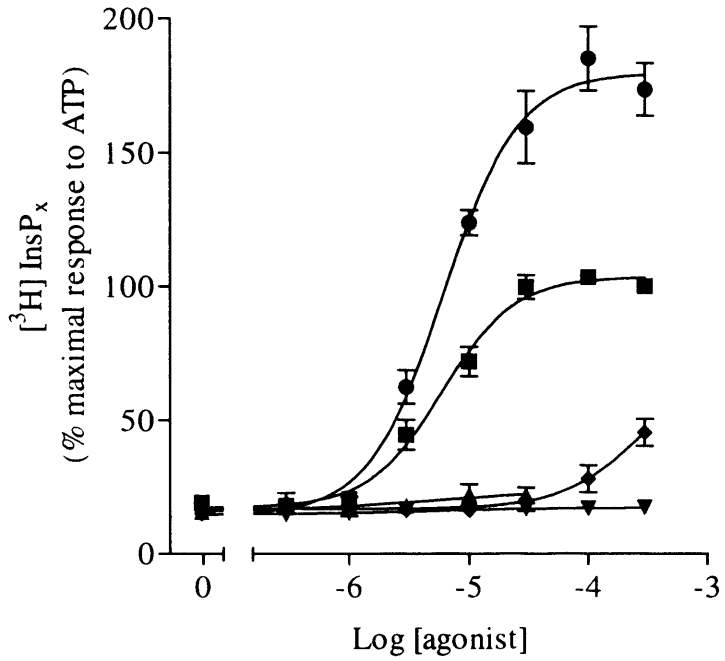


Figure 6.7

Agonist concentration-response curves of [^{3}H]InsP$_x$ accumulation in SHR-VSMCs.

Cells were preincubated in 10 mM LiCl for 10 minutes and then with increasing concentrations of UTP (●), ATP (■), 2MeSATP (▲), α,βMeATP (▼), and ADP (◆), for 15 minutes. Data are the mean ± SEM percent of the maximal response to UTP, and were from three separate experiments, each performed in triplicate.

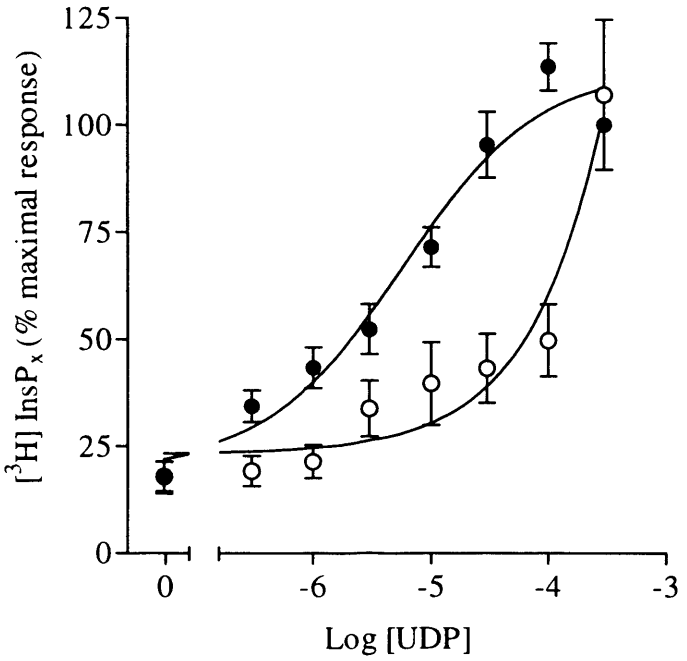


Figure 6.8

SHR-VSMC concentration-response curves to UDP in the presence and absence of hexokinase.

Stock UDP (5 mM) was preincubatedwith 50 units/ml hexokinase and 110 mM glucose. Cells were preincubated in 1 unit/ml hexokinase, 22 mM glucose and 10 mM LiCl (○) or with 10 mM LiCl only (●) for 10 minutes. Cells were then incubated in hexokinase-treated UDP (○) and untreated UDP (●) for 15 minutes. Data are the mean ± SEM of the maximal response from 3 separate experiments, each performed in triplicate.

6.3.3 ATP antagonism

ATP appears to act as a partial agonist at the SHR-VSMCs, as was observed with the cloned human $P2Y_4$ receptor in section 4.5. Cells were exposed to increasing concentrations of ATP in the presence of UTP (30 μM) to determine whether UTP and ATP were acting at the same receptor site. Figure 6.9 shows that ATP at concentrations of 30, 100, and 300 μM reduced the response to 30 μM UTP to 96.6 ± 1.4 %, 83.3 ± 2.0 %, and 64.9 ± 12.2 % of that to 30 μM UTP alone (significant to $P<0.05$ by analysis of variance). The response to 300 μM ATP alone was 55.5 ± 4.2 % of that to 30 μM UTP.

6.3.4 [^{3}H]thymidine

Previous studies have shown that proliferation of SHR-VSMCs can be stimulated by angiotensin II (AII) (Morton et al, 1996; Wilkie et al, 1996). A preliminary study measuring [^{3}H]thymidine incorporation was performed to investigate the proliferative properties of UTP (300 μM) and ATP (300 μM), which were compared to those of AII (100 nM). Figure 6.10 shows there was a 2.9 ± 0.7, 3.1 ± 1.1 and 4.3 ± 0.5 fold over basal increase in [^{3}H]thymidine incorporation with ATP, UTP, and AII, respectively.

6.4 Discussion

Several studies have demonstrated smooth muscle PIP_2 hydroysis in response to extracellular nucleotides (Strobaek et al, 1996; Yu et al, 1996). In this chapter, the presence of P2Y receptors was examined in cells derived from two different parts of the vasculature, measuring [^{3}H]$InsP_x$ accumulation and [^{3}H]thymidine incorporation.

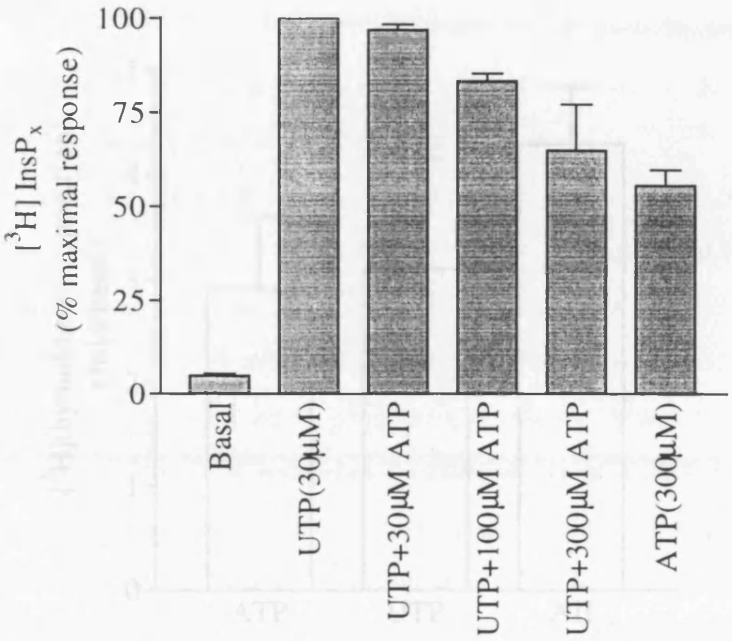


Figure 6.9

Total [^{3}H]inositol (poly)phosphate accumulation in SHR-VSMC.

Cells were pre-incubated with 10 mM LiCl for 10 minutes. Cells were then incubated for 15 minutes in 30 μM UTP alone, 300 μM ATP alone, or 30 μM UTP with increasing concentrations of ATP. Data are the mean ± SEM response to 30 μM UTP alone from 3 separate experiments, each performed in triplicate.

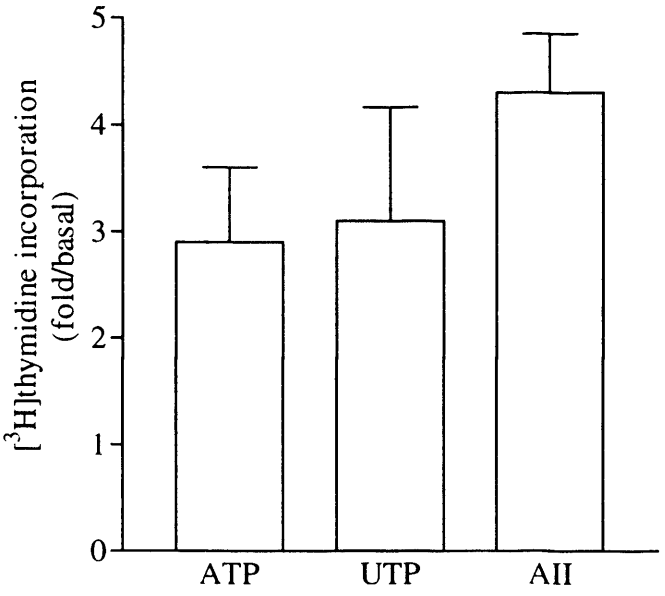


Figure 6.10

[^{3}H]thymidine incorporation into SHR-VSMCs in response to ATP, UTP and angiotensin II.

Cells were incubated with ATP (300 μM), UTP (300 μM), and angiotensin II (AII; 100 nM) for one hour, and were then incubated for a further 19 hours in the absence of agonist before responses were terminted. Data are the mean ± SEM fold over basal responses from 3 experiments, each performed in triplicate.

6.4.1 Human saphenous vein smooth muscle cells

The preliminary results presented in this chapter show that the hSV-SMCs gave small [^{3}H]InsP$_x$ responses on stimulation with nucleotides. This appears to be due to a general unresponsiveness of the cells to G protein-coupled receptor stimulation, because AII, known to stimulate PLC in many other vascular smooth muscle cells (e.g. Socorro et al, 1990; Lucchesi et al, 1996; Morton et al, 1995), gave no significant stimulation of [^{3}H]InsP$_x$ accumulation. It must be noted that the hSV-SMCs were derived from elderly patients requiring coronary bypass surgery, and so may have become less responsive through age.

However, despite poor stimulations and a very limited range of nucleotides, a tentative characterisation of the possible P2Y receptors present on hSV-SMCs can be discussed. It is possible that P2X receptors are present on these cells, because [^{3}H]InsP$_x$ accumulation and not intracellular Ca^{2+} was measured, so action at P2X receptors would not be expected to generate a response. However, it has been shown that VSMCs loose their P2X receptors on culture, while a P2Y response of similar characteristics is found in both intact tissues and cultured cells (Pacaud et al, 1995). Nevertheless, there is no P2X contibution to the [^{3}H]InsP$_x$ response in these cells, because no responses were seen to 2MeSATP and α,β-MeATP. Adenosine was inactive, so the presence of PLC-coupled P$_1$ purinoceptors can be ruled out. ATPγS, UTP, and ATP all gave stimulations of a similar potency and of similar maximal response. UDP and ADP at 300 μM gave responses that were approximately 80 % of those to the nucleoside triphosphates. Several conclusions can be drawn from these results. The inactivity of 2MeSATP rules out the presence of a P2Y$_1$ receptor, and suggests that a UTP selective receptor is present. Of the three functional uridine nucleotide-preferring P2Y receptors, the P2Y$_2$ is the most likely candidate. Agonist potencies at the cloned human P2Y$_2$ receptor (Figure 3.11) show that ATP and UTP are equipotent, with ADP and UDP being less active, the profile observed with the hSV-SMCs. The P2Y$_6$ receptor can be discounted, as UDP is much less potent than UTP, and the P2Y$_4$ is unlikely because ATP acts as a full agonist (compare with Figure 3.12).

However, many tissues have been shown to co-express P2Y receptors (e.g. Wilkinson et al, 1994), and the presence of two P2Y receptors may explain this

agonist profile. Nevertheless, the presence of a receptor for ATP (the $P2Y_2$) and a separate receptor for UTP (the $P2Y_4$) is unlikely because an increased response to UTP over ATP would be expected. It therefore appears that a single P2Y receptor, the $P2Y_2$, is present on hSV-SMCs, but a more thorough investigation must be performed to unequivocally demonstrate this.

Few studies have examined the presence of P2Y receptors in human-derived VSMCs. Recently, Strobaek et al (1996) have shown that ATP, UTP, and 2MeSATP stimulate a Ca^{2+} response in smooth muscle cells derived from human large coronary arteries (hLCA-SMCs). They propose that both $P2Y_1$ and $P2Y_2$ receptors are present, although their data does not rule out the presence of a uridine nucleotide-sensitive receptor other than the $P2Y_2$. The results presented in this chapter show that whilst it is possible that hSV-SMCs express a $P2Y_2$ receptor, a $P2Y_1$ receptor is unlikely to be present, because 2MeSATP was inactive.

However, increases in Ca^{2+} were measured with the hLCA-SMCs, whilst [^{3}H]$InsP_x$ accumulation was measured with the hSV-SMCs. While at first this might seem a small point, it must be considered in the light of a recent study by Albert et al (1997). They showed that rat brain endothelial cells possess both $P2Y_1$ and $P2Y_2$ receptors, and that the $P2Y_2$ receptors are coupled to PLC and Ca^{2+} increases. The $P2Y_1$ receptors, however, were apparently atypical with respect to their coupling mechanism, in that they are described as stimulating an increase in intracellular Ca^{2+} in the absence of a detectable increase in $Ins(1,4,5)P_3$ levels. It is therefore possible that responses observed to 2MeSATP in the hLCA-SMCs were due to this atypical coupling, which would not be observed with [^{3}H]$InsP_x$ measurements in the hSV-SMCs.

6.4.2 Spontaneously hypertensive rat vascular smooth muscle cells

Agonist stimulated $PtdIns(4,5)P_2$ hydrolysis in SHR-VSMCs has been demonstrated for several G protein-coupled receptors, e.g. angiotensin II (Nabika et al, 1985), 5-HT (Turla & Webb, 1990), vasopressin (Nabika et al, 1985). In this

chapter, the responses to several nucleotides were examined in order to determine the nature of the P2Y receptors present on SHR-VSMCs.

There are several explanations for the pattern of nucleotide potencies observed with these cells. The fact that 2MeSATP is inactive discounts the presence of a $P2Y_1$ receptor, and the potent action of UTP suggests $P2Y_2$, $P2Y_4$, or $P2Y_6$ receptors. The $P2Y_6$ receptor was first cloned from cultured rat aorta VSMCs (Chang et al, 1995), and was described as being preferentially stimulated by UTP. The human homologue has since been cloned (Communi et al, 1996), and was shown to be more sensitive to UDP that UTP. This finding was confirmed by Nicholas et al (1996) when characterisation of the rat $P2Y_6$ receptor was performed with careful monitoring of nucleotide interconversion, and Harden et al (1997) conclude that the $P2Y_6$ receptor is highly selective for UDP compared to UTP. Although the presence of high concentrations of $P2Y_6$ mRNA in rat aorta suggests that the $P2Y_6$ receptor is present in these cells, it does not prove that the receptor is functionally expressed. Therefore, since UTP was more potent than UDP, and in the presence of hexokinase the potency of UDP was dramatically reduced, it is unlikely that the responses observed in SHR-VSMC were at $P2Y_6$ receptors.

Although it is possible that the responses observed in SHR-VSMCs were at a novel receptor subtype, the results obtained will be discussed in two separate hypotheses utilising P2Y receptors that have been cloned and well characterised. These hypotheses are:

1. *Two separate P2Y receptors coexist on SHR-VSMCs, one selective for UTP and one selective for both UTP and ATP.*
2. *A single receptor is responsible for the UTP- and ATP-induced responses.*

Although it is difficult to determine which of these theoretical situations exists, arguments for and against both of these hypotheses will be discussed.

The first hypothesis states that *two receptors coexist, one selective for UTP and one selective for both UTP and ATP*. The EC_{50} values for UTP and ATP are very similar, consistent with the presence of a $P2Y_2$ receptor. The fact that UTP elicits a greater response than ATP is consistent with the presence of a second receptor in addition to the $P2Y_2$ receptor; one that is predominantly activated by UTP (i.e. the

$P2Y_4$ receptor). However, ATP also activated the cloned $P2Y_4$ receptor (Figure 3.12), albeit to a lesser degree than the $P2Y_2$ receptor, and gave a response that was 57.7 ± 2.12 % of that to UTP. The maximal response achieved by ATP in the SHR-VSMCs was only 44.3 ± 5.3 % of that to UTP, so it is unlikely that ATP was acting at both a $P2Y_2$ and $P2Y_4$ receptor.

The second hypothesis states that *one single receptor is responsible for the ATP- and UTP-induced responses*. This receptor must be one at which ATP and UTP are equipotent, but at which UTP is almost twice as maximally effective. As has been discussed previously, the $P2Y_2$ fits the first part of the profile, whilst the $P2Y_4$ receptor fulfils the second requirement. However, the relative potencies may provide a clue to the receptor involved. While it is accepted that many factors can affect the potencies of agonists, such as differences in host cell transduction capabilities, the potency of a partial agonist would not be affected by increased receptor number, whereas the potency of a full agonist would be increased. Thus, if the receptor number was larger in the $P2Y_4$ transfected 1321N1 cells than in the SHR-VSMCs, the potency of UTP would appear higher in the 1321N1 cells while that of ATP would remain the same. When the position of the curve to ATP on the cloned $P2Y_4$ receptor (Figure 3.12) is compared to that observed in the SHR-VSMCs, it can be seen that that EC_{50} values are very similar (16.2 μM and 8.31 μM, respectively). However, the EC_{50} value for UTP is much lower in the 1321N1 cells than in the SHR-VSMCs, consistent with a larger receptor reserve in 1321N1 cells. The fact that ADP had no effect even in the absence on hexokinase also suggests that the receptor involved is of the $P2Y_4$ subtype. The final piece of evidence substantiating the $P2Y_4$ receptor is that provided by the co-addition of UTP and ATP. The results obtained with the 1321N1 cells and the SHR-VSMCs look remarkably similar. However, the concentration of ATP required to inhibit the UTP response in the 1321N1 cells was higher than that in the SHR-VSMCs. This can again be explained by difference in receptor number. If the concentration-response curve to UTP on the SHR-VSMCs lay close to, or upon the occupation curve of the receptor, then ATP would effectively reduce the response to UTP at lower concentrations that in a system where the UTP response had 'spare' receptors, as suggested for the 1321N1 cells.

It therefore seems likely that the responses observed to UTP and ATP in the SHR-VSMCs were at a single receptor, the $P2Y_4$. Further studies using antagonists would provide more information, and may allow a more conclusive classification.

6.4.3 Tissue differences in receptors

There are many conflicting reports of the P2Y receptor populations present in vascular smooth muscle cells in the literature. This is also apparent in the results presented in this chapter, for it seems that while the hSV-SMCs possess a $P2Y_2$ receptor, responses to ATP and UTP in the SHR-VSMCs were possibly at a $P2Y_4$ receptor. This may, in part, be due to the nature of the vessels studied. Large arteries such as the aorta are under constant high pressures, whereas veins are under less pressure and are more elastic. This difference in environment may affect the expression of P2 receptors. However, there is no clear evidence in the literature substantiating this suggestion.

In addition to these physiological differences, expression of P2 receptors on VSMCs has been shown to change with prolonged culture of cells. For example, in a study by Pacaud et al (1995), it was shown that while P2X receptors were expressed by primary cultures of aortic SMCs, these receptors were lost upon culturing. In contrast, a $P2Y_1$ response was only observed in cultured cells that had been stimulated with serum to induce cell cycle progression. A response was also observed to UTP, which had similar characteristics in both intact tissues and cultured cells. Studies on whole tissues, primary cultures, and relatively highly passaged cells all contribute to the literature on P2 receptor presence in VSMCs, and thus may also contribute to the inconsistencies in receptor classification.

Finally, the differences in apparent receptor expression may be a direct result of the use of the hypertensive model. The literature contains relatively few examples of P2Y receptor characterisation in VSMCs. Pharmacological techniques have suggested that responses to UTP are mediated via $P2Y_2$ receptors (e.g. Ralevic & Burnstock, 1991; Erlinge et al, 1995), while molecular cloning techniques have isolated both $P2Y_2$ and $P2Y_6$ clones from VSMCs (Chang et al, 1995). The results obtained with the hSV-SMCs are consistent with other reports in the literature, but there have been no reports describing the presence of a $P2Y_4$ receptor, as hypothesised

for the SHR-VSMCs. A possible explanation for this observation is that the difference in receptor subtype may be due to the hypertensive model studied. Indeed, recent work in our laboratory, conducted in parallel with the present study, have shown that VSMCs derived from WKY rats (a normotensive control) appear to possess a $P2Y_2$ receptor, that is, that ATP and UTP are equipotent and achieve a similar maximal response. Further work must be carried out before any firm conclusions can be drawn, but these findings are potentially very exciting.

6.4.4 Nucleotide induced proliferation

There is a growing body of evidence implicating P2Y receptors in cell proliferation e.g. $P2Y_2$ receptor-induced proliferation of sheep chondrocytes (Kaplan et al, 1996) and MCF-7 breast cancer cells (Dixon et al, 1997). In a detailed study, Yu et al (1996) demonstrated $P2Y_1$ receptor stimulated proliferation of rat aortic smooth muscle cells. The $P2Y_1$ receptors were coupled via a pertussis toxin-insensitive G protein to PLC, and subsequent ATP-induced Raf-1 and MAPK activations involved both PKCα and PKCδ. Stimulation of $P2Y_1$ receptors also caused accumulation of c-fos and c-myc mRNAs. Both Reactive blue 2 and staurosporine significantly blocked this increase by ATP. PKCδ has also been implicated in the activation of the MAPK cascade in ATP and UTP induced mesangial cell proliferation (Pfeilscifter & Huwiler, 1996). In a study by Malam-Souley et al (1996), it was shown that stimulation of $P2Y_2$ receptors on rat aortic smooth muscle cells induced transcription of the genes for chemotactic proteins. They suggest that by the production of these proteins, nucleotides may contribute to vascular or blood cell migration and proliferation and consequently to the genesis of arterial diseases.

Thus, the demonstration that ATP and UTP can stimulate mitogenesis in hSV-SMCs may have broad-reaching clinical implications. As discussed previously, in situations of compromised endothelium (as occurs during surgery), nucleotides in the blood (released from platelets and endothelial cells) can act directly upon the smooth muscle cells, causing proliferation. The development of specific P2Y antagonist may present a method of preventing the contribution nucleotides make to the proliferation of smooth muscle cells after surgery, and in the pathogenesis of atherosclerosis.

Special Note

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Chapter 7: The generation of GST fusion proteins

7.1 Introduction

A fusion protein is constructed by splicing the cDNA of a protein of interest to that of another, typically larger protein. When this DNA is expressed in bacteria, a fusion protein is synthesised. This chapter describes the production of glutathione s-transferase (GST) -fusion proteins to two different regions of the bovine $P2Y_1$ receptor for use in the generation of antibodies. Despite their potential usefulness, antibodies have not yet been produced to P2Y receptors, despite attempts by several groups to do so. Antibodies to a P2Y receptor would have several uses. Firstly, antibodies to each of the cloned receptors would be an invaluable tool for the verification of pharmacologically classified receptors. Although the detection of mRNA can suggest the presence of a particular receptor, the binding of a specific antibody to a protein of the correct molecular weight in cell membranes would be a more useful indication of which receptor subtype was present. Also, in the absence of reliable binding assays, antibodies could be used to estimate the level of expression of cloned and transfected P2Y receptors, and then to compare this to receptor number in native systems. Chemical modification of the receptors could be studied using immunoprecipitation. There is evidence that rapid receptor desensitisation is at least partly due to phosphorylation of serine/threonine residues on the C terminus of some receptors, e.g. those for platelet activating factor (Takano et al, 1994) and substance P (Sasakawa et al, 1994). Rapid desensitisation was observed with the cloned P2Y receptors (Section 5.3), and antibodies could be used to study the changes in receptor during these early events. The bovine $P2Y_1$ receptor was chosen to make fusion antibodies against because in bovine aortic endothelial cells, it was shown that responses to the $P2Y_1$, but not the $P2Y_2$ receptor, were reduced on activation of PKC (Purkiss et al, 1994).

7.1.1 Fusion proteins

Fusion proteins were chosen for several reasons. Firstly, the immunogenicity of a receptor fragment is often increased by fusing it to a larger protein. Immunogenicity can be further increased by coupling an antigen to large particles

such as red blood cells or beads, thereby rendering it more prone to phagocytosis by antigen presenting cells (Harlow & Lane, 1988). The purification step for GST fusion proteins involves the binding of the molecule to glutathione-sepharose (GS) beads, and then elution with 10 mM glutathione. However, if the fusion protein is not eluted it can be injected into the animals coupled to the beads, possibly resulting in an increased immunogenicity.

Secondly, the fusion proteins can be used for other purposes. Once the antisera has been produced and collected, specific antibodies can be purified by running the antisera down a column packed with GST fusion protein-bound GS beads. Specific antibodies would be retained by the fusion protein, and after washing can be eluted with high salt concentrations.

If it was established that the bovine $P2Y_1$ receptor was phosphorylated upon activation, the fusion protein could also be used as a substrate to determine the specific kinase responsible. Tobin et al (1996) gave a detailed account of the purification of the protein kinase responsible for the phosphorylation of the m3-muscarinic receptor, and a similar method could be applied to P2Y receptors. The suitability of the fusion protein as a substrate for the receptor kinase could be assessed by its ability to inhibit phosphorylation of the intact receptor in membrane preparations.

7.1.2 GST fusion vectors

GST fusion vectors are designed for inducible, high-level intracellular expression of genes or gene fragments as fusions with Schistosoma japonicum glutathione S-transferase. The GST fusion proteins can be easily detected using antibodies to GST. Many GST fusion vectors are available. Some are engineered to contain an expanded multiple cloning site (MCS), facilitating the cloning of cDNA inserts from libraries in lambda vectors, and others contain just two or three restriction sites. The vectors contain a *tac* promotor for chemically induced, high-level expression, a *lac* gene for use in *E. coli*, and an ampicillin restistance gene for selection of transformed colonies. The pGEX-2T vector that was chosen for these experminents contains restriction sites for BamHI and EcoRI, with an overlapping site for SmaI. The recognition sequences for EcoRI and BamHI are G↓AATTC and

G↓GATCC, respectively, where ↓ is the point of digestion. This vector also contains a thrombin protease recognition site for cleavage of the desired protein from the fusion product.

7.1.3 Receptor region

Several important factors govern the choice of receptor region. Firstly, the hydrophobicity of the fragment must be considered. A fusion protein with hydrophobic regions may be insoluble and thus compartmentalised into inclusion bodies in *E. coli*, hindering protein recovery. However, a fusion protein with hydrophobic domains would probably be more immunogenic than a soluble protein. Another consideration is the required specificity of the antibody to be produced. If the antibody is required to be specific for a receptor subtype across different species, then a region that is conserved across each of the species homologues should be chosen. Also, a fragment must not contain consensus sites for the restriction enzymes specific to the linker site in the vector. For example, the pGEX-2T vector used for this work contains BamHI and EcoRI restriction enzyme sites, so the fragment chosen must not contain consensus sites for these enzymes.

The C-terminus of G protein-coupled receptors has been shown to be important in the regulation of receptor activity in a number of studies. Most G protein-coupled receptors have a C-terminus that is rich in serine and threonine residues, and specific G protein-coupled receptor kinases (GRKs) phosphorylate certain of these residues, resulting in rapid desensitisation. A variety of truncated receptors without these residues have been constructed, and have shown varying degrees of resistance to agonist-induced desensitisation e.g. the receptors for platelet activating factor (Takano et al, 1994) and substance P (Sasakawa et al, 1994). The C-terminus of the bovine $P2Y_1$ contains 2 consensus phosphorylation sites for PKC, as well as others for PKA and caesine kinase. The C-terminus is also of interest because this region is poorly conserved between other P2Y receptors, increasing the probability of generating an antibody specific for the $P2Y_1$ receptor. Two regions were chosen, one purely hydophylic, and other with a hydrophobic domain:

1. C-terminus region including 6 base pairs into the 7th transmembrane region (7TM) (144 base pairs).
2. From the last 9 base pairs of the 6TM to the C-terminus, including the 3rd intracellular loop and 7TM (279 base pairs).

In order to amplify the required regions of the receptor, PCR primers were produced that were specific for those regions. Figure 7.0 illustrates the sections of receptor chosen for each fusion protein.

7.2 Polymerase chain reaction (PCR)

7.2.1 Primer Design

G-C base pairs have three hydrogen bonds while A-T have only two, so primers were designed to a G-C rich region of the receptor to increase annealing strength. The primers were designed with restriction enzyme sites at the 5' end to enable digestion of the fragment ends and ligation into the vector. Finally, three extra cytosine residues were added to facilitate the binding of the restriction enzymes. The primers chosen for each fragment were:

Fragment 1.

Forward primer: CCC GGA TCC GCA GGA GAT ACT TTC AGA AGG
BamHI A G D T F R R

Reverse primer: CCC GAA TTC TCA CAA GCT TGT ATC TCC
EcoRI Stop L S T D G

Fragment 2.

Forward primer: CCC GGA TCC ATG AAT TTG AGG GCC CGG CTG
BamHI M N L R A R L

Reverse primer: CCC GAA TTC TCA CAA GCT TGT ATC TCC
EcoRI Stop L S T D G

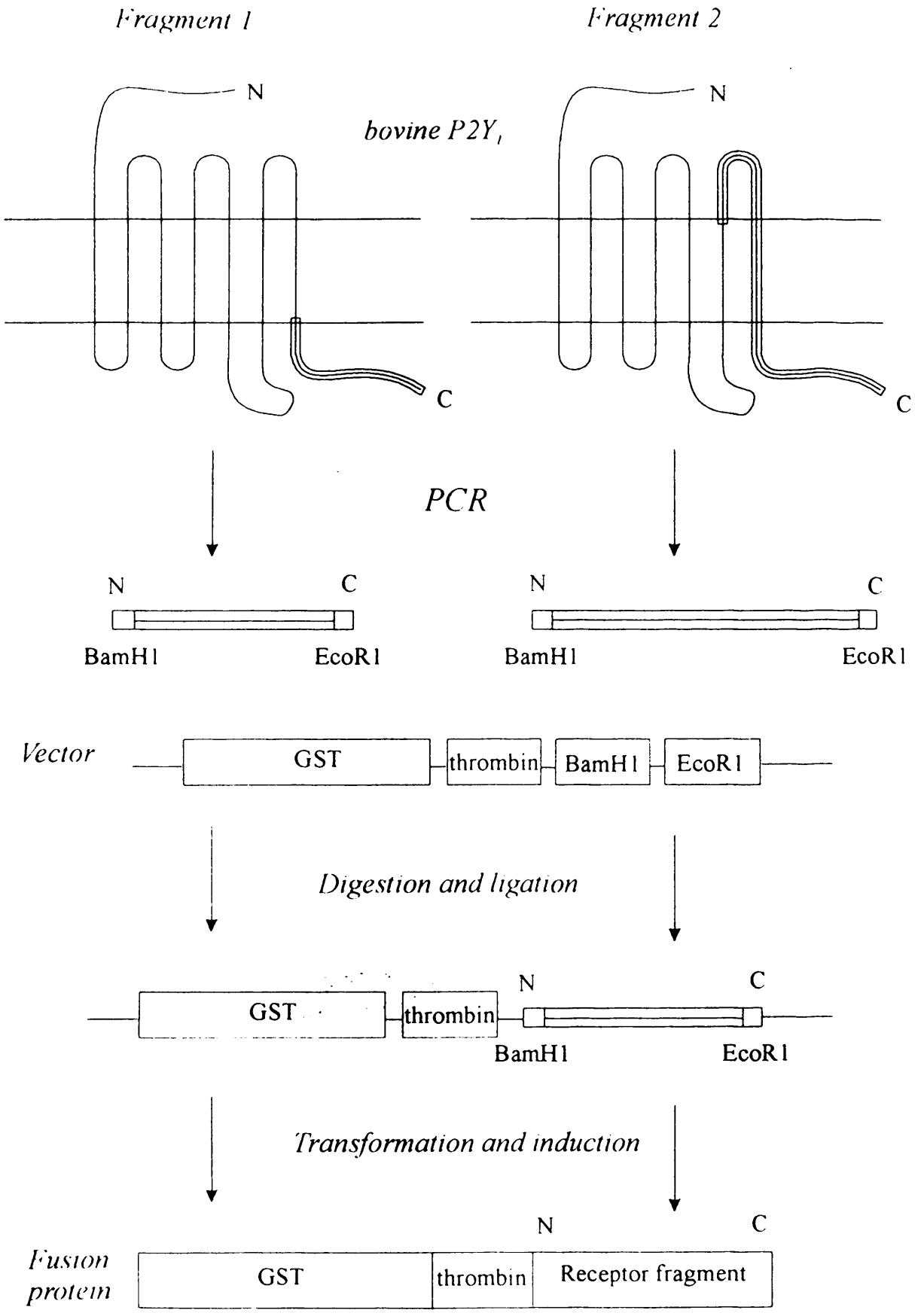

Figure 7.0
Schematic summary of the production of GST-P2Y$_1$ fusion proteins

7.2.3 Method

PCR was used to amplify the two regions of interest of the bovine $P2Y_1$ receptor. The bovine $P2Y_1$ template DNA was a gift from Duncan Henderson (Astra Charnwood), and consisted of the DNA encoding the bovine $P2Y_1$ receptor ligated into the *Not*I and *Hin*dIII polylinker cloning sites of the vector pRc/CMV (Invitrogen) (Henderson et al, 1995).

The PCR reaction mixture (100 µl final volume) consisted of 10 µl template DNA, 16 µl dNTPs (1,25 mM), 10 µl 10 × reaction buffer (100 mM Tris-HCl (pH 9.0), 15 mM $MgCl_2$, 500 mM KCl), 0.5 µl *Taq* DNA polymerase, 6.25 µl primers (1.0 µM final concentration). The mixture was cycled 25 times using the following programme: 1 minute denaturing step at 97 °C, 2 minute primer annealing step at 50 °C, and a 3 minute primer extension step at 72 °C (PCR machine from Perkin Elmer, Norwalk, CT, U.S.A).

When the reaction had finished, a small amount of the PCR reaction mixture was run on a 1 % agarose gel with a 100 bp DNA ladder (Gibco) to ensure the correct fragments were produced. The agarose gel consisted of 1 % agarose in Tris-acetate/EDTA buffer (40 mM Tris-acetate, 1 mM EDTA) with 0.1 µl/ml ethidium bromide, and was run for 45 minutes at 100 V. Figure 7.1 shows that for each reaction there was one clean band of DNA, with a smear of oligonucleotides at the leading edge of the gel. Fragment 1 was between the 100 and 200 bp markers, and fragment 2 was at the 300 bp marker. Including the primers, the predicted size of fragments 1 and 2 were 162 bp and 297 bp, respectively. The PCR products were purified and concentrated into 50 µl water using the Wizard™ Minipreps DNA purification system (Promega, Madison, WI, U.S.A).

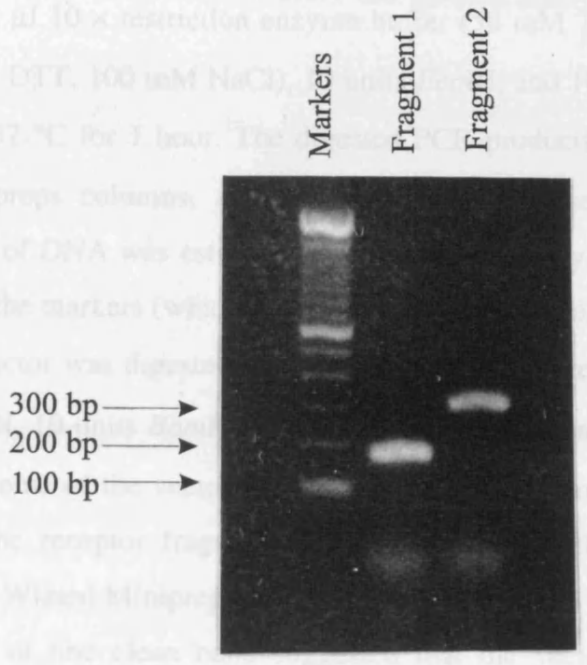

Figure 7.1

Agarose gel (1 %) of the PCR products fragment 1 and fragment 2.

The markers are a 100 base pair DNA ladder. See Figure 7.0 for representation of receptor fragments 1 and 2.

7.3 Insertion of the PCR products into the vector.

7.3.1 Digestion

Both the PCR products and the vector were digested with *Eco*RI and *Bam*HI restriction enzymes to provide complementary 'sticky' ends for ligation of the products into the vector. The PCR products were digested in a mixture containing 25 µl of DNA, 3 µl 10 × restriction enzyme buffer (50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 100 mM NaCl), 10 units *Eco*RI, and 10 units *Bam*HI, which was incubated at 37 °C for 1 hour. The digested PCR products were then purified using Wizard Minipreps columns, and run on a 1 % agarose gel (Figure 7.2A). The concentration of DNA was estimated for each fragment by comparing band intensity with those of the markers (which were at known, varying concentrations).

The vector was digested in a 50 µl reaction mixture containing 2 µl of DNA, 10 units *Eco*RI, 10 units *Bam*HI for 2 hours at 37 °C to ensure all of the vector was digested. If some of the vector remained intact, some transformed colonies that did not contain the receptor fragment would survive the selection. The vector was purified using Wizard Minipreps columns and run on a 1 % agarose gel (Figure 7.2B). The presence of one clean band suggested that the vector had been successfully digested, and there was no indication of coiled or supercoiled DNA.

7.3.2 Ligation

The PCR products were ligated into the vector using T4 DNA ligase (Promega). The ratio of molar concentrations of the insert DNA and vector has a profound effect on the efficiency of this enzyme, so two ligation ratios were used, 1:1 and 3:1 insert:vector. The ligation mixture (10 µl final volume) consisted of 1 µl buffer (×10), 100 ng pGEX-2T, 10 units T4 DNA ligase, 10 nM ATP, and PCR product (100 ng for 1:1, and 300 ng for 3:1), and was incubated at 37 °C for 1 hour. A reaction mixture containing no PCR product was also incubated at 37 °C for 1 hour to provide a negative control.

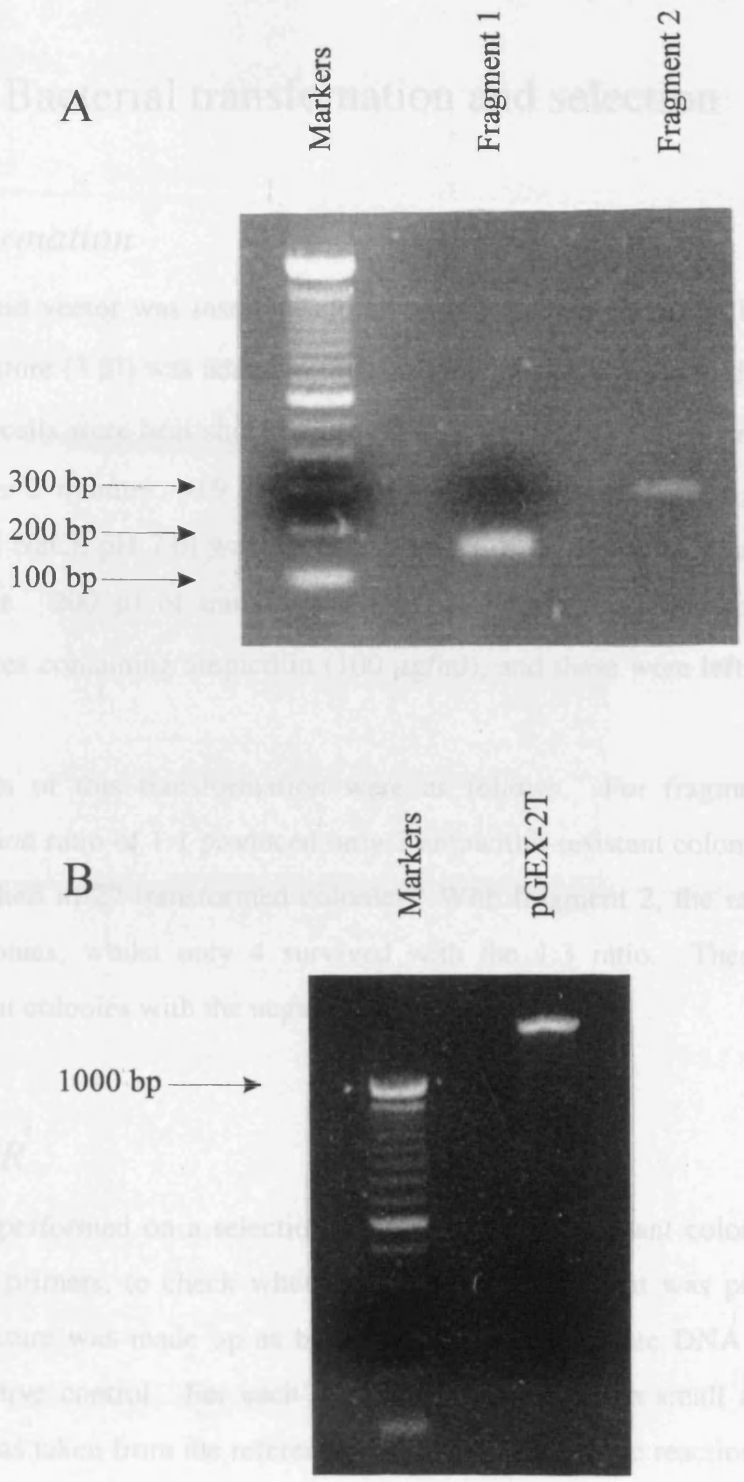

Figure 7.2

Agarose gel (1 %) of DNA after digestion with EcoR1 and BamH1.

PCR products fragment 1 and fragment 2 (**A**) and the pGEX-2T vector (**B**), were incubated for 1 hour at 37 °C with the EcoRI and BamHI (10 units each), and were purified as described in the text before electrophoresis. The markers are a 100 base pair DNA ladder.

7.4 Bacterial transfomation and selection

7.4.1 Transformation

The plasmid vector was inserted into competent *E. coli* DH5α cells (Gibco). DNA ligation mixture (3 µl) was added to 90 µl of cells, which were placed on ice for 30 minutes. The cells were heat shocked for 45 seconds at 42 °C, and were returned to ice for a further 2 minutes. 0.9 ml LB broth (10 mg/ml tryptane, 5 mg/ml yeast extract, 10 mg/ml NaCl; pH 7.0) was added and the cells were placed on a shaker at 37 °C for 1 hour. 200 µl of transformed bacteria were spread onto prewarmed bacterial agar plates containing ampicillin (100 µg/ml), and these were left overnight at 37 °C.

The results of this transformation were as follows. For fragment 1, the vector:insert ligation ratio of 1:1 produced only 2 ampicillin-resistant colonies, whilst the 1:3 ratio resulted in 27 transformed colonies. With fragment 2, the ratio of 1:1 produced 19 colonies, whilst only 4 survived with the 1:3 ratio. There were 5 ampicillin-resistant colonies with the negative control.

7.4.2 Test PCR

PCR was performed on a selection of the ampicillin resistant colonies using the original PCR primers, to check whether the receptor fragment was present. A PCR reaction mixture was made up as before, except that template DNA was only added to the positive control. For each colony that was tested, a small amount of bacterial matter was taken from the reference plate and placed in the reaction mixture. The PCR protocol used was as described previously. Figure 7.3 shows the results of the test PCR on a 1 % agarose gel. For fragment 1, 7 out of 11 colonies had a band of the same size as the positive control. These colonies were 131, 132, 133, 134, 136, 137, and 1310. With fragment 2, all but one colony had a band of the same size as the positive control. Positive colonies were 212, 213, 214, 215, 216, 217, 219, 2110, and 2111, with colony 218 giving a weaker signal.

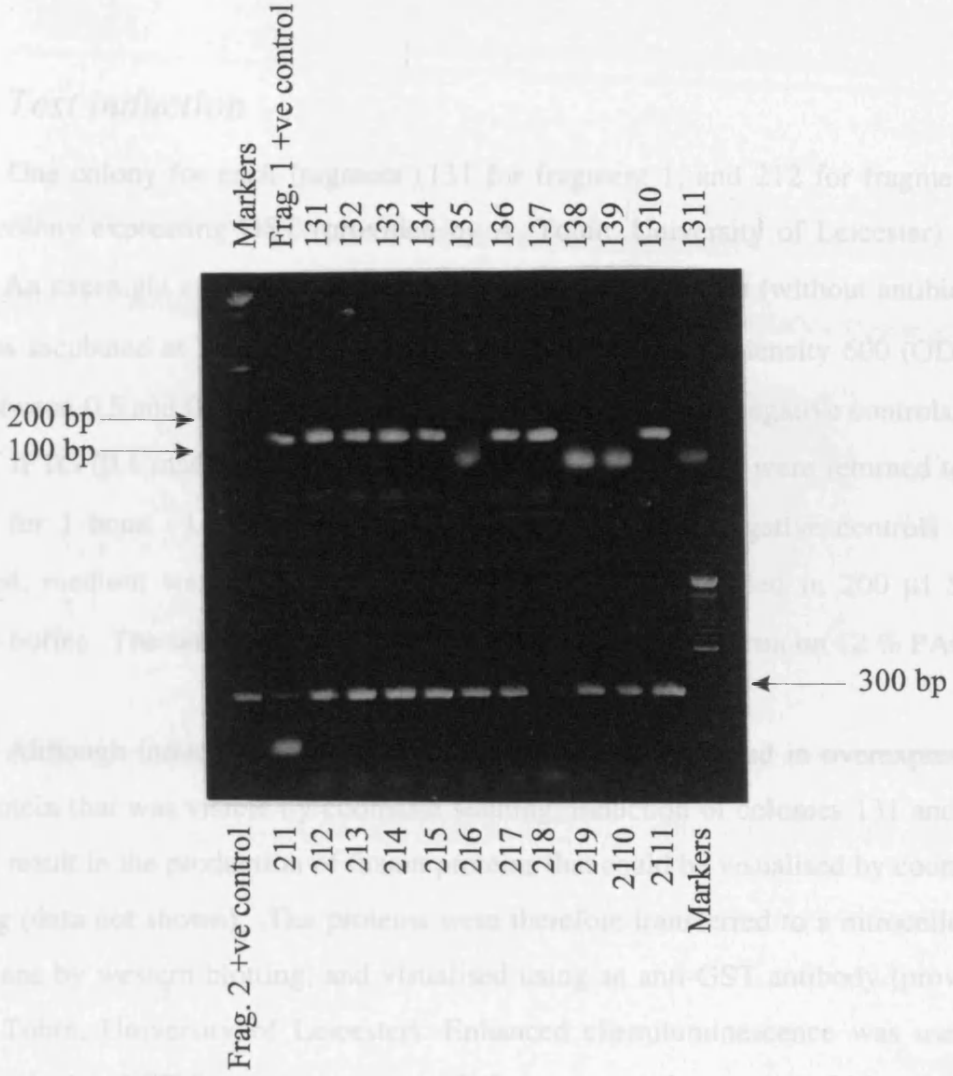

Figure 7.3

Agarose gel (1 %) of the test PCR of ampicillin resistant, transformed DH5α *E. coli* cells.

A selection of transformed colonies were tested using PCR with the original primers (see text for details). The positive controls were with original bovine $P2Y_1$ receptor template DNA, and contained no bacterial matter. The markers are a 100 base pair DNA ladder.

7.5 Protein overexpression and purification

7.5.1 Test induction

One colony for each fragment (131 for fragment 1, and 212 for fragment 2) and a colony expressing GST (provided by A. Tobin, University of Leicester) were tested. An overnight culture (250 µl) was added to 5 ml LB broth (without antibiotic), and was incubated at 37 °C for about 3 hours until the optical density 600 (OD600) was between 0.5 and 0.8. 1 ml of each culture was removed as negative controls, and 4 µl of IPTG (0.1 mM) was added to the remaining 4 ml. Both were returned to the shaker for 1 hour. 1 ml of IPTG treated cultures and the negative controls were pelletted, medium was aspirated, and the cells were resuspended in 200 µl SDS-sample buffer. The samples were boiled for 5 minutes, and were run on 12 % PAGE.

Although induction of the colony expressing GST resulted in overexpression of a protein that was visible by coomasie staining, induction of colonies 131 and 212 did not result in the production of fusion proteins that could be visualised by coomasie staining (data not shown). The proteins were therefore transferred to a nitrocellulose membrane by western blotting, and visualised using an anti-GST antibody (provided by A. Tobin, University of Leicester). Enhanced chemiluminescence was used to visualised the GST-fusion proteins, utilising a secondary, anti-rabbit antibody conjugated to horseradish peroxidase and ECL reagent (Amersham International, Bucks, U.K.).

Figure 7.4 shows the results of this test induction. Induction of the colony expressing GST resulted in the production of a protein that ran slightly faster than the 31 kDa marker (Lanes 1 and 2). GST has a molecular weight of 27.3 kDa, consistent with the induced protein being GST. Lanes 3 and 4 show non-induced and induced colony 131 respectively. The clear band at about 32-33 kDa in both lanes is consistent with the predicted molecular weight of fusion protein 1 (approximately 32.7 kDa). Although the inclusion of IPTG increased the level of expression, there was substantial production in the absence of IPTG (lane 3). In both cases there was a

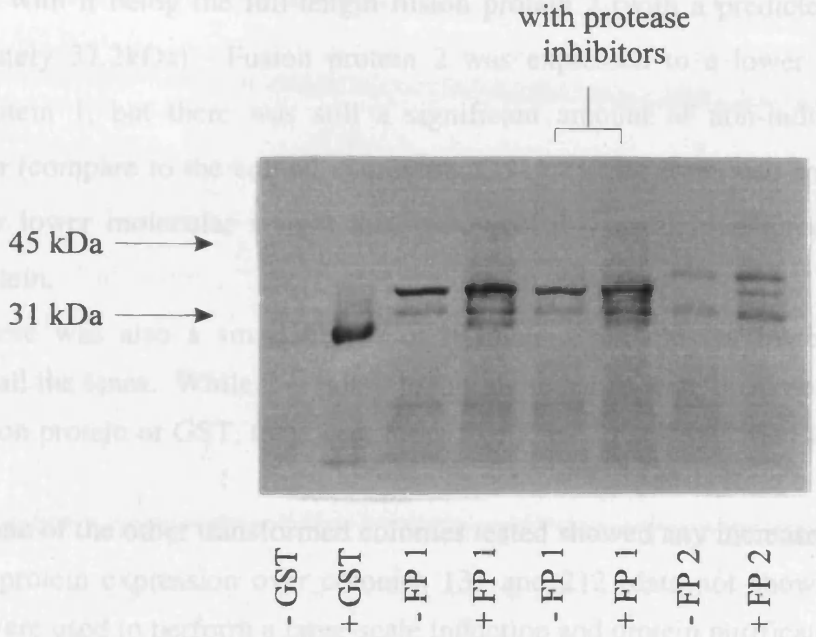

Figure 7.4

Western blot of the induction of colonies 131 (fusion protein 1) and 212 (fusion protein 2) in the absence and presence of protease inhibitors.

The two colonies and a control colony expressing GST were induced for 3 hours. Cells were pelletted, resuspended in SDS-sample buffer, and run on 12 % SDS-PAGE. Proteins were transfered to a nitrocellulose membrane by western blotting, probed with an anti-GST antibody (1:5000 dilution), and were visualised using an anti-rabbit secondary antibody (1:5000 dilution) with ECL reagent. See text for details of protease inhibitors. - is non-induced, + is induced, and fusion protein is abbreviated to FP.

band of approximately 31 kDa that was probably a breakdown product of the fusion protein. As with the intact protein, induction of colony 131 resulted in an increased production of this breakdown product.

Lanes 5 and 6 show induced and non-induced colony 212, respectively. The band of highest molecular weight is situated between the 45 and 31 kDa markers, consistent with it being the full-length fusion protein 2 (with a predicted weight of approximately 37.2kDa). Fusion protein 2 was expressed to a lower degree than fusion protein 1, but there was still a significant amount of non-induced protein production (compare to the colony expressing GST). There were also some proteins of slightly lower molecular weight that were probably breakdown products of the fusion protein.

There was also a small degree of binding to proteins of lower molecular weight in all the lanes. While it is possible that these bands were breakdown products of the fusion protein or GST, they were more likely due to non-specific binding of the antibody.

None of the other transformed colonies tested showed any increase in the level of fusion protein expression over colonies 131 and 212 (data not shown), so these colonies were used to perform a large-scale induction and protein purification.

7.5.2 Protein over-expression and purification

An overnight culture was diluted 1:30 into 200 ml LB, and was returned to the shaker for 3 hrs or until the OD600 was 0.5 to 0.8. The cells were induced by the addition of IPTG (0.1 mM), and returned to the shaker for 1 hr. A second 1 ml aliquot was removed from the induced cultures and was spun at 13000 × g for 1 minute. The cells were resuspended in 200 µl SDS sample buffer which was boiled for 5 minutes. The negative controls were treated in the same way.

The remaining induced cultures were centrifuged at 13000 × g for 10 minutes at 4 °C, and cells were resuspended in 10 ml ice cold Tris-EDTA (10 mM Tris base, 1 mM EDTA, pH 7.5) buffer (TE) with the protease inhibitors leupeptin (10 µg/ml), aprotinin (2 µg/ml), PMSF (2 mg/ml), pepstatin A (1 µM), iodoacetamide (2 mM), and benzamimide (1 mM). Cell suspensions were probe-sonicated on ice for three 10

second pulses, and were then centrifuged at 20,000 rpm for 10 minutes. The supernatant was removed and run down glutathione sepharose (GS) columns.

7.5.3 Glutathione sepharose (GS) columns

Two 1 ml aliquots of a suspension of glutathione sepharose 4B beads (Pharmacia Biotec, Sweden) were added to Biorad polythene columns (15 ml total volume), and were washed with 5 volumes of PBS. Samples were added to the columns, the eluent collected, and the columns washed with a further 5 volumes of PBS. If required, the fusion proteins can be eluted with 10mM glutathione. However, for the purpose of antibody production, the proteins were left bound to the beads. The results of the large-scale protein purification of fusion protein 1 and 2 are shown in Figure 7.5 and Figure 7.6 respectively.

7.3.5.1 Fusion protein 1

Lanes 1 and 2 show the induced and non-induced colony that expresses GST. The coomasie stained SDS-PAGE gel shows that the non-induced colony did not produce visible quantities of GST. However, upon induction by IPTG, a strong band corresponding to GST was observed. In contrast, a band corresponding to GST was easily observed in non-induced cells using western blotting with an anti-GST antibody showing that this method of visualisation was much more sensitive. The western blot of the induced GST-expressing cells was severely overloaded, resulting in the appearance of breakdown products. Several bands of higher molecular weight were also visible, and these were probably due to the overloading. Lane 3 shows the expression of fusion protein 1 in the whole, induced culture. There was no visible production of the protein as determined by coomasie staining, but it was visible on the western blot. At this early stage in the purification process, the protein was still largely intact, with very little breakdown evident. However, after cell lysis by probe sonication, and pelleting of the cell debris (lane 4), the band corresponding to the whole fusion protein all but disappeared, and was replaced by bands of lower molecular weight, presumably breakdown products. The strongest of these bands was of a similar size to GST, and suggests that the receptor fragment was digested from

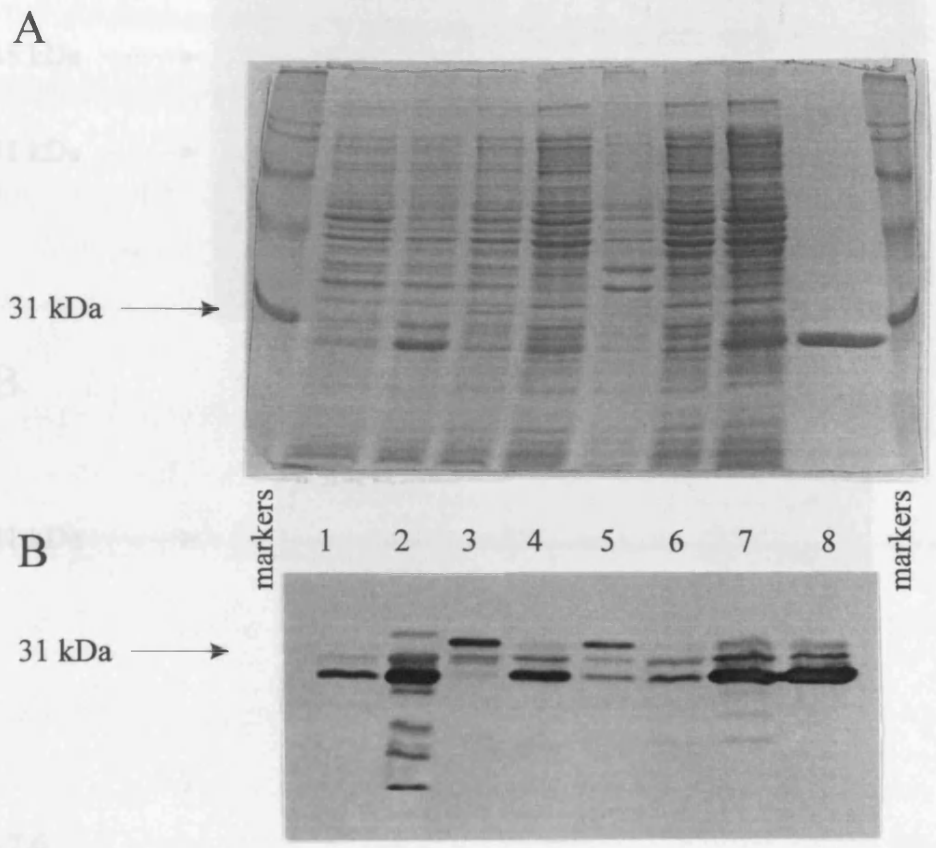

Figure 7.5

Large scale induction and purification of fusion protein 1.

Colony 131 and a control colony expressing GST were induced as described in the text, and fusion protein 1 was purified on glutathione-sepharose (GS) columns. Results are shown as both a Coomasie blue-stained 12 % SDS-PAGE (**A**) and a anti-GST probed western blot (**B**). 1, non-induced culture expressing GST; 2, induced culture expressing GST; 3, whole induced culture of colony 131; 4, cytosolic fraction of colony 131 after lysis of the cells by probe sonication; 5, pellet fraction after cell lysis; 6, eluent of cytosolic fraction from GS cloumns; 7, GS beads before washing; 8, GS beads after washing.

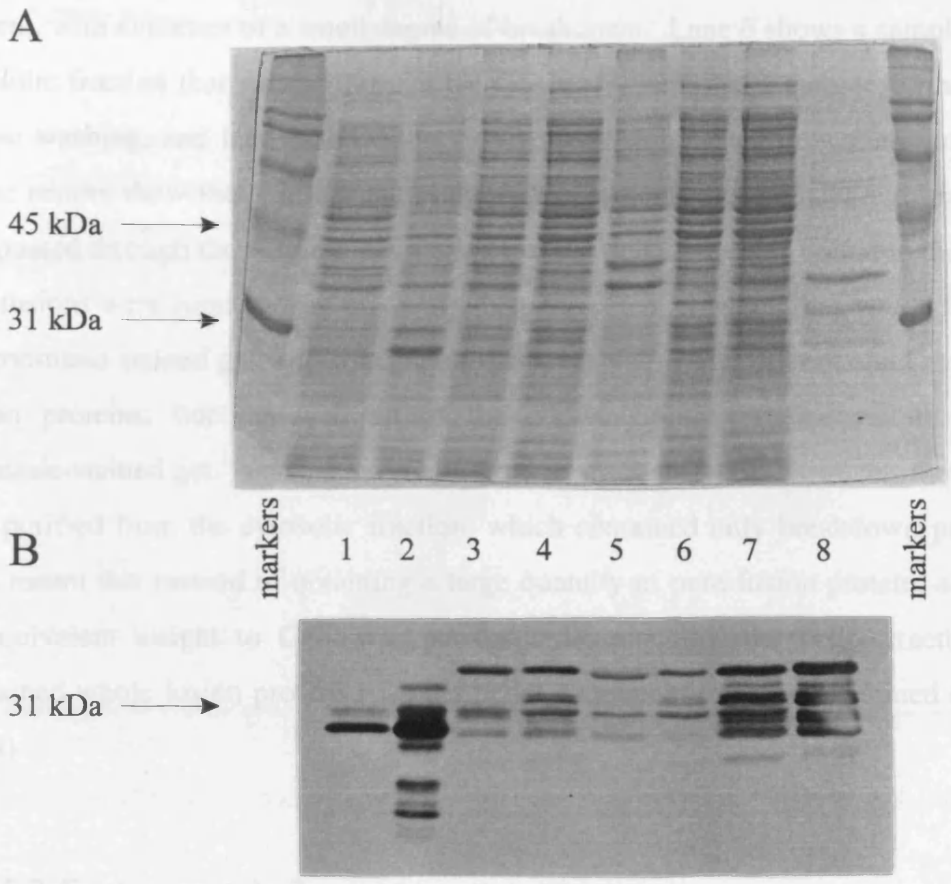

Figure 7.6

Large scale induction and purification of fusion protein 2.

Colony 212 and a control colony expressing GST were induced as described in the text, and fusion protein 1 was purified on glutathione-sepharose (GS) columns. Results are shown as both a Coomasie blue-stained 12 % SDS-PAGE (**A**) and a anti-GST probed western blot (**B**). 1, non-induced culture expressing GST; 2, induced culture expressing GST; 3, whole induced culture of colony 212; 4, cytosolic fraction of colony 212 after lysis of the cells by probe sonication; 5, pellet fraction after cell lysis; 6, eluent of cytosolic fraction from GS cloumns; 7, GS beads before washing; 8, GS beads after washing.

GST. Lane 5 shows the sample taken from the pellet produced when the lysed cells were spun down after probe sonication. In this fraction, the whole fusion protein was present, with evidence of a small degree of breakdown. Lane 6 shows a sample of the cytosolic fraction that passed through the GS beads, lane 7 is a sample of the beads before washing, and lane 8 shows the beads after washing with 5 volume of water. These results show that while some of the fusion proteins did not adhere to the beads and passed through the column, the majority of the fusion proteins bound to the beads. The fusions were concentrated on the beads to the extent at which they were visible on the coomasie stained gel, and washing of the beads with water did not elute any of the fusion proteins, but removed all of the contaminating proteins visible on the coomasie-stained gel. Thus, a very pure protein was achieved. However, the protein was purified from the cytosolic fraction, which contained only breakdown products. This meant that instead of obtaining a large quantity of pure fusion protein, a protein of equivalent weight to GST was purified. Despite this, the pellet fraction still contained whole fusion protein 1, so the pellet was solubilised and examined (section 7.5.4).

7.3.5.2 Fusion protein 2

As was observed with fusion protein 1, induction of the cells expressing GST resulted in the production of a protein of the correct molecular weight which was visible on a coomasie stained gel. Lane 3 shows that when the whole induced colony was examined, a protein of similar molecular weight as that predicted for fusion protein 2 was observed. There were also two bands of lower molecular weight which were probably breakdown products. However, unlike fusion protein 1, when the cells from colony 212 were lysed by probe sonication, fusion protein 2 remained largely intact. However, there was an additional breakdown band in the cytosolic fraction. Lane 5 shows that a detectable amount of fusion protein remained in the pellet fraction after probe sonication, and that the same breakdown products were present. When the cytosolic fraction was passed through the column, only a small amount failed to be retained by the GS beads (as shown in lane 6). After washing, only two bands were visible on the coomasie stained gel, that of the whole intact fusion protein

2, and one of its breakdown products. The western blot showed that there were also additional breakdown products.
Thus, unlike fusion protein 1, whole fusion protein 2 was purified on the GS beads.

7.5.4 Recovery of the fusion proteins from the pellet

High level expression of proteins in *E. coli* often results in cytoplasmic granules that can be separated from crude cell lysates by centrifugation. High concentrations of foreign proteins are sequestered in these inclusion bodies, which must be solubilised to obtain the fusion proteins. Marston (1987) has described a number of effective solubilisation reagents, including 8 M urea. Once the protein has been recovered from the inclusion bodies, it must be refolded by dialysis in decreasing concentrations of urea.

The pellet was resuspended in 10 ml 8 M urea. After 30 minutes at room temperature, the suspensions were centrifuged at 20000 rpm for 10 minutes. The supernatant was placed in dialysis membrane and was dialysed against 2 litres of PBS with decreasing concentrations of urea (i.e. 2M, 1 M, and 0.5 M urea). The supernatant was then dialysed against PBS for 48 hours with frequent changes of buffer. The samples were then run down the GSH columns and were examined using SDS PAGE and western blotting.

Figure 7.7A shows the results of solubilisation of the inclusion bodies of cells from colony 131. Lanes 2 and 3 show the pellet before and after urea treatment, respectively. Urea treatment removed virtually all of the fusion proteins, with only a small amount of one of the breakdown products remaining. Lane 4 shows this fraction after dialysis of the urea, and it is clear that in the process, all of the whole fusion protein 1 was broken down to a band of the same molecular weight as GST. Consequently, all further purification steps with fusion protein 1 contained only the degraded protein.

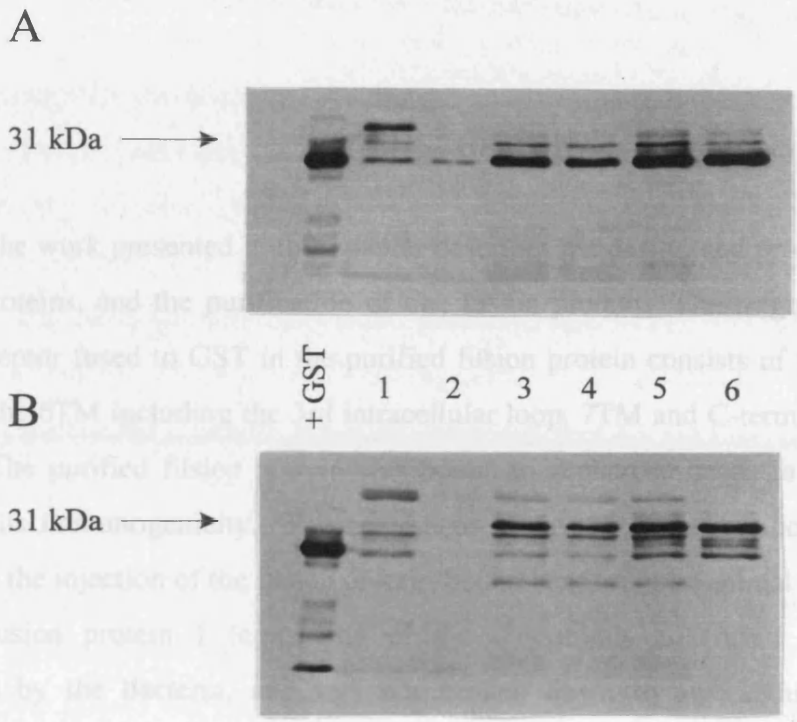

Figure 7.7

Western blot of the purification of the fusion proteins from the pellet fraction.

The pellets from the large scale induction of colonies 131 (**A**) and 212 (**B**) were resuspended in 8 M urea for 30 minutes at room temperature, centrifuged, and the supernatent was dialysed againsts PBS as sedcribed in the test. The fusion proteins were purified on glutathione-sepharose (GS) columns. 1, pellets before treatment; 2, pellets after urea treatment; 3, dialysis samples; 4, eluent of dialysed samples from GS cloumns; 5, GS beads before washing; 6, GS beads after washing.

Figure 7.7B shows the same method applied to cells from colony 212. Solubilisation of the pellet with 8 M urea resulted in the complete removal of all fusion protein 2 and its metabolites. However, like fusion protein 1, during the dialysis proceedure virtually all of the whole fusion protein 2 was degraded. As with fusion protein 1, all further purification steps resulted only in the purification of these breakdown products.

7.6 Discussion

The work presented in this chapter describes the design and production of two fusion proteins, and the purification of one fusion protein. The fragment of bovine $P2Y_1$ receptor fused to GST in the purified fusion protein consists of the last 9 base pairs of the 6TM including the 3rd intracellular loop, 7TM and C-terminus (279 base pairs). The purified fusion protein was bound to sepharose beads in an attempt to increase its immunogenicity. The next stage in the process of antibody production would be the injection of the fusion protein-bound beads into an animal.

Fusion protein 1 (consisting of the C-terminus tail fused to GST) was produced by the bacteria, and was not broken down to any significant degree. However, when the cells were lysed by sonication, the fusion protein was completely degraded. There are two possible explanations for this observation, and it is likely that both were responsible to some degree. Firstly, many protease enzymes are localised to intracellular compartments such as lysosomes. Sonication would break apart some of these lysosomes, releasing the cocktail of proteases which would rapidly degrade the fusion protein. It is also possible that the fusion protein was compartmentalised into inclusion bodies. This would increase the stability of the fusion protein as it would not be in contact with cytosolic proteases. Sonication may break apart some of these bodies, leaving the protein open to attack by proteases. Both these explanations are strengthened by the observation that the fusion protein remained whole in the pellet fraction after sonication.

Those inclusion bodies and lysosomes that were not lysed by sonication were pelleted with the cell debris. When this pellet was examined, it was found to contain

whole fusion protein. Treatment with 8 M urea removed all of the whole fusion protein from the pellet fraction (Figure 7.7), but this treatment would also have solubilised any proteases present in the pellet. Whilst in 8 M urea, the proteases would be rendered inactive due to the unfolding of their protein structure. However, as the samples were dialysed and all urea was removed, it is possible that the proteins refolded into their original structure. This appears to have occurred, because even at 4 °C there was complete degradation of the fusion protein in the dialysis membrane.

In contrast to fusion protein 1, fusion protein 2 was less liable to breakdown. It is possible that the degree of solubility of each protein may be a factor in their predisposal to degradation. Fusion protein 1 consists only of hydrophilic residues found in the cytosol, whereas fusion protein 2 consists of an additional transmembrane region of hydrophobic residues. It is therefore likely that fusion protein 1 was more soluble than fusion protein 2, and would consequently be more prone to degradation by proteases. This suggestion is strengthened by the observation that as soon as fusion protein 1 was solubilised (e.g. directly after its removal from the pellet fraction) it was broken down.

Several measures could be taken to try to reduce the proteolysis of the fusion proteins. Firstly, the inclusion of protease inhibitors at every step of the purification process may reduce the degradation. In the procedure described above, protease inhibitors were included in the intial lysis of the cells, but were not used during the dialysis step. However, as breakdown of fusion protein 1 occured even in the presence of protease inhibitors, a larger range of inhibitors should be utilised.

Secondly, the stability of the fusion proteins may have been enhanced by the use of a lon^- E.coli strain. Lon^- bacteria have a mutation that renders the major protease La inactive. This mutation has been shown to prevent in vivo proteolytic processing (e.g. Allet et al, 1988). However, lon deficiency is not always of benefit. For example, in a study by Minus & Bailey (1995), it was shown that although lon deficiency slightly increased the stability of amylase during the late stationary phase of cell growth, the specific productivity of the cells was only about 40-60 % of the non-lon^- strain. Therefore, as the majority of fusion protein breakdown occured once the cells were lysed, it is not clear whether the use of lon^- cells would increase the overall stability of the fusion proteins to a significant degree.

Although significant progress was made in this project, a large body of work still remains. The next stage in the production of antibodies to the bovine $P2Y_1$ receptor would be the injection of the fusion protein-bound sepharose beads into an animal. Although mice, rats, hamsters and guinea-pigs are also used, rabbits represent the animal of choice for polyclonal antibody production in most cases. Rabbits are easy to keep and handle, provide a large volume of serum, and secondary anti-rabbit antibodies are widely produced. Several animals should always be used for any immunisation scheme, as even genetically identical animals will elicit different antibodies to the same antigen. The beads would be mixed with an adjuvant and administered by intradermal injections, the normal route for insoluble matter. After a minimum of 2 or 3 weeks, the antigen should be reintroduced. Levels of antibody in the serum increase exponentially to reach a peak at about 10-14 days, and high levels of antibody typically persist for 2-4 weeks. Further injections can be given, often resulting in affinity maturation of the antibody. The serum should be sampled regularly during the whole immunisation regime, and collected when antibody titre reaches a peak. The antibodies can then be purified by passing down a column containing the fusion protein-bound beads. Antibodies specific for the fusion protein will be retained on the column, and can be eluted at high salt concentrations.

Chapter 8: Discussion

The relatively recent introduction of molecular cloning techniques into the field of P2 receptor research has resulted in the isolation of cDNAs coding for seven P2X receptors and up to six P2Y receptors. This has revolutionised the study of these receptors, allowing the examination of receptors in isolation and in carefully controlled conditions. The aims of this thesis were firstly to characterise several of the cloned P2Y receptors transfected into 1321N1 astrocytoma cells, and secondly to use this information to aid the classification of the P2Y receptors present on two vascular smooth muscle cell types.

This chapter will highlight some of the important issues in the field of cloned P2 receptor characterisation, including a discussion into the validity of the study of transduction mechanisms in transfected systems. It will also attempt a general review of the current status of P2Y receptor classification, with a further section on the various ways cloned receptors have aided the classification of native receptors.

8.1 Important issues

8.1.1 The choice of host cell type

The study of cloned P2Y receptors requires a host cell that possesses no natively expressed P2Y receptors, so that the receptor can be studied in isolation. Although this point seems obvious, several mistakes have been made in the choice of cell type. Many of the cloned receptors have been transiently transfected into *Xenopus laevis* oocytes for preliminary characterisation, but the mammalian cells utilised for stable transfection have been more varied.

Non-adherent cells such as Jurkat human T lymphocytes (Henderson et al, 1995) and K562 human leukemia cells (Parr et al, 1994) have been used to measure increases in intracellular Ca^{2+} levels in response to P2Y receptors. However, in such assays the cells are constantly stirred. This mechanical aggitation causes the release of endogenous ATP and the subsequent desensitisation of the transfected receptor. For example, Henderson et al (1995) found that only the inclusion of apyrase reversed the desensitised state of the transfected bovine $P2Y_1$ receptor in Jurkat cells. However, the

enzyme apyrase degrades P2Y agonists, necessitating thorough washing and further mechanical dispruption of the cells prior to the addition of agonist. C6-15 rat glioma cells were used to first characterise the $P2Y_6$ receptor (Chang et al, 1995), but these cells also show small responses to nucleotides in the absence of transfection.
The COS-7 cells utilised by Akbar et al (1997) to host the putative $P2Y_7$ receptor clone have since been shown to respond to P2Y agonists in the absence of the $P2Y_7$ DNA (Yokomizo et al, 1997). COS-7 cells were also used to host the 6H1 (the so-called $P2Y_5$) receptor (Webb et al, 1995), at which specific binding was reported. In the light of the findings by Yokomizo et al, and the controversy surrounding binding assays for P2Y receptor, it seems unlikely that the observed binding was at the 6H1 receptor.

A clonal line of 1321N1 human astrocytoma cells that did not respond to P2Y agonists was first used to host the human $P2Y_2$ receptor (Parr et al, 1994) and the turkey $P2Y_1$ receptor (Filtz et al, 1994). Since then, 1321N1 cells expressing each of the cloned P2Y receptors have been produced. The 1321N1 cell line remains the most popular choice for the study of cloned P2Y receptors for several reasons. The cells grow rapidly, and high levels of receptor expression are relatively easy to obtain. They are also adherent, and so are suited to a more varied range of biochemical measurements. This, however, does not mean that they are without fault.
Like many other cell types, 1321N1 cells release ATP into the extracellular space in response to mechanical sheer stress. The exact physiological significance of this phenomenon is unclear, although some possible roles have been discused previously (Chapter 3). Release of endogenous ATP must obviously be avoided, and some preliminary work presented in this thesis has described the development of an assay procedure that avoids this phenomenon.

8.1.2 Nucleotide interconversion

An issue that is rapidly becoming one of the most important factors in the study of P2 receptors is that of nucleotide interconversion. Using shorter stimulation times and an ectonucleotidase inhibitor it was shown that any ectonucleotidase activity in the cells used in this study did not significantly alter observed agonist potencies, in contrast to

many other studies (e.g. Kennedy & Leff, 1995). However, when hexokinase was used, it was found that a considerable amount of the response to nucleoside diphosphates was via their conversion to triphosphates, as reported by Nicholas et al (1996). The enzyme responsible for this phosphorylation is an ecto-nucleoside diphosphokinase (NDPK), which transfers the γ-phosphate from a nucleoside triphosphate to a diphosphate. In a recent study by Lazarowski et al (1997), the activity of this enzyme was measured and was found to exceed that of the extracellular nucleotidase activity by up to 20-fold. Although most of the work undertaken in this area has been on 1321N1 cells, ecto-NDPK is not limited to this tumour cell line. For example, similar extracellular conversion has been observed in polarised human airway epithelial cells (Lazarowski et al, 1995), and in pig aortic endothelial cells (Pearson et al, 1980). These findings have many implications, not only for the characterisation of receptors, but also for the question of the physiological relevance of such a conversion. It is interesting to speculate that high NDPK activity, coupled with the endogenous release of ATP, is an important mechanism for maintaining nucleoside triphosphate levels in the extracellular space of the periphery. This idea is extremely novel, as work to date has focused almost entirely upon the rapid removal of nucleoside triphosphates from the extracellular space.

These findings suggest that our approach to P2-signalling must be revised. The possible physiological relevance of two opposing pathways for nucleotide interconversion will be discussed by forming a hypothesis. It is unlikely that two such opposing pathways would be present in the same cell or tissue type, as this would result in wasteful cycling. Thus, the hypothesis is that *in any one tissue, only one of these pathways is prevelant, and that the tissue distribution of each pathway is dependent upon the function of nucleotides in that system.*

For example, in fast excitatory P2X-mediated synapses, the response to ATP must be transient and rapidly terminated. Indeed, if the post-synaptic P2X receptors were open for any length of time, the excessive Ca^{2+} influx would cause cell death (see Edwards, 1996). Like the signal in cholinergic neurons, termination of the response at P2X receptors is dependant upon the rapid breakdown of neurotransmiter (although rapid desensitisation of certain receptors may also occur). Such rapid breakdown of ATP has been demonstrated in synaptic clefts of the sympathetic neurons that innervate the

guinea-pig vas deferens (Kennedy et al, 1996; Westfall et al, 1996). In addition, Todorov et al (1997) have recently demonstrated that stimulation of these nerves releases not only neuronal ATP, but also soluble nucleotidases that hydrolyse the released ATP to adenosine. It is therefore apparent that in this situation, the predominant ecto-enzymes are nucleotidases.

In contrast, it is possible that in some tissues extracellular nucleoside triphosphates are required for much longer periods of time. Examples of such long-term (or trophic) actions of nucleoside triphosphates are becoming more widespread. For example, much interest has focused on the mitogenic roles of extracellular ATP, such as that observed in astrocytes, where ATP greatly enhances mitogenesis induced by polypeptide growth factors (Neary et al, 1994b). This is consistent with the observation that in 1321N1 astrocytoma cells, levels of nucleoside triphosphate are maintained by high NDPK activity and low ectonucleotidase activity (Harden et al, 1997; this thesis). Indeed, it is possible that cell death and the consequent release of endogenous nucleotides may act as a paracrine signal to nearby cells, causing cell proliferation and replacement, and that this signal is maintained for long enough periods by ecto-NDPK.

Thus, it is conceivable that nucleotides are required for different time periods in different tissues, and that differential distribution of these ecto-enzymes reflects the diverse role of extracellular nucleotides.

8.2 Transduction mechanisms

In addition to the pharmacological characterisation of the cloned receptors, work in this thesis has attempted to examine some of the events that occur downstream of agonist binding, including G protein coupling, the kinetics of PLC activation, and the effects of PKC. Although the results are discussed in detail in chapter 5, several issues pertaining to the usefulness of cloned systems in the study of downstream events must be addressed.

The usefulness of cloned receptors resides in the ability to study them in isolation. Ideally, the receptors would be expressed in membranes and studied using

radioligand-binding assays, as well as in intact cells. However, as this can not yet be achieved, measurements are made close to the receptor, to avoid excessive modulation of the response by other factors. Despite this, several factors can effect responses measured even at the level of PLC activation, and these have been discused elsewhere (see chapter 5). With this in mind, can the investigation of components further downstream, e.g. PKC, be justified?

Perhaps the best way to approach this issue is to firstly define the question being asked. This issue was approached in light of the finding that in BAECs, responses at the $P2Y_2$ receptor were unaffected by PKC stimulation, whereas responses at the $P2Y_1$ receptor were inhibited. Therefore, the experiments were performed only to test whether each receptor was modulated in a different manner, not to examine the nature of this phenomenon in 1321N1 cells. The results in this thesis showed that PKC activation effected both receptors to a similar degree. Two possible explanations for these results were discussed in detail in Chapter 5, but briefly, either each receptor was phosphorylated in the same way by PKC, or the negative feedback by PKC was not determined by the specific receptor, but was due to inhibition of other components of the transduction pathway.

Further study of the actions of PKC on cloned receptors should only be examined if the first case is proven, as in this instance, the actual receptors are involved. PKC phosphorylation of the receptor protein could then be measured. However, if the second suggestion were shown to be correct and regulation occurs at the level of other components in the transduction pathway, any further examination would provide information on the host cell, and not the receptors. This example illustrates the caution that must be exercised when examining cloned and transfected receptors. Each experiment must be undertaken with a clear objective, and care must be taken not to over-interpret results.

8.3 Review of P2Y classification

The advent of molecular cloning has resulted in the last few years being amongst the most productive periods in the relatively short history of P2 receptor research. As the

pace quickens with each new receptor cloned, an up to date review of the present status of P2Y receptor subtypes will be discussed.

Although the cloning of seven separate P2Y receptors has been described, the validity of some of these reports has been questioned. As was discussed in chapter 1, similarity in both sequence and functional properties has lead to the suggestion that the $P2Y_3$ receptor is in fact the chick orthologue of the rat and human $P2Y_6$ receptor, rather than a genuinely distinct subtype. Initially, it was argued that because the amino acid identity was only 60 % (less than the 83 % similarity between the chick and the human $P2Y_1$ receptors), the $P2Y_3$ receptor must represent a novel subtype. However, this sequence identity is higher than that found between various P2Y subypes, typically 35-40 %, and if the very similar agonist potency profiles are also taken into consideration, it seems likely that the $P2Y_3$ receptor is the chick orthologue of the rat and human $P2Y_6$ receptor.

The controversy surrounding the binding assay and cells used to classify the orphan receptor 6H1 as the $P2Y_5$ receptor has also been discussed elsewhere in this thesis. However, further evidence opposing this assertion has very recently been published. A human seven transmembrane receptor was cloned that exhibited close structural homology to the chicken $P2Y_5$ clone (Janssens et al, 1997), but when this receptor was transfected into several different cell types (including 1321N1), none of the 40 nucleotides tested elicited a cAMP, $InsP_X$, or Ca^{2+} response. The authors conclude that either the receptor is activated by an uncommon nucleotide, or that despite its structural similarity, the $P2Y_5$ receptor is not a member of the P2Y receptor family.

Finally, in a report by Yokomizo et al (1997), the so-called $P2Y_7$ receptor was shown to actually correspond to a receptor for leukotriene B_4. Thus, only four of the seven cloned receptors appear to be novel P2Y receptors. This highlights the imbalance between the rates of cloning new potential receptors and their detailed pharmacological characterisation.

Thus, ironically, it seems that of all the cloned metabotropic receptors once termed 'purinoceptors', only one is preferentially activated by purines. A second receptor is activated by both purine and pyrimidine nucleotides, with the remaining two receptors being activated exculsively by pyrimidine nucleotides. The modern classification system suggested by IUPHAR overcomes this inadequacy, but should the receptors

for UTP and UDP (i.e. the $P2Y_4$ and the $P2Y_6$ receptors) constitute a separate pyrimidinergic subgroup of receptors within the P2Y family?

Review of the sequences of the $P2Y_4$ and $P2Y_6$ subtypes reveals a 40 % amino acid identity, a value that is lower than the 51 % identity between the $P2Y_4$ and $P2Y_2$ receptors. In fact, this similarity is only marginally superior to the 35 % identity between the $P2Y_4$ or $P2Y_6$ subtypes and the $P2Y_1$ receptor, which has no affinity for UTP or UDP. Furthermore, the $P2Y_4$ and $P2Y_6$ receptors share a seven amino acid consensus sequence located in TM3 of all the cloned P2Y receptors (see section 1.5.2). It therefore appears that there is no structural basis for the subdivision of P2Y receptors into purinoceptors and pyrimidinoceptors (see Communi & Boeynaems, 1997, for review).

These four clones appear to be sufficient to account for most of the G protein-coupled responses observed to nucleotides throughout the body, with two exceptions. These are the platelet receptor for ADP and the adenylyl cyclase linked receptor in C6-glioma cells, both of which will be discussed in the next section.

The arrival of P2 receptor clones has had a large impact in the field. Two examples that highlight the value of cloned receptors will be discussed in the section, those of native receptor classification and advances in drug discovery.

8.4 Characterisation of native receptors

Above all, cloned and transfected receptors for the first time permit the designation of a particular pharmacological profile to a known molecular structure. This has enabled the characterisation of defined proteins, the results of which have been used to aid the classification of native receptors.

The detailed examination and characterisation of cloned P2Y receptors has made an invaluable contribution to the classification of P2 receptors in many native systems. In some cases (e.g. endothelial cells), the cloning of P2Y receptors has served to reinforce ideas already proposed, whereas in others (e.g. platelets), the cloning has contributed to the suggestion of new theories. Several examples will be

discussed in this section, highlighting the impact P2Y receptor cloning has had with respect to the classification of native receptors.

8.4.1 Endothelial Cells

Endothelial cells have been the focus of many studies and the subtypes of P2Y receptors present have been well characterised. They have long been known to respond to nucleotides, and in 1993 two groups published data in support of the coexpression of two P2Y receptors (Wilkinson et al, 1993; Motte et al, 1993). The subtypes were termed P_{2Y}, at which ATP and ADP acted, and P_{2U}, for ATP and UTP. In 1994, Purkiss et al showed that these two receptors were differentially coupled to G proteins. They found that pertussis toxin reduced the response to the P_{2U} receptor, but had no effect on the P_{2Y} receptor. Later, characterisation of the cloned of the $P2Y_1$ and $P2Y_2$ receptors showed that they corresponded to the previously described P_{2Y} and P_{2U} receptors. Thus, a defined molecular structure was asigned to the receptors present in endothelial cells. In addition, transfection of these receptors into 1321N1 cells has shown that the effect of pertussis toxin on the individual receptors was consistent with that observed in endothelial cells (this thesis).

This example illustrates how the cloning of P2Y receptors has served to confirm the existance of receptors that had been previously proposed. However, in other tissues, transfected receptors have had a more profound influence on the classification of native receptors.

8.4.2 Vascular smooth muscle

An ongoing interest in the laboratory of the vascular aspects of P2-signalling lead to the examination of the P2Y receptors present on two vascular smooth muscle cell types. As was discussed previously in Chapter 6, it seems likely that the P2Y receptor present in smooth muscle cells derived from the aorta of spontaneously hypertensive rat is of the $P2Y_1$ subtype. The detailed discussion will not be repeated in this section, but it is clear that information obtained from the cloned receptors allowed the direct comparison of concentration response curves and experiments involving co-

addition of UTP and ATP, and as such played a critical role in reaching the conclusion.

Most of the smooth muscle P2Y receptors for UTP characterised to date have been classed as $P2Y_2$ receptors, indeed, it is possoble that the receptor present in human saphenous vein smooth muscle cells (hSV-SMCs) is of the $P2Y_2$ subtype. However, the notion that certain smooth muscle cells possess receptors specifically for UTP is not a new one. There are several examples of smooth muscle cells that appear to express receptors specific for uracil nucleotides, e.g. in the dog maxillary internal vein (Saiag et al, 1992), and in isolated rat aorta (Garcia-Velasco et al, 1995). It is possible that now the cloned $P2Y_4$ and $P2Y_6$ receptors have been pharmacologically characterised, the receptors for uracil nucleotides will be found in many more tissues.

8.4.3 Hepatocytes

Hepatocytes provide an example of a different use of cloned receptor information, that of tissue distribution. ATP has been shown to induce liver glycogenolysis in an $Ins(1,4,5)P_3/Ca^{2+}$ mediated manner (Charest et al, 1985), an effect that was insensitive to pretreatment with pertussis toxin (Okajima et al, 1987). The receptor at which ATP acted was classified as a P_{2Y} receptor (Keppens & Wulf, 1986), based on the proposals of Burnstock & Kennedy (1985). However, in 1991, Keppens et al suggested that in addition to the $P2Y_1$ receptor, another P2 subtype was present on the surface of rabbit and guinea-pig hepatocytes. This suggestion arose from data showing that 2MeSATP-stimulated an increase in glycogenolysis that was not mediated by the PLC/$Ins(1,4,5)P_3$ pathway.

The advent of P2 receptor cloning saw the undertaking of many tissue distribution studies. Rice et al (1995) showed that there was no $P2Y_2$ receptor mRNA present in liver, whilst Tokyama et al (1995) demonstrated $P2Y_1$ receptor mRNA in both rat and mouse liver, consistent with the earlier pharmacological studies. In 1994, Valera et al cloned the $P2X_1$ receptor, and found that it was activated preferentially by 2MeSATP. In addition, mRNA for the $P2X_1$ receptor was later found in liver (Longhurst et al, 1996).

Thus, although other reports have shown Ca^{2+} mobilisation responses to 2MeSATP which are not dependent upon Ca^{2+} entry or Ins(1,4,5)P_3 generation (e.g. Frelin et al, 1993; Vigne et al, 1994; Albert et al, 1997), it is also possible that the $P2X_1$ receptor on hepatocytes is responsible for the non Ins(1,4,5)P_3-mediated stimulation of glycogenolysis by 2MeSATP.

8.4.4 Platelets

The exact nature of the ADP receptor present on platelets has been a constant source of speculation since it was first described in 1962 by Born. Many studies have shown that ADP is the most potent agonist, with ATP acting as an antagonist (e.g. Hourani & Hall, 1994), and the receptor has been named the P_{2T} receptor (Gordon et al, 1986).

Recently, Greco (1997) carried out an experiment to see if the characteristic P_{2T} response could be confered to a null cell on injection of RNA from a cell expressing the P_{2T} receptor. He found that the megakaryoblastic cell line, CMK 11-5, responded with an increase in intracellular Ca^{2+} mobilization in response to ADP but not to ATP (which acted as an antagonist) and concluded that a P_{2T} receptor was responsible. He then took RNA from the CMK 11-5 cells and injected it into *Xenopus oocytes*, and found that this confered a response to ADP that was inhibited by ATP, and was unlike any response at other P2Y receptors. Thus, the RNA encoding an ADP receptor was present in these cells. However, although many groups have attempted it, the P_{2T} receptor has yet to be cloned, fuelling speculation that the responses to ADP in platelets are mediated by a known and cloned P2Y receptor, and not by a separate P_{2T} subtype.

Leon et al (1997) have provided some strong evidence that the receptor responsible for mediating the ADP response is actually the cloned $P2Y_1$ receptor. When the human $P2Y_1$ receptor was transfected into Jurkat cells, they found it displayed a similar pharmacology to that of the P_{2T} receptor. Using HPLC-purified nucleotides, they found that 2MeSADP and ADP were full agonists, while ATP was an antagonist. Following this finding, they examined the distribution of $P2Y_1$ receptor mRNA, and found that it was present in platelets and megakaryoblastic cell lines. As a $P2Y_1$ receptor response has not previously been described before in platelets, despite

the presence of $P2Y_1$ receptor mRNA, they suggest that the ADP response described in platelets is at the $P2Y_1$ receptor. These data are consistent with a previous report by Henderson et al (1995). When they transfected the bovine $P2Y_1$ receptor into Jurkat cells, they found that ATP was a partial agonist, achieving only 60 % of the maximal stimulation obtained with ADP.

However, although these data are convincing, the results from Leon et al (1997) and Henderson et al (1995) contrast sharply to those obtained by other groups. For example, Schachter & Harden (1997) have shown that ATP is a full agonist at the human $P2Y_1$ receptor transfected into 1321N1 cells. Also, results obtained with the bovine homologue of the $P2Y_1$ receptor in 1321N1 cells (this thesis) have shown that ATP and ATPγS are full agonists. This is surprising, and suggests that the different agonist potencies may, in part, be due to differences in host cell.

As this whole issue seems to rest upon different degrees of ATP agonism, it may be useful to first define the terms 'full' and 'partial' agonism. A 'full' agonist is a ligand that elicits the same maximal response as the most 'potent' agonist tested. The maximal response to a 'partial' agonist may still plateau, but at a lower level of response to that of a full agonist. Thus, a compound that appears to be a full agonist when compared with less effective agonists may actually appear as a partial agonist when compared to more effective ligands.

A possible explanation of the different effect of ATP at receptors in 1321N1 cells and Jurkats may be differences in receptor number. The effect an increase in receptor number can have on the potency of agonists has been discussed previously in detail (Chapters 3 and 4). In a system with low levels of expressed receptor, the potency of agonists with appear to decrease. This can occur to the extent that an agonist that behaves as a full agonist in a system with a high receptor reserve can appear to be a partial agonist in a system with no spare receptors. So, if transfection of the cloned receptor into Jurkat cells resulted in a lower level of receptor expression than in 1321N1 cells, ATP may be expected to behave differently.

Thus, the potency of agonists in transfected systems may not be particularly useful in determining whether the $P2Y_1$ receptor is the P_{2T} receptor. Far more important is the effect of selective antagonists. The compound ARL 67085 has been described as a highly potent, competitive antagonist of ADP-induced aggregation (Humphries et al, 1994), and in the same study they showed that this compound had

little or no effect at $P2Y_1$ or $P2Y_2$ receptors. This suggests that there is still a further P2Y receptor subtype to be cloned.

8.4.5 C6-glioma cells

The use of cloned P2Y receptors has also enabled a more thorough description of the $P2Y_1$-like receptor coupled to adenylyl cyclase in C6-glioma cells. Boyer et al (1993) described a P2Y receptor linked to adenylyl cyclase in C6 glioma cells with a similar agonist profile to the $P2Y_1$ receptor that activates PLC in turkey erythrocyte membranes. However, this response at the C6-glioma cells was blocked by pretreatment with pertussis toxin, unlike the coupling of the cloned turkey $P2Y_1$ receptor in 1321N1 cells (Chapter 5, this thesis), and the $P2Y_1$ in turkey erythrocytes. They also found that the P2 antagonist, PPADS, had no effect on the adenylate cyclase linked $P2Y_1$-like receptor on C6 glioma cells (Boyer et al, 1994), despite being an antagonist at the cloned turkey $P2Y_1$ receptor in 1321N1 cells (Chapter 4, this thesis). There is therefore evidence that different P2Y subtypes mediate coupling to adenylate cyclase and phospholipase C, and thus the existance of a novel P2Y subtype.

Further evidence for a novel P2Y subtype was produced in 1996 using the cloned human $P2Y_1$ receptor. Boyer et al (1996b) expressed the human $P2Y_1$ receptor in C6 glioma cells, and found that this conferred 2MeSATP stimulated inositol lipid hydrolysis into the cells, which was sensitive to treatment with PPADS. This strongly suggests that the human $P2Y_1$ receptor differs from the $P2Y_1$-like receptor expressed natively in C6 glioma cells. Work by Berti-Mattera et al (1996) has also demonstrated the existence of $P2Y_1$-like receptors coupled to adenylyl cyclase in Schwann cells. Like the receptor in C6-glioma cells, responses at this receptor are inhibited by preincubation with pertussis toxin. The presence of $P2Y_2$ receptor linked to PLC in the same cell type (Ansselin et al, 1997; Berti-Mattera et al, 1996) shows that the Ca^{2+} signalling apparatus are present, and that the $P2Y_1$-like receptor does not couple to it.

Webb et al published a paper in 1996 in opposition to the novel receptor theory. They described the characterisation of a receptor coupled to adenylate cyclase inhibition in B10 rat brain microvascular endothelial cells, and found that nucleotides bound to this

receptor with a profile similar to the cloned $P2Y_1$ receptor, but that PPADS was not effective. This was consistent with the response being at the same $P2Y_1$-like receptor in C6 glioma cells. However, when mRNA from the B10 and C6 glioma cells was examined, only the cloned $P2Y_1$ receptor was found. The authors therefore suggest that the presence of the cloned $P2Y_1$ receptor can account for all of the observations in B10 and C6 glioma cells, and that it can couple in different native cell types to either adenylate cyclase inhibition or to phospholipase C activation.

However, Schachter et al (1997) have recently shown that when the rat $P2Y_1$ receptor was transfected into C6 glioma cells, it behaved exactly as the human and turkey homologues, and did not couple to adenylate cyclase. It therefore appears that there is at least one further P2Y subtype to be cloned.

8.4.6 Human airway epithelial cells

Particular interest has been shown in the P2 receptors present on human airway epithelial cells largely due to their capacity to overcome the defective chloride secretion found in patients with cystic fibrosis. Initial studies concluded that a $P2Y_2$ receptor was responsible for this effect (Mason et al, 1991), and in 1994, Parr et al cloned a $P2Y_2$ receptor from human airway epithelial cells. However, several reports have recently suggested the presence of further P2Y receptor subtypes. A study by Hwang et al (1996) showed that in addition to the $P2Y_2$ receptor on the apical side of the cells, there was a receptor at the basolateral region of the epithelial cells that responded to ADP. Also, Lazarowski et al (1997) have described a response to UDP that was unaffected by hexokinase and was therefore acting at a receptor other than the $P2Y_2$ subtype. However, the response elicited by UDP was half of that to UTP, so the UDP receptor must be expressed at lower levels than the $P2Y_2$ receptor. Thus, in this example, the conclusions were drawn using information on nucleotide interconversion gained directly from cloned receptor systems.

8.5 Drug discovery

P2 receptors represent therapeutic targets for a significant number of disease states, some of which are reviewed in section 1.9. It is therefore not surprising that many of the largest pharmaceutical companies have shown an interest, and participated in P2 receptor research. The advent of molecular cloning and transfection of P2 receptors has had major implications in the drug discovery process. Before the availability of transfected systems, the only way to study P2 receptors in isolation was to search for a tissue or cell type in which only the P2 receptor of interest was expressed, a far from simple task. For example, rabbit ear artery has long been used as a preparation in which to study P2X receptors (O'Connor et al, 1990; Humphries et al, 1994). However, this tissue also responds to P_1 purinoceptor agonists, causing relaxation via endothelial cells (Kennedy & Burnstock, 1985), thus necessitating the removal of the endothelium before experimentation. Also, the guinea-pig aorta has long been used to examine relaxation to $P2Y_1$ receptor agonists, acting at endothelial cells (Humphries et al, 1994). Thus, in contrast to the rabbit ear artery, the endothelium is retained in this tissue during experimentation. The examination of the pharmacology of a receptor by measuring the response of a completely different cell type is clearly not satisfactory. The advent of molecular cloning of the P2 receptors has, however, made easier the measurement of second messengers close to the receptor itself (e.g. $Ins(1,4,5)P_3$), and allows the study of receptors in isolation from one another.

The availability of cloned $P2Y_4$ and $P2Y_6$ receptors are particularly important, because until the advent of molecular cloning, only a handful of tissues had been reported that specifically respond to uracil nucleotides. These tissues were often poorly characterised pharmacologically due to the co-expression of other P2 receptors. For example, Connolly & Harrison (1995) described a receptor for pyrimidines in the rat isolated superior cervical ganglion that mediated depolarisation. However, they also reported the presence of a P2X receptor on the same tissue, also mediating depolarisation. In addition, Johnson & Hourani (1994) have described a suramin-insensitive receptor for UTP on rat duodenum. However, this was also co-expressed with a suramin-sensitive receptor for ATP, preventing satisfactory characterisation of the pyrimidine receptor.

Transfected cells are also better suited to the high-throughput screening technology employed by pharmaceutical companies. Instead of the use of organ-bath apparatus, which was very labour intensive and allowed a maximum of only several hundred compounds to be tested in a day, several thousand compounds can be screened against transfected cells in a fully automated manner overnight. This has revolutionised the drug discovery process.

Another potential benefit of cloned receptors is the possibility of molecular modelling of the agonist-binding site, enabling the so-called 'rational' design of drugs. Mutants of the receptors are constructed in which residues that are considered to play a part in agonist binding are replaced with a different residue, or are deleted. These then give information about the structure of the agonist binding site, facilitating the modelling of new drugs. A preliminary investigation has already demonstrated several residues important for agonist binding at the $P2Y_2$ receptor (Erb et al, 1994), and this has been discussed in Chapter 1. However, very recently a further study has been performed on the human $P2Y_1$ receptor (Jiang et al, 1997). They found that neutralisation of postitive residues on the exofacial side of TM3 and TM7 markedly reduced the potency of 2MeSATP, suggesting that these residues are critical determinants of the ATP binding pocket. Because changes in the potency of 2MeSADP and 2-hexylthioAMP paralleled those of 2MeSATP, it was also concluded that the β and γ phosphates of the nucleotides are less important than the α phosphate in ligand/ $P2Y_1$ receptor interactions. However, T221A and T222A mutant receptors exibited much larger reductions in 2MeSATP potency, indicative of a greater role of these TM5 residues in γ phosphate recognition. There therefore appears to be differences in $P2Y_1$ and $P2Y_2$ receptor, as Erb et al (1994) showed that residues in TM6 and TM7 appear to coordinate the phosphate group of uridine nucleotides. The further characterisation of the structure function differences among P2Y receptor subtypes promises to aid in the design of highly potent and selective ligands.

8.6 Conclusions

P2Y receptors are found in almost every cell of the body and play many physiological roles. The work presented in this thesis has contributed to several aspects of the growing field of P2 receptor research, comprising detailed antagonist pharmacology, investigation into receptor coupling in cloned systems, generation of fusion proteins to facilitate antibody production, and preliminary investigations into P2 subtypes present on vascular smooth muscle cells. In addition, important issues such as nucleotide conversion and endogenous release have been addressed.

Now that the cloned receptors have been characterised using agonists and antagonists, the next logical step would be to utilise the structural information contained within the sequence of each receptor to help determine which regions are responsible for certain functions. The generation of fusion proteins to regions of the bovine $P2Y_1$ receptor and the subsequent production of antibodies will be invaluable in further investigations. For example, they could be used to determine whether phosphorylation of the receptor is involved in the rapid desensitisation observed in Chapter 5. However, perhaps the most exciting advance in the field of receptor research to date is the potential for mutation of specific residues that are believed to be important in roles such as agonist-binding, G protein-coupling, and receptor desensitisation. The information provided using these techniques will help us to fully understand the mechanisms of action of receptors, and will aid in the design of specific drugs for the treatment of disease.

Receptor	Agonist potency	Effect of antagonists			Effect of pertussis toxin	Effect of PKC activation
		suramin	PPADS	NF023		
turkey P2Y1	2MeSATP>ADP>ATP= ATPγS, not UTP	$pA_2 = 5.7$	$pA_2 \approx 6.0$	$pA_2 \approx 5.3$	no effect	inhibition
bovine P2Y1	2MeSATP>ADP>ATP, not UTP	$pA_2 \approx 5.3$	not calculated	not done	not done	inhibition
human P2Y2	ATP=UTP, not ADP, UDP or 2MeSATP	$pA_2 \approx 4.3$	no effect	non-competitive	approximately 20 % reduction	inhibtion
human P2Y4	UTP>>ATP (partial) > UDP, not ADP, 2MeSATP	no effect	no effect	non-competitive	no effect	inhibition

Table 8.1 Summary of the properties of four cloned P2 receptors transfected into 1321N1 cells.

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