Signal Transduction of Transfected and Native P2Y receptors

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by

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Contents

Abstract

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Five mammalian G protein-linked P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$) have been cloned, sequenced and pharmacologically characterised. Native P2Y receptors are reported to couple to PLC, tyrosine phosphorylation, p42/p44 MAPK and cyclic AMP. This thesis looks at the turkey $P2Y_1$, human $P2Y_2$, human $P2Y_4$ and rat $P2Y_6$ receptors transfected into the human astrocytoma null cell line 1321N1 to investigate signalling pathways linking G protein receptor activation to tyrosine phosphorylation, MAPK and mitogenesis. This was compared with results for the native primary cell preparation of rat brain microvascular endothelial cells.

Previous work by others and us has established that all 1321N1 transfected P2Y receptors are strongly linked to an increase in PLC activation. Neither the turkey $P2Y_1$ or human $P2Y_2$ receptors were coupled to an increase in overall tyrosine phosphorylation assessed by PY20 antibody western blot. Pervanadate alone gave large increases in tyrosine phosphorylation, but no further increase in tyrosine phosphorylation was observed with co-addition of 2MeSATP to the turkey $P2Y_1$ transfectants. Co-addition of UTP and a sub-maximal concentration of pervanadate on the human $P2Y_2$ receptor gave a reduction in tyrosine phosphorylation compared to pervanadate alone. This indicated possible activation of tyrosine phosphatase activity by the human $P2Y_2$ receptor activation.

Turkey $P2Y_1$ and human $P2Y_2$ receptors were both shown to activate p42/p44 MAPK assessed by phospho-MAPK antibody western blotting and a nonapeptide kinase assay. Both turkey $P2Y_1$ and human $P2Y_2$ receptor activation of MAPK was inhibited by the MEK inhibitor PD 98059. Human $P2Y_4$ and rat $P2Y_6$ receptors showed no activation of MAPK. Both turkey $P2Y_1$ and human $P2Y_2$ MAPK activation was PKC dependant; inhibited by Ro 31-8220 and Go 6850, but not Go 6976 a calcium sensitive PKC isoform inhibitor. PKC isoforms (ε or λ) may be involved in this signalling pathway. Some experiments investigating Pyk2 and Shc involvement in P2Y signalling are presented.

P2Y agonists alone or with platelet derived growth factor modulated thymidine incorporation in P2Y transfectants. 2MeSATP on turkey $P2Y_1$ transfectants increased incorporation and co-addition with PDGF led to an additive effect. UTP was ineffective on human $P2Y_2$ transfectants, while PDGF increased incorporation. UTP was also ineffective on human $P2Y_4$ transfectants but caused decreased incorporation when co-incubated with PDGF, compared with PDGF alone. Rat $P2Y_6$ showed no increase with UDP but an increase with addition of PDGF.

Rat brain endothelial cells showed activation of MAPK that was characteristic of a pyrimidine activated receptor, being either $P2Y_2$ or $P2Y_4$. The activation of MAPK was dependant on extracellular calcium and was not modulated by cAMP. Small MAPK responses to endothelin but not to histamine and large responses to 10% FCS were also recorded. Activation of adenylate cyclase occurred on addition of ATP, but not UTP, indicating the presence of a $P2Y_1$-like receptor.

These data show startling differences in the way various P2Y isoforms are coupled to MAPK and mitogenesis.

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Publications from thesis

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Abbreviations

α,β-MeATP	α,β–methylene adenosine triphosphate
β,γ-MeATP	β,γ–methylene adenosine triphosphate
$[^3H]InsP_X$	total [^{3}H]inositol (poly)phosphates
$[Ca^{++}]i$	intracellular calcium ion concentration
2MeSATP	2-Methylthio-adenosine triphosphate
a.a.	amino acid
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ATPγS	Adenosine 5'-0-(2-thiodiphosphate)
BAECs	Bovine Aortic Endothelial Cells
BSA	Bovine Serum Albumin
BSS	Balanced Salt Solution
BW-A522	3-(3-iodo-4-aminobenzyl)-8-(4-oxyacetate)-1-propylxanthine
cAMP	cyclic Adenosine monophosphate
CGS-21680	2-((carboxyethyl)phenethylamino)-5'carboxyamidoadenosine
CHO	Chinese hamster ovary
CPA	N^6-cyclopentyladenosine
d.p.m.	disintegrations per minute
DAG	sn-1,2-diacylglycerol
DMEM	Dulbecco's modified Eagles medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DPCPX	1,3-dipropyl-8-cyclopentylxanthine
ECL	Enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol bis(beta-aminoethylether)-N,N'- tetraacetic acid
ERK	Extracellular Regulated Kinase
et al	*et alia*
FCS	Foetal Calf Serum
G Protein	Guanine nucleotide-binding protein
GDP	Guanosine diphosphate

Go 6850	Bisindolylmaleimide I; GF 109203X; 2-[1-(3-dimethylamino propyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide
Go 6976	Indolocarbazole compound
GTP	Guanosine triphosphate
HEK	Human embryonic kidney
HEPES	N-2 hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HRP	Horseradish peroxidase
log	logarithm to base 10
MAPK	Mitogen Activated Protein Kinase
MEK/MAPKK	Mitogen Activated Protein Kinase Kinase
MEKK	Mitogen Activated Protein Kinase Kinase Kinase
NECA	1-(6-amino-9H-purin-9-yl)-1-deoxy-N-ethyl-β-D-ribofuramide
PBS-T	Phosphate Buffered Saline with Tween
PD 98059	2'-amino-3'-methoxyflavone
PDGF	Platelet derived growth factor
PGI_2	Prostaglandin I_2 / Prostacyclin
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
$PtdIns(4,5)P_2$	phosphatidylinositol (4,5) bisphosphate
PTX	pertussis toxin
RBECs	Rat brain microvascular endothelial cells
Ro 31 8220	3-[1-[3-(amidinothio)propyl-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)maleimide methane sulphonate
R-PIA	R-N^6-(phenylisopropyl)adenosine
SCH 58261	5-amino-7-(phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine
SDS PAGE	Sodium dodecylsulphate Polyacrylamide Gel Electrophoresis
Sos	Son of sevenless
TBS-T	Tris Buffered Saline with Tween
TCA	trichloroacetic acid

Chapter 1

Introduction

1.1 Characterisation of responses to ATP - History of P2 receptors

Adenosine-5'-triphosphate (ATP) was initially known for its role as an intracellular source of energy. However, Drury and Szent-Gyorgi first described in 1929 early characterisation of cellular responses by action of adenosine compounds on slowing the guinea-pig heart by intravenous injection. This marked the beginning of a series of discoveries of the action of adenosine nucleotides and nucleosides on various different tissues.

It was not until 1972 that Burnstock suggested ATP was one of the main compounds involved in neurotransmission. This formed the basis of the 'purinergic nerve hypothesis' which described nerves using ATP as a neurotransmitter. This was in neurons using non-adrenergic and non-cholinergic (NANC) transmission, or as a co-transmitter with noradrenaline, acetylcholine or other neurotransmitters (Burnstock et al., 1976). After further pharmacological characterisations, Burnstock proposed in 1978 to divide purinoceptors, as they became termed, into P1 receptors and P2 receptors. P1 receptors had an agonist potency order of adenosine>>ATP, were selectively antagonised by methylxanthines and lead to activation of adenylate cyclase. P2 receptors had an agonist potency order of ATP >> adenosine, were not antagonised by methylxanthines, and stimulated prostaglandin synthesis. This nomenclature was accepted but as further receptor-binding and biochemical studies progressed it became apparent further subdivision was necessary.

Burnstock and Kennedy in 1985 reviewed the studies for P2-purinoceptors and concluded that there was basis for a further subdivision into subtype 1 (P_{2X}), mediating contraction in the vas deferens and bladder, and subtype 2 (P_{2Y}), mediating relaxation in guinea-pig taenia coli and rabbit portal vein. The two subtypes could be

separated on their apparent agonist profiles: α,β-methyleneATP, β,γ-methyleneATP > ATP = 2-methyl-thioATP (P_{2X}) and 2-methyl-thioATP >> ATP > α,β-methylene ATP, β, γ–methyleneATP (P_{2Y}). Gordon later presented evidence for further subdivisions of P2 receptors in a review article in 1986. This proposed further subdivision building on Burnstock and Kennedy's proposed nomenclature creating classes P_{2X}, P_{2Y}, P_{2T} and P_{2Z}. Haslam and Cusack, (1981) observed purinergic responses on platelets responding to ADP, leading to platelet aggregation, while ATP acted as an antagonist, defining the pharmacological profile of the P_{2T} purinoceptor subtype. The receptor subtype P_{2Z} was proposed, keeping alphabetical order, based on evidence from macrophages, mast cells and lymphocytes of a non-selective pore responsive to the tetrabasic form of ATP, ATP^{4-}.

Cellular responses for UTP had been observed for some time, generating the term pyrimidinoceptor (reviewed by Seifert and Schultz, 1989). The term purinoceptor became inadequate as UTP in some circumstances was acting alone or at the same receptor as ATP (von Kugelen and Starke, 1991; O'Connor et al., 1991,1992; Keppens et al., 1992) which became termed as the P_{2U} or P_{2N} receptor (Dubyak and El-Moatassim, 1993). The use of the alphabet nomenclature continued with addition of P_{2D}, a receptor for diadenosine polyphosphates (Hilderman et al., 1991; Pintor et al., 1993; Pintor and Miras-Portugal, 1993). In 1992 a meeting of the NC-IUPHAR (International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification) purine receptor nomenclature committee agreed that the alphabetical naming of P2 receptors was unhelpful, making addition of new subtypes difficult and inconsistent with NC-IUPHAR rules (Abbrachio et al., 1993; Fredholm et al., 1994). Receptor nomenclature is normally based on receptor antagonist pharmacology and structure (Kenakin et al., 1992). However P2 receptors

had no antagonists, few defining ligands and little structural information known, unlike P1 receptors which were already being cloned. For this reason the meeting proposed to keep the tentative alphabet system until more data was available. The first cloning paper was published for the chick P_{2Y} receptor (Webb et al., 1993) followed shortly after by the mouse P_{2U} receptor (Lustig et al., 1993). There followed massive increase in cloning efforts until it became evident from data collected on receptor structure and pharmacology that a new nomenclature system was required. In 1994 Abbracchio and Burnstock proposed a nomenclature system based on numbering receptor subtypes. P2X receptors, which are ligand-gated, and P2Y receptors, which are G protein-linked receptors (Dubyak, 1991), were proposed as two distinct structural families of P2 receptors each of which could be subdivided into numbered subtypes. Analysis of pharmacological profiles at that time identified seven different subclasses, which were proposed to be renamed $P2Y_1$ through to $P2Y_7$. This allowed for new subclasses to be added at a later date more easily and also incorporated pyrimidine receptors making usage of the term P2 purinoceptor out of date. The numbered system was accepted by IUPHAR after discussion and further receptor subtypes included (Fredholm et al., 1997). The numbered nomenclature system is used throughout this thesis and is further discussed along with cloning and recent nomenclature additions in later sections of this introduction.

1.2 Nucleotide sources and metabolism

Intracellular ATP has been recognised for many years as an energy source and is stored at high intracellular concentrations (~5mM). Extracellular ATP levels remain low due to low membrane permeability and ectonucleotidases which rapidly breakdown ATP to adenosine. The discovery of receptors responsive to ATP raises the question of sources of extracellular ATP and its metabolism. The following sections discuss the various sources of extracellular nucleotides followed by a discussion on ATP metabolism.

1.2.1 Exocytosis

ATP may be released from intracellular stores via vesicular release to provide extracellular ATP. The concentrations of ATP required extracellularly to activate P2Y receptors is in the 20-100μM range which is achievable for example by degranulation, but dependent on the volume of the extracellular space and adjacent ectonucleotidases present. Vesicular release can occur in neuronal and non-neuronal cells.

Neuronal cells have been shown to co-store ATP along with other neurotransmitters. Pioneering work by Holton, published in 1959, established that a release of ATP occurred on nerve stimulation in the rabbit ear artery. In 1972 Burnstock hypothesised that ATP was the neurotransmitter in NANC nerves. Edwards, and co-workers, investigated the role ATP plays in synaptic transmission in the CNS in 1992. Pre-synaptic stimulation led to postsynaptic responses that could not be blocked by conventional transmitter inhibitors. Suramin, a non-selective P2

antagonist, did inhibit and along with α,β–methylene ATP desensitisation pointed towards a role of ATP release and P2 receptors in synaptic transmission. Further work since the proposed 'purinergic nerve' hypothesis has shown ATP also to be co-released with noradrenaline and acetylcholine neurotransmitter granules at the synapse (Burnstock, 1976; Sneddon and Burnstock, 1984; Richardson and Brown, 1987; Von Kugelgen and Starke, 1991).

Non-neuronal release occurs from platelets that contain large amounts of stored ATP and ADP (Carty et al., 1981) and are known to release these stores when activated (Born and Kratzer, 1984). This release, due to the high number of platelets, would occur in the 50μM range in serum, which may reach higher levels at local areas of release. Platelet activation and promotion and modulation of thrombosis is discussed further in section 1.9.

Mast cells are activated by ATP which increases membrane permeability (Cockcroft and Gomperts, 1980), induces secretion of histamine and also additional ATP making interpretation difficult (Trams et al., 1980).

Adrenomedullary chromaffin cells contain high mM concentrations of ATP and ADP in granules that are released on cell stimulation (Rojas et al., 1985). This release may play a role in the main function of chromaffin cells, which is to release catecholamines.

1.2.2 Transmembrane transport

Intact cells that have no exocytotic release pathway are still able to release nucleotides extracellularly. This is proposed to occur via transmembrane transporters for nucleotides (Sedaa et al., 1990). The ABC (ATP binding cassette)

group of transporter proteins are possible pumps for nucleotides such as the multidrug resistance (MDR) protein or cystic fibrosis transmembrane conductance regulator (CFTR) (Abraham et al., 1993; Reisin et al., 1994). The role that the CFTR plays in purinergic signalling and cystic fibrosis (Schwiebert et al., 1995) is discussed later in section 1.10.

1.2.3 Cell lysis

As cells store ATP at high mM concentrations any cell rupture would cause a massive release of high concentrations of intracellular ATP to the extracellular environment. An example of this occurrence would be in blood vessel injury when endothelial cell lysis would release ATP aiding the wounding response. P2 receptors localised on vascular endothelial, smooth muscle and circulatory cells would be activated resulting in closing the wound and preventing pathogen invasion. This is further discussed in section 1.9.

1.2.4 Shear stress

Release from cells due to shear stress occurs not by exocytotic release but by some other mechanism with cells remaining viable. In vasculature increased blood flow has been linked to an increase in ATP released from endothelial cells (Ralevic et al., 1992; Milner et al., 1990a;1990b) which may then cause vasodilation in response to the increased cell stress. In cell culture ATP may be released from vascular endothelial cells and smooth muscle cells (Pearson and Gordon, 1979). Burnstock in 1989 described release of ATP from vascular endothelial cells during

hypoxia or ischaemia that caused initial vasodilation. Mechanical stretching of vascular smooth muscle cells caused a time and strength-dependent rise in ATP levels in the extracellular media (Hamada et al., 1998) activating the Jun N-Terminal kinase (JNK)/ Stress Activated Protein Kinase (SAPK) cascade.

UTP is released due to fluid shear stress in vascular endothelial cells (Saiag et al., 1994). Lazarowski in 1995 showed release of ATP from 1321N1 astrocytoma cells that are used for the expression and functional assay of cloned P2Y receptors. The same group demonstrated in 1997 that mechanical movement of extracellular cell medium caused a large release of UTP also in 1321N1 cells (Lazarowski et al., 1997a). Harden discussed this further when metabolism of ATP and UTP was considered with particular reference to the cloned P2Y receptors (Harden et al., 1997). These problems of metabolism are discussed in the following sections.

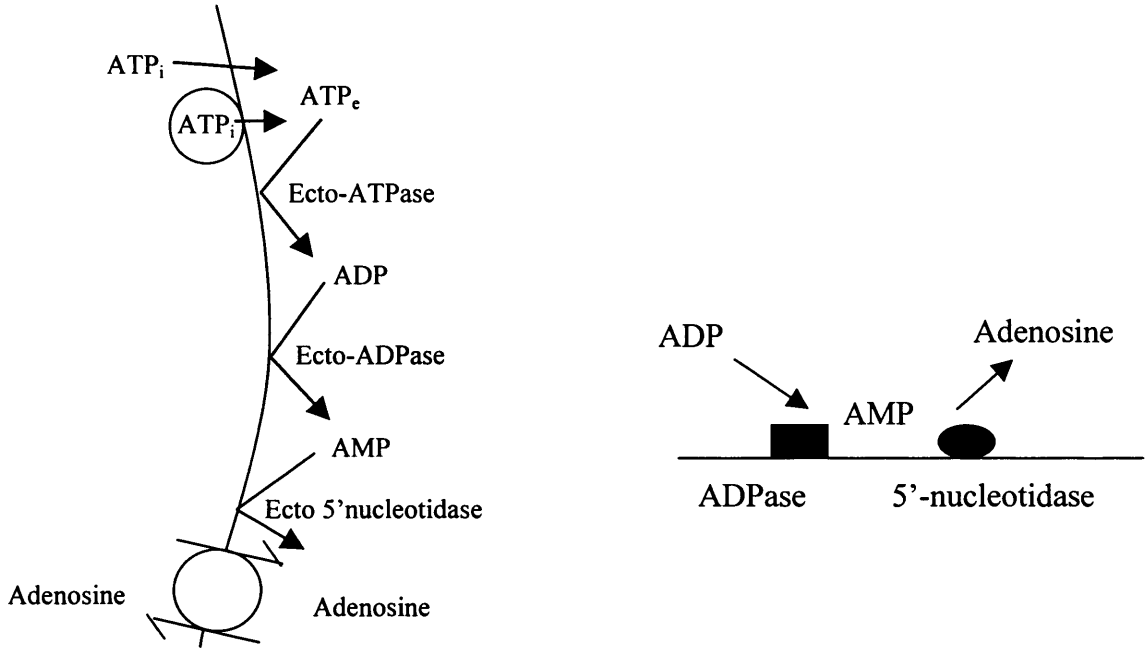
1.2.5 Nucleotide Metabolism

ATP, ADP and adenosine may act on extracellular receptors to produce similar or very different cell signalling. AMP has little effect at either P1 or P2 receptors. The cellular production and inter-conversion of these adenine-based compounds is important in maintaining a cell's energy state and modulation of cell activation. Uridine nucleotides can be converted to uridine, though no uridine receptor has yet been cloned. Responses to uridine are therefore likely to be due to the presence of uridine nucleotides. Breakdown of uridine nucleotides to uridine therefore poses less of a problem than adenosine nucleotide conversion. However conversion of UTP to UDP, and vice versa, is important as completely different P2Y receptors may be activated. Having discussed previously the sources of adenosine

and uridine nucleotides the enzymatic conversion of these compounds must now be discussed.

1.2.5.1 Ectonucleotidases

ATP is sequentially degraded to ADP then AMP and then to adenosine by the ectonucleotidase pathway (Figure 1.1A). Three ecto-enzymes are responsible for this dergadation being ATPase, ADPase and 5'-ectonucleotidase. This mechanism of ATP removal means that ATP is only available transiently for receptor activation and produces adenosine, which may be necessary for activation of P1 receptors.



A. **B.**

Figure 1.1 **A.** Ectonucleotidase cascade for conversion of ATP to adenosine. **B.** Depicts the delivery of AMP between ADPase and 5'-nucleotidase.

EctoATPase removes the terminal or γ phosphate group off nucleotide triphosphates to produce nucleotide diphosphates. ATP is the prefered substrate of

ectoATPase (Ziganshin et al., 1994). EctoATPase is found in a variety of cell types including endothelial (Pearson and Gordon, 1985), smooth muscle (Pearson et al., 1985), cochlear hair cells (Mockett et al., 1994), myocytes (Meghji et al., 1992) and cholinergic nerve terminals (Richardson et al., 1987). EctoATPase is a glycoprotein that contains an extracellular catalytic domain, transmembrane anchor region and a cytoplasmic region containing sites that undergo phosphorylation for possible regulation of ATPase (Rees-Jones and Taylor, 1985).

EctoADPase removes the β phosphate group of ADP to produce AMP. ATP levels, causing inhibition of EctoADPase (Pearson and Gordon, 1985; Meghji, 1993), regulate output of AMP. ADPase preferentially delivers AMP to 5' ectonucleotidase (Figure 1.1B) rather than into the bulk phase. This enables efficient production of adenosine. This can vary with cell-type, as can the efficiencies of all three ectoenzymes. In endothelial and aortic smooth muscle cells with similar enzyme activities there is a difference in the rate of adenosine production. Endothelial cells do not appear to use the preferential movement of localised nucleotides as predictions of adenosine production matched levels of nucleotides observed in the bulk phase. On aortic smooth muscle cells this was not the case as hydrolysis of ATP to ADP and AMP occurred more efficiently than predicted (Gordon et al., 1986; 1989).

5' ectonucleotidase (ecto-monophosphatase) catalyses the production of adenosine from AMP. 5' ectonucleotidase exhibits feed forward inhibition from high levels of ADP and ATP. This inhibition is overcome by the preferential delivery system mentioned above. This system was also observed in ventricular myocytes (Meghji et al., 1992). Here ADP supplied from ATPase was more

efficiently processed than ADP supplied from bulk phase additions leading to the conclusion of preferential supply between ectoenzymes.

1.2.5.2 Nucleoside diphosphokinase

It has become increasingly important not only to consider the breakdown of ATP to adenosine but also the phosphorylation of nucleotides by ecto-kinases. One such kinase is the nucleoside diphosphokinase. This enzyme transfers the γ-phosphate group from a nucleotide triphosphate to a nucleotide diphosphate. This enzyme activity was noticed by Pearson and colleagues in 1980, when conversion of [^{3}H]ADP led to production of [^{3}H]ATP, showing the presence of nucleoside diphosphokinase on pig aortic endothelial cells.

Recently this enzyme became important when considering the cloned receptors transfected into 1321N1 cells as ecto-nucleoside diphosphokinase activity was discovered (Lazarowksi et al., 1997b; Nicholas et al., 1996a). The presence of this enzyme, which exhibits a higher affinity than any nucleotidase present, means that addition of UDP in the presence of ATP does not lead to production of UMP but the conversion to UTP and ADP. This is ~5% conversion of UDP to UTP on addition of 100nM UDP to 1321N1 cells (Harden et al., 1997). However if the medium was changed and the cell stressed, endogenous release of ATP from 1321N1 cells cause the conversion to increase to ~16%. This was also noticed with the addition of 100nM ATP. This discovery proved important so that the real rank order of potency for nucleotides on P2Y receptors cloned into 1321N1 cells could be uncovered.

1.2.5.3 Effects of nucleotide metabolism on P2Y receptors

As the last few sections have described, inter-conversion of nucleotides has obscured the real agonist profiles of the P2Y receptors. This is extremely important to correct as no selective antagonists have been discovered for the mammalian $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ receptors discovered. This makes identification of receptor subtypes in tissues difficult as more than one P2Y receptor or P2X receptors may exist on the tissue, and if nucleotidases and P1 receptors are also present then action of adenosine must be considered. The presence of nucleotidases has also caused problems since action of potential antagonists (e.g. suramin) may act as inhibitors of nucleotidases. Ecto-nucleotidases have also prevented accurate radio-receptor binding data, as ligands binding to receptors also bind to ecto-nucleotidases.

Inhibition of ecto-nucleotidases (section 1.2.5.5) and production of hydrolysis resistant ATP analogues (section 1.2.5.4) have helped address the issue of agonist profiles on receptors responsive to ATP and ADP. Triphosphate regeneration systems using creatine phosphokinase and phospho-creatine as the phospho-donor enable solutions of ATP to remain ADP free. High Pressure Liquid Chromotography (HPLC) can be used to generate pure solutions of nucleotides. This is particularly useful as commercially available nucleotides are often contaminated with other nucleotides. Apyrase (ATP or ADP→ AMP) has been useful in removing nucleotides from the cell medium removing released endogenous nucleotides and reducing any associated receptor desensitisation. Apyrase however must be washed out from cells (causing endogenous release) if nucleotides are to be used as agonists. This is not true however for hydrolysis resistant compounds such as ATPγS and

UTPγS. Apyrase may also be used to remove contamination from these resistant nucleotides.

Conversion of UDP to UTP by the ecto-nucleoside diphosphokinase (Lazarowski et al., 1997b) was investigated on 1321N1 cells. Previous pharmacological profiles had placed UDP as a full agonist at $P2Y_2$ receptors (Lazarowski et al., 1995) but after analysis using purified UDP and hexokinase and glucose (UTP→UDP + glucose-6P) UDP was discovered to be inactive at $P2Y_2$ (Nicholas et al., 1996b). The profile for $P2Y_2$ was of equal activation by ATP and UTP but not by UDP or ADP. The $P2Y_4$ receptor also was reported (Nicholas et al., 1996b) to have a different profile than initially thought (Communi et al., 1995a; Nguyen et al., 1995). $P2Y_4$ was activated most potently by UTP, less potently by ATP but not at all by nucleotide diphosphates. The $P2Y_6$ receptor was initially thought to be UTP selective (Chang et al., 1995) however further analysis showed it to be selective for UDP over UTP. This was not due to UTP contamination of UDP as hexokinase treatment did not effect the dose response curve. Therefore $P2Y_6$ is a UDP preferring nucleotide receptor with UTP as a full but less potent agonist.

1.2.5.4 Resistant nucleotides

Resistant nucleotides were developed to overcome the problems of nucleotide breakdown observed in tissues so that receptor subtypes could be identified more clearly. α,β–MethyleneATP and β,γ–MethyleneATP are both nucleotide resistant analogues selective for P2X receptors (Welford et al., 1986). ATPγS has been widely used and commercially available for some time. It is equipotent in most tissues with ATP when ectonucleotidase activity is low. A more recent addition,

though not commercially available, is UTPγS (Lazarowski et al., 1996). This can be produced by the enzyme nucleoside diphosphokinase using either GTPγS or ATPγS as the γS donor to UDP. UTPγS was shown to be resistant to hydrolysis by apyrase, alkaline phosphatase, acid phosphatase and incubation on epithelium cells. UTPγS has been used on $P2Y_2$ receptors transfected into 1321N1 cells and observed to be equipotent with UTP and ATP.

1.2.5.5 Ectonucleotidase inhibitors

Suramin is a compound well known for its multitude of effects. Hourani and Chown in 1989 described the action of suramin as a non-competitive inhibitor of ATP breakdown in the guinea-pig urinary bladder. Ziganshin and co-workers in 1996 describe the action of suramin in *Xenopus* oocytes being competitive inhibitor of Ca^{++}/Mg^{++}-dependent ectonucleotidase. Suramin, as well as being a simple competitive antagonist at P2X receptors (Leff et al., 1990), can also inhibit ectonucleotidases in a blood cell assay (Crack et al., 1994). The action of an antagonist however may result in the 'self cancellation' of any potentiating effect of inhibiting ectonucleotidases by its rightward shifting properties. Meghji and Burnstock (1995) discuss inhibition of ectonucleotidases on endothelial cells, including suramin, but no better ectonucleotidase inhibitor is discovered. NF023, a structure based on suramin, has also been observed to decrease ATP breakdown on human blood cells to 51% of control values (Beukers et al., 1995). Another P2 antagonist that also inhibits ectonucleotidases is Reactive red 2 (Bultmann and Starke, 1995). Reactive red 2 reduced ATP degradation on vas deferens and taenia coli by up to 95% but is a strong antagonist of P2X receptors in rat vas deferens and

P2Y receptors in guinea-pig taenia coli. Pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) is also a P2 antagonist that shows properties of ectonucleotidase inhibition in the rat vas deferens. PPADS reduced ATP breakdown to 80.7±10.2% of the initial levels (Khakh et al., 1994)

ARL 67156 (6-N,N-diethyl-β,γ-dibromomethylene-D-ATP) is the most useful current ectonucleotidase inhibitor. Also known as FPL 67156 this compound inhibits ectoATPase in a human blood cell assay (Crack et al., 1995), but also has weak agonist effects at $P2Y_2$ receptors and weak antagonist action at P2X and P2T receptors. These attributes make it the most effective inhibitor to date.

1.3 P1 receptors nomenclature and subtypes

It is important when considering cellular responses to ATP that receptors for adenosine are also considered, as breakdown of ATP leads to production of adenosine. This means that transient levels of ATP may activate P2 receptors initially but after adenosine is produced as a metabolic product, P1 receptors may also be activated. Similarly on cells that contain multiple P2 receptor subtypes metabolic breakdown products, such as ADP, may activate different subtypes, varying activation of signalling systems with time.

P1 receptors were initially defined as responsive to adenosine, antagonised by methylxanthines and linking to adenylate cyclase (Burnstock, 1978). Subdivision of P1 receptors led to subtypes A_1 and A_2 (Van Calker et al., 1979) being recognised on the basis of stimulation or inhibition of adenylate cyclase (also named R_i and R_a respectively). The numbered nomenclature was accepted by IUPHAR and has not changed (Fredholm et al., 1994). 5'-N-ethylcarboxy amidoadenosine (NECA) was

found to be selective for the A_2 subtype above adenosine at the A_1 subtype. Further subdivision of the A_2 subtype occurred when observations using NECA discovered both high affinity and low affinity binding sites that were designated A_{2A} and A_{2B} (Daly et al., 1983). Extensive cloning of P1 receptors led to the molecular characterisation of the A_1 (Libert et al., 1989; 1991) A_{2A} (Libert et al., 1989; Maenhaut et al., 1990) and A_{2B} receptors (Stehle et al., 1992; Rivkees and Reppert, 1992). Cloning efforts also led to the structure of the A_3 subtype (Meyerhof et al., 1991), which when functionally expressed (Zhou et al., 1992) linked to an inhibition of adenylate cyclase but was not antagonised by xanthine compounds.

Selective antagonists of P1 receptors have aided in the progression of identification of P1 subtypes as well as specific ligands such as CGS21680. The compounds used for agonists are mainly adenosine based and antagonists are mainly xanthine based. These useful compounds are summarized in Table 1.1

	Agonist Profile	Selective Antagonists	Adenylate Cyclase
A_1	CPA>NECA>>CGS21680	DPCPX>8PT	Negative
A_{2A}	CGS21680=NECA>CPA	SCH 58261*	Positive
A_{2B}	NECA>CGS21680	No selective available	Positive
A_3	NECA=R-PIA>CGS21680	BW-A522+	Negative

Table 1.1. Useful compounds in P1 receptor analysis.

References: * Ongini et al., 1999 + Fozard and Hannon, 1994

Although reports exist that suggest further subdivision of P1 adenosine receptors further numbering is only recommended on the basis of structurally different receptors with distinctive pharmacology (Fredholm et al., 1997). Cloning

has helped in the isolation of receptor subtypes aiding the unravelling of a complex pharmacology of mixed receptor populations. However, because cloning of receptor subtypes has occurred over different species, it creates a problem in identifying the difference between which receptors are pharmacologically different, which are species homologs and which are the same receptor in a different cell line. This is usually resolved by much debate and the use of the main tools of pharmacology i.e. agonists and antagonists rather than secondary messenger systems. By development of highly selective agonists/antagonists P1 receptor function can be properly assessed in native cell systems enabling resolution of the P1 subtypes. Antagonists at P1 receptors also prevent confusion between P2 subtype signalling present in the same cell due to agonist breakdown.

1.4 P2 receptor nomenclature

Abbrachio and Burnstock in 1994 proposed a numbered P2X and P2Y nomenclature consisting at that time of $P2X_{1-4}$ and $P2Y_{1-7}$. Current P2 receptor cloning has expanded this nomenclature into $P2X_{1-7}$ and $P2Y_{1-11}$ (Burnstock and King, 1998). These cloned and sequenced subtypes are not all pharmacologically characterised as yet. Some may turn out to be orphan receptors with a genomic sequence but no function, others may be closely related receptors unresponsive to nucleotides. There is still some debate over the presence of further subtypes identified in native tissues, the pharmacology of which is unaccounted using the transfected cloned P2Y receptor pharmacology for comparison (King et al., 1998). The functional analysis of the transfected P2Y receptor family still has a large role to play in characterisation of native tissue responses to nucleotides.

1.5 Cloning of P2Y receptor subtypes

P2Y receptors are members of the seven transmembrane domain containing G protein linked receptor superfamily. Cloning of P2Y receptors was achieved by the polymerase chain reaction (PCR) being utilised with degenerate primers based on transmembranes II and VI of G protein-coupled receptors. A Guinea-pig brain partial cDNA was isolated which is related to RDC1, a receptor most closely related in amino-acid sequence to canine adenosine receptors. P1 adenosine receptors are not closely related based on amino-acid sequence to P2Y receptors, which are more closely related to interleukin-8 and thrombin receptors. However this method provided the RDC-like sequence with which a complementary DNA library for chick brain was screened. This screen yielded the first published P2Y clone, now known as chick $P2Y_1$ (Webb et al., 1993). Other P2Y receptors were cloned on a similar basis using degenerate primers based on transmembranes III and VII of available P2Y receptor sequences. A current sequence homology dendogram of cloned P2Y receptors is represented in Figure 1.2.

1.5.1 $P2Y_1$

As described above, chick $P2Y_1$ was the first P2Y receptor cloned and published in 1993. The chick brain was used as it expresses large numbers of receptors at the time the chick hatches. Being 362 amino acids in length it is fairly small in size and hydropathicity analysis exhibited the classical seven hydrophobic domains. Expression of the receptor in COS-7 cells and *Xenopus* oocytes by Simon et al., (1995) showed activation of PLC and Ca^{++} by the agonist 2-MeSATP.

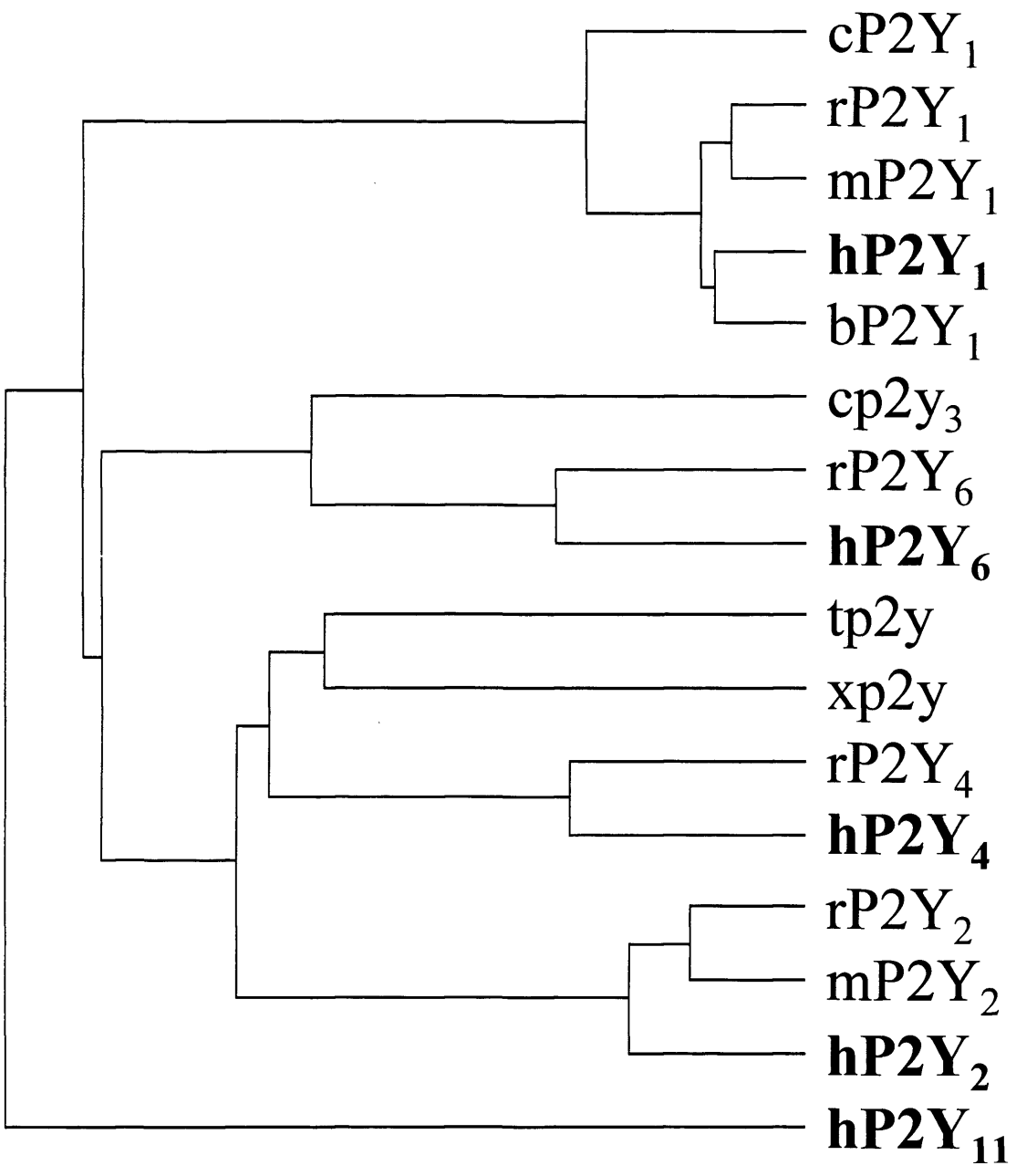


Figure 1.2
Dendogram of current P2Y receptor sequences.
Lines on the dendogram are representative of the % amino acid identity between the receptors. Receptors of similar identity are grouped into families. Human receptors are shown in bold.

Different species homologs of the $P2Y_1$ receptor were identified when Filtz et al., (1994) cloned the turkey homologue. This receptor was cloned and transfected into the null cell line 1321N1. Filtz and collaborators showed 2-MeSATP activation of PLC, which was pertussis toxin insensitive, indicating a possible role of G_q protein in signal transduction.

Mammalian homologs of $P2Y_1$ were soon identified with mouse and rat sequences discovered by Tokuyama et al., (1995). Rat $P2Y_1$ was detected via northern blot analysis in heart, brain, lung, liver, muscle, kidney, in small amounts in the spleen and absent from the testis. Mammalian homologs are different to avian sequences by addition of 11 amino acids in the mammalian sequence as well as substitution of different amino acids, mainly in transmembranes IV and V. Bovine $P2Y_1$ was also isolated from bovine aortic endothelial cells using the chick $P2Y_1$ cDNA sequence as a probe (Henderson et al., 1995). Translated sequence comparisons showed an 86% identity to the chick $P2Y_1$ receptor sequence. Though there were noted differences in sequence, areas of similarity (basic residues TMIII:5, TMVI:20 and TMVII:6) were identified that were thought to be of importance in ligand binding. Functional analysis of the bovine $P2Y_1$ receptor (increased cytosolic Ca^{++}) gave a rank order of potency of 2MeSATP=ADP >ATP>>α,βmethyleneATP, UTP.

The human $P2Y_1$ receptor has been cloned from several tissues; placenta (Leon et al., 1997), erythro-leukemia cell line (Ayyanathan et al., 1996), brain (Schachter et al., 1996), prostate and ovary (Janssens et al., 1996) and platelets (Jin et al., 1998). Tissue distribution studies in human showed expression in heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas. Chromosomal

localisation experiments identified the human $P2Y_1$ receptor gene presence on chromosome 3, position q25 (Ayyanathan et al., 1996).

Recent characterisation of the $P2Y_1$ receptor has included a review of the agonist potency profile with regards impurities and breakdown products, role of ATP as an antagonist at the $P2Y_1$ receptor and the proposal that $P2Y_1$ is the same as the P_{2T} receptor.

Work on rat C6 glioma cells describe a $P2Y_1$-like receptor linked to inhibition of adenylate cyclase (Boyer et al., 1993). This had a similar agonist profile to the $P2Y_1$ receptor in turkey erythrocytes. PPADS was not an antagonist at the C6 glioma $P2Y_1$-like receptor but it was at the erythrocyte $P2Y_1$ receptor (Boyer et al., 1994). PPADS was therefore able to separate actions by the rat C6 glioma $P2Y_1$-like adenylate cyclase linked receptor and the turkey $P2Y_1$ PLC linked receptor, providing evidence that different types of '$P2Y_1$-like' receptor may differentially link to different secondary messenger systems. In support of this Schachter and co-workers (1996) transfected the human $P2Y_1$ receptor into the C6 glioma cell line and showed activation of PLC, which was PPADS sensitive. PLC activity was not enhanced by activation of the endogenous C6 glioma '$P2Y_1$-like' receptor.

Webb and colleagues reported that the rat C6 glioma cell and B10 rat brain capillary endothelial cell line P2Y receptors, that are both linked to adenylate cyclase, are rat $P2Y_1$ (Webb et al., 1996a). This questioned the work by Schachter and co-workers that the $P2Y_1$ receptor couples to PLC and that the rat 'P2Y-like' receptor on C6 glioma cells, distinct from $P2Y_1$, couples to adenylate cyclase inhibition. Schachter and co-workers had used the human $P2Y_1$ receptor for transfection and not the rat $P2Y_1$ receptor, proposed by Webb et al., to link to

adenylate cyclase. Schachter et al., (1997a) responded to this by transfecting the rat $P2Y_1$ receptor sequence into rat C6 glioma cells, showing signalling to PLC and not to adenylate cyclase like the endogenous P2Y-like receptor. Therefore the rat $P2Y_1$ receptor is distinct from the rat C6 glioma P2Y-like receptor. Schachter et al., accounts for the discrepancies between their work and Webb et al., by inadequate controls in RT-PCR experiments and use of [^{35}S]-dATPαS binding data, which is questionable based on previous work showing non-specific binding (Schachter and Harden, 1997b).

Leon and co-workers (1997) expressed the human $P2Y_1$ receptor in Jurkat cells and using HPLC purified agonists stimulated a rise in cytosolic calcium. The agonist order of potency uncovered 2MeSADP and ADP to be agonists and ATP and its derivatives to be weak competitive antagonists. Hechler et al., (1998a) also showed this to be the case for rat $P2Y_1$ and human $P2Y_1$ receptors with purified agonists. Both studies commented on the similarity of this agonist profile and the profile of the P_{2T} receptor expressed on platelets. Webb et al., (1996a) also supported this proposal by reporting the rat $P2Y_1$ receptor is coupled to adenylate cyclase inhibition, as is the P_{2T} receptor.

The suggestion that ATP and its derivatives are weak antagonists at the $P2Y_1$ receptor was investigated further by Palmer et al., (1998). Using purified agonists on human $P2Y_1$ transfected into 1321N1 cells and native $P2Y_1$ receptors in HEK 293 cells they showed a rank order of potency of 2MeSADP>ADP>2MeSATP>ATP. ATP was not acting as a weak antagonist and the activity of ATP compounds was not due to conversion to ADP during the experiment. The explanation of the difference between these results and those observed for Jurkat transfected receptors was suggested to be due to differences in receptor reserve. Down-regulation of human

$P2Y_1$ receptors in 1321N1 cells was achieved with adenosine-5'-0-(2-thiodiphosphate) for 24 hours. After receptor down-regulation the concentration-effect curve for ADP was rightward-shifted by 10-fold and ATP agonist activity abolished. Thus with a lower receptor reserve, as may occur in Jurkat cells, ATP is ineffective as an agonist.

The hypothesis that the $P2Y_1$ receptor is the P_{2T} or $P2Y_{ADP}$ receptor was further discussed by Fagura et al., (1998). They concluded that washed platelets contained a $P2Y_1$ receptor population that mediates ADP-induced increases in $[Ca^{++}]_i$. This population is distinct from the $P2Y_{ADP}$-receptor. ADP on platelets is therefore capable of activating $P2Y_{ADP}$ and $P2Y_1$ receptors present. Activation of these receptors are important in mediating shape-change and aggregation but the precise mechanisms are as yet unknown.

1.5.2 $P2Y_2$

Lustig et al., (1993) first describe the cloning of the mouse $P2Y_2$ receptor, previously known as P_{2U}, from a mouse neuroblastoma cell line (NG108-15). The pharmacological agonist profile for $P2Y_2$ is ATP=UTP>ATPγS>>2MeSATP. The $P2Y_2$ receptor is responsive to UTP, and not suprisingly shares only 40% identity with chick $P2Y_1$. Co-localisation of the $P2Y_2$ and $P2Y_6$ receptor genes at human chromosome 11q13.3-14.1 has been shown establishing a gene cluster thought to suggest a relatively recent expansion of the gene family by gene duplication (Pidlaoan et al., 1997)

Initial reports placed UDP as an agonist at $P2Y_2$ receptors but Nicholas et al., (1996a) showed that this was due to contamination of UDP with UTP or cell-surface

conversion via nucleoside diphosphokinase. The $P2Y_2$ receptor is therefore most potently responsive to ATP and UTP. The human $P2Y_2$ receptor was cloned by Parr et al., (1994) from human airway epithelial cells. It was of particular interest as it shows promise for a type of therapy for cystic fibrosis (section 10). Other $P2Y_2$ receptors cloned to date include human bone (Bowler et al., 1995) and rat lung (Rice et al., 1995), pituitary (Chen et al., 1996a) and endothelium (Godecke et al., 1996). Linking to PLC activation and a rise in intracellular Ca^{++}, $P2Y_2$ receptors are widely distributed throughout the body, being identified in the heart, liver, lung, kidney, placenta, skeletal muscle, brain, spleen and testis (Lustig et al., 1996).

Like the $P2Y_1$ receptor, subtypes of $P2Y_2$ are also thought to exist based on native tissue observations. $P2Y_2$ receptors have been described that are competitively inhibited by suramin in PC12 cells (Murrin and Boarder, 1992) and rat aortic rings (Dainty et al., 1994). However in bovine aortic endothelial cells and canine tracheal epithelium responses at the $P2Y_2$ receptor proposed are insensitive to suramin (Wilkinson et al., 1993; Dainty et al., 1994). These issues may be resolved by considering $P2Y_4$ receptors as well as $P2Y_2$ receptors. $P2Y_4$ receptors from the rat have a similar agonist profile as $P2Y_2$ receptors (Webb et al., 1998a; Bogdanov et al., 1998) but are insensitive to suramin (Charlton et al., 1996b). It is possible, therefore, that responses previous designated as suramin insensitive P_{2U} receptors, may be due to the presence of $P2Y_4$ receptors.

1.5.3 $P2Y_3$

The chick $P2Y_3$ receptor was cloned by Webb et al., (1996c) and reported to be activated by nucleoside diphosphates. The chick $P2Y_3$ receptor was expressed in

Jurkat cells and assayed with various nucleotides for calcium responses. The rank order of potency was UDP>UTP>ADP>ATP with ATP being only a partial agonist. Suramin and reactive blue 2 both were able to antagonise the calcium responses of $P2Y_3$ receptors. Sharing only 39% identity with $P2Y_1$ the $P2Y_3$ receptor is expressed in brain, spinal cord, kidney, lung and in the spleen. A recent report by Li et al., (1998) suggested that the chick $P2Y_3$ is the avian homolog of the mammalian $P2Y_6$ receptor. The mammalian $P2Y_6$ shares 65% identity with the chick $P2Y_3$ receptor making it the closest P2Y receptor relative. The turkey $P2Y_3$ receptor was isolated by Li and co-workers and expressed in 1321N1 cells. This was functionally compared to 1321N1 cells transfected with rat $P2Y_6$. UDP was the most potent agonist at both receptors with UTP being of lower potency but remaining a full agonist. ATP and its derivatives were slightly more active at the turkey $P2Y_3$ receptor than at the rat $P2Y_6$. To further establish $P2Y_3$ as $P2Y_6$ a $P2Y_3$ probe was used on two human genomic libraries at low stringency identifying the human $P2Y_6$ receptor. It can therefore be stated that the human genome does not contain a receptor that is more homologous to the avian $P2Y_3$ receptor than the $P2Y_6$ receptor.

1.5.4 $P2Y_4$

The human $P2Y_4$ receptor was cloned by Communi et al., (1995) and Nguyen et al., (1995). As shown on the dendogram (Figure 1.2) $P2Y_4$ is most closely related to the $P2Y_2$ receptor. Using a fluorescent probe, Nguyen et al., (1995) established the chromosomal location of human $P2Y_4$ receptor to be in region q13 of the X chromosome. Stam et al., (1996) cloned the human $P2Y_4$ receptor from the pancreas, which at the time they termed $P_{2P,}$ but is identical in sequence to that published by

Communi et al., (1995). Webb et al., (1998a) and Bogdanov et al., (1998) published the cloned rat brain $P2Y_4$ receptor. The rat $P2Y_4$ shows 83% sequence identity with the human $P2Y_4$ receptor and represents a sequence homologue of the human pyrimidinoceptor. Communi et al., (1996a) expressed human $P2Y_4$ in 1321N1 cells and assayed for activation of PLC. UTP and UDP both stimulated the formation of inositol phosphates with equal potency above ATP a partial agonist and ADP relatively inactive. However Nguyen et al., (1995) also expressed their clone in 1321N1 and reported a similar agonist profile, except ATP was not an effective agonist. Bogdanov et al., 1998 reported that the rat $P2Y_4$ receptor shows an agonist potency order of ITP=ATP=ADP(pure)=UTP=ATPγS=2-MeSATP=Ap4A>UDP (pure) with ADP, ATPγS, 2-MeSATP and UDP being partial agonists. Therefore the $P2Y_4$ receptor has UTP as a full and potent agonist but ATP has been reported as a full, partial or ineffective agonist. To resolve this problem transfected human and rat $P2Y_4$ receptors were compared in the 1321N1 cell line (Kennedy et al., 1999a). Human $P2Y_4$ receptors responded maximally to UTP for both Ca^{++} and IP levels detected. ATP was ineffective at increasing Ca^{++} levels, acting as a weak antagonist, and achieved small increases in IP levels due to ATP dependent production of UTP in the cell media. Therefore ATP is ineffective at human $P2Y_4$. The rat $P2Y_4$ receptor displayed UTP to be more potent than ATP but both being full agonists for both Ca^{++} and IP levels. These results show that ATP is a potent agonist at the rat $P2Y_4$ receptor, unlike the human $P2Y_4$ receptor. The agonist profile of rat $P2Y_4$ is more like the P2U receptor subtype than human $P2Y_4$. The rat $P2Y_4$ receptor was reversibly antagonized by Reactive blue 2 but not by suramin, which inhibits the $hP2Y_2$ receptor (a known P2U receptor). Therefore the rat $P2Y_4$ and the $P2Y_2$ subtypes appear to be distinct forms of the P2U receptor, defined by ATP and UTP

being equal in potency, but can be distinguished as suramin-insensitive and suramin-sensitive P2U receptors, respectively. This may reflect the native receptors seen in PC12 cells and bovine aortic endothelial cells (as discussed in section 1.5.2).

Observations of nucleotide metabolism with implications to the $P2Y_4$ receptor were also discussed by Nicholas et al., (1996b). Action of UDP on $P2Y_4$ receptors was discovered to be due to impurities of stocks with UTP, and cell-surface conversion of UDP to UTP by nucleoside diphosphokinase. Treatment of stock solutions with hexokinase and glucose or HPLC purification to produce pure UDP resulted in the conclusion that UDP was ineffective at $P2Y_4$, making them most potently activated by UTP.

1.5.5 $P2Y_5$

The $P2Y_5$ receptor was first cloned as 6H1 by Kaplan et al., (1993) and described as being P2Y like in a paper by Webb et al., (1996d). The inclusion of 6H1 into the P2Y family was considered based on its sequence similarity (~30%) and ligand binding displacement experiments with [^{35}S]dATPαS. Binding data for this ATP compound was later thought to be untrustworthy due to non-specific binding (Schachter and Harden, 1997). Li et al., (1997) reported that having cloned the turkey homolog of $P2Y_5$ and made stable expression clones in 1321N1 cells no nucleotide evoked responses were observed from these cells. To date no functional data has been acquired for the $P2Y_5$ receptor and it remains as either a pseudo-receptor or a receptor for which a natural ligand has yet to be found.

1.5.6 $P2Y_6$

The $P2Y_6$ receptor was first isolated from a rat aortic smooth muscle cell cDNA library by Chang et al., (1995). The rat $P2Y_6$ shows 44 and 38% identity with rat $P2Y_2$ and chick $P2Y_1$ receptors respectively. Cloned into C6 glioma cells, rat $P2Y_6$ shows a rank order of potency for PLC activation of UTP>ADP=2MeSATP> ADPβS>ATP=ATPγS. Preliminary use of antagonists suramin and reactive blue 2 showed inhibition of rat $P2Y_6$ responses. Tissue distribution of rat $P2Y_6$ included lung, stomach, intestine, spleen, mesentery, heart and aorta. Communi et al., (1996b) published the human $P2Y_6$ sequence and expressed it in 1321N1 cells. Human $P2Y_6$ was also cloned from placenta where it is produced in different lengths possibly due to alternate splicing (Maier et al., 1997) and human T cells (Southey et al., 1996). 1321N1 expression of human $P2Y_6$ led to PLC activation agonist potency order of UDP>5-bromo-UTP>UTP>ADP>2MeSATP>>ATP. Nicholas et al., (1996b) altered this rank order after investigations with stable purified nucleotides. UTP was discovered only to be active due to contamination or breakdown to UDP. This means that UDP is the most potent activator of $P2Y_6$ with UTP, ATP and ADP being weakly active.

1.5.7 $P2Y_7$

The $P2Y_7$ receptor was first cloned by Akbar et al., (1996) from human erythro-leukemic cells. Cloned into COS-7 cells the apparent rank order of potency was ATP=ATPγS>2MeSATP>βγ-MeATP>ADP=UTP. However, Herold et al., (1997) transfected the receptor into 1321N1 cells and were unable to stimulate the

$P2Y_7$ receptor with nucleotides. The reason became apparent when Yokomizo et al., (1997) published cloning and functional analysis of a leukotriene B_4 receptor with identical sequence to the $P2Y_7$ receptor. Nucleotide signalling observed by Akbar et al., (1996) was thought to have occurred due to the presence on intrinsic purinoceptors on COS-7 cells. Therefore $P2Y_7$ is now termed as a leukotriene B_4 receptor and not a P2Y receptor.

1.5.8 $P2Y_8$

Bogdanov et al., (1997) described the cloning of a novel nucleotide receptor isolated from the neural plate of *Xenopus* embryos. This receptor, for which no other species homologs have been cloned so far, was expressed highly in embryos and functionally linked to PLC. A rank order of agonist potency was ATP=UTP =ITP=CTP=GTP for PLC activation. Until mammalian homologs of $P2Y_8$ exist then the receptor will remain less important.

1.5.9 $p2y_9$

The $p2y_9$ receptor was cloned from human genomic DNA by Bohm et al., (1997) and Janssens et al., (1997). Cloned from a sequenced-tagged site that was similar to the receptor $P2Y_5$ Janssens and co-workers carried out transfections of the $P2Y_5$-like receptor into COS-7, 1321N1 and CHO-K1 cells. No functional responses were found for 40 nucleotides tested over 4 assays. It can be concluded that the $p2y_9$ receptor may be activated by a rare nucleotide or it is not a nucleotide receptor and has some other unknown ligand. It is therefore labelled in lowercase lettering to display its unknown P2 function.

1.5.10 $p2y_{10}$

The $p2y_{10}$ receptor was cloned by Bohm et al., (1997) and based on sequence similarity was placed tentatively in the P2Y family. No Functional studies have yet established if $P2Y_{10}$ is in fact a P2Y receptor.

1.5.11 $P2Y_{11}$

Human $P2Y_{11}$ was cloned from placenta by Communi et al., (1997b) and exhibits 33% amino acid identity with the human $P2Y_1$ receptor its closest homolog. The $P2Y_{11}$ receptor has unusual gene structure in that it contains an intron not seen in other P2Y receptors. The $P2Y_{11}$ receptor unlike other P2Y receptors is also only expressed in the spleen and in HL60 cells. Transfected into 1321N1 cells the human $P2Y_{11}$ shows a rank order of potency of ATP>2MeSATP>>>ADP with UTP and UDP being inactive. Expressed in 1321N1 cells and CHO-K1 cells the $P2Y_{11}$ receptor shows secondary messenger coupling to activation of PLC and adenylate cyclase, a novelty in the P2Y family.

1.5.12 Other P2Y receptors

Boyer et al., (1997) described cloning an avian P2Y receptor which shares a high homology to the mammalian $P2Y_4$ receptor. However it does not behave like a $P2Y_4$ receptor. Stable expression of turkey p2y in 1321N1 cells led to the observation that activation of the receptor lead to inositol phosphate responses with a agonist potency order of ATP=UTP>AP_4A which is similar in order to the $P2Y_2$ receptor.

1.5.13 Summary of P2Y characterisation

The previous sections have described the efforts to identify different P2Y receptor subtypes. So far 5 mammalian P2Y receptors have been cloned and characterised and are summarised in Table 1.2 below. The agonist profiles are based on purified agonists and where necessary appropriate precautions were made for agonist inter-conversion. Antagonists listed in table 1.2 are of limited use in defining the difference between P2Y subtypes and high-light an area that is being thoroughly investigated.

	Agonist Profile	Coupling	Localisation	Antagonists
$P2Y_1$	2MeSADP>ADP> 2MeSATP>ATP	PLCβ/IP_3/Ca^{2+}	Heart, brain, lung, liver, muscle, kidney, in small amounts in the spleen and absent from the testis	PPADS, suramin, N6MABP
$P2Y_2$	ATP=UTP>ATPγS>> 2MeSATP	PLCβ/IP_3/Ca^{2+}	Heart, liver, lung, kidney, placenta, skeletal muscle, brain, spleen and testis	suramin
$P2Y_4$	UTP>ATP*. UDP ineffective	PLCβ/IP_3/Ca^{2+}	Brain, heart, placenta, skeletal muscle, vas deferens, spleen and spinal cord	RB2
$P2Y_6$	UDP>>UTP=ATP= ADP	PLCβ/IP_3/Ca^{2+}	Lung, stomach, intestine, spleen, mesentery, heart and aorta	RB2>PPADS >suramin
$P2Y_{11}$	ATP>2MeSATP>>> ADP. UDP and UTP are ineffective	PLCβ/IP_3/Ca^{2+}/AC	Spleen and HL60 cells	suramin

Table 1.2 Characteristics of functional mammalian P2Y receptors.

1.6 Agonists at P2Y receptors

In section 1.2 the sources of natural ligands of P2 receptors were discussed. To help characterise the different subtypes of P2 receptors more clearly, better agonists were developed. Most of these are based around ATP as a basic structure. A few of these compounds are shown in Figure 1.3. ATP can be modified in a few different ways. Additions can be made to the second carbon of the adenine ring to

give for example 2MeSATP, 2-cyanohexylthioATP or 2-(4-nitrophenylethylthio) ATP or the sixth carbon on the ring to give N^6-Methyl-ATP. Additions on the ribose group to give for example BzATP or 3'-deoxy-3'-acetylamino-ATP. Most substitutions occur on the phosphate backbone that may confer additional specificity or resistance to nucleotidase degradation. Examples of this include ADP-β-F, ATPγS or α,β-methylene-ATP (for other examples see Fredholm et al., 1994 and Windscheif, 1996).

1.7 Antagonists at P2Y receptors

As well as specific agonists it is equally important to develop subtype specific antagonists. So far little progress has been made in defining highly specific antagonist in the public domain.

Suramin is a particularly non-specific sulphonic acid derivative antagonist based on napthalene. It has been used in many studies on P2 receptors since first used (Dunn and Blakeley, 1988) but has many biological effects (Voogd et al., 1993). Action at P2 receptors is complicated by its ability to inhibit ectonucleotidases (Hourani and Chown, 1989). Suramin and its analogues, such as NF023, are also able to inhibit the formation of G protein ternary complex (agonist/receptor/G protein) by binding to the Gα subunit preventing spontaneous GDP exchange and receptor to Gα binding (Beindl et al., 1996). Suramin can also interfere with growth factors and their receptors thus affecting proliferation (Nakajima et al., 1991). Suramin though non-specific still remains a useful compound in P2 receptor research.

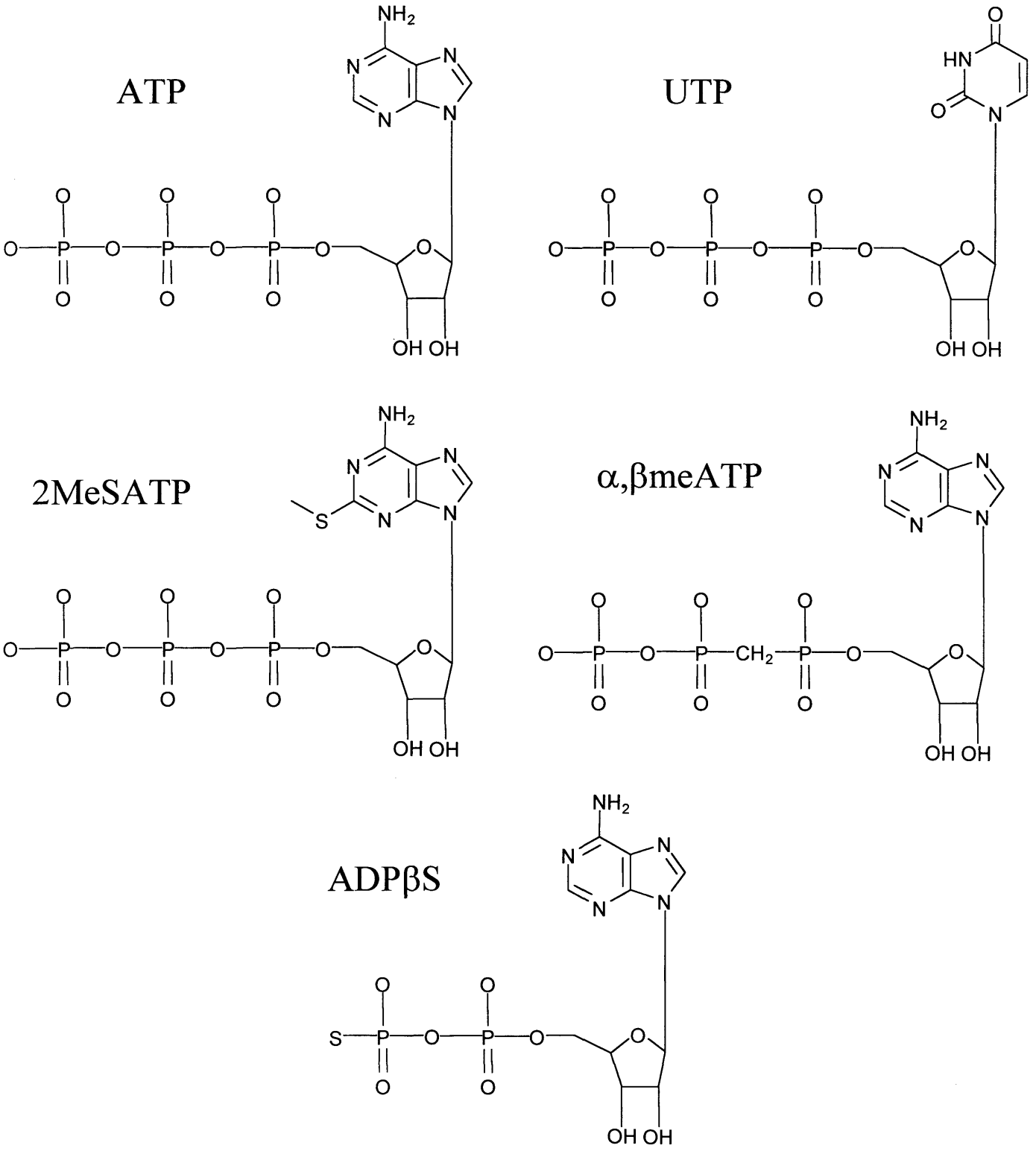


Figure 1.3
Structures of some nucleotide analogues.

The use of suramin has pointed to $P2Y_2$ subtype receptors based on antagonist sensitivity (Dainty et al., 1994; Wilkinson et al., 1994). Suramin antagonises P2X and P2Y receptors (Hoyle et al., 1990) indicating a lack of specificity in some tissues. Charlton et al., 1996a investigated the use of suramin as a potential $P2Y_1$ and $P2Y_2$ antagonists in transfected cell systems. Suramin inhibited $P2Y_1$ receptor activation (pA_2 5.77±0.11) with a higher potency than at the $P2Y_2$ receptor (pA_2 4.32±0.13). This reflected earlier work by Wilkinson et al., (1993; 1994), on bovine aortic endothelial cells. Charlton et al., (1996b) showed that suramin is not an antagonist at the cloned $P2Y_4$ receptor.

Pyridoxal-phosphate-6-azophenyl-2'-4'-disulphonic acid (PPADS) is another well known P2 antagonist used initially as a P2X antagonist (Lambrecht et al., 1992). PPADS is a useful antagonist in bovine aortic endothelial cells. Brown et al., (1995) showed selectivity of PPADS for $P2Y_1$ receptors above $P2Y_2$ receptors. However, Ho et al., (1995) described the antagonistic action of PPADS on astrocyte $P2Y_2$ receptors and not $P2Y_1$. It is difficult to explain this discrepancy.

Reactive blue 2 and reactive red 2 are both anthraquinone dye based antagonists with selectivity for P2Y receptors over P2X receptors. Reactive red 2 showed potent antagonism of $P2Y_1$ receptors present on guinea-pig taenia coli (Bultmann and Starke, 1995)

Adenosine-3'-phosphate-5'-phosphosulphonate (A3P5PS) was investigated by Boyer et al., (1996) as a turkey $P2Y_1$ receptor antagonist developing a pA_2 of 6.46±0.17. It also showed a small degree of agonist activity at the turkey $P2Y_1$ receptor, but not at the human $P2Y_1$ receptor. This antagonist appears to be specific to PLC coupled $P2Y_1$ receptors as it is ineffective as an antagonist and unreactive as an agonist at the C6 glioma $P2Y_1$-like adenylate cyclase coupled receptor, or on

cloned $P2Y_2$, $P2Y_4$ or $P2Y_6$ in 1321N1 cells. A more recent report however outlines the non-specific actions of this compound making it less useful for intact tissue work (Bultmann et al., 1998). Another derivative, N6-methyl 2'-deoxyadenosine 3',5'-bisphosphate (N6MABP), has been examined at the phospholipase C-coupled $P2Y_1$ receptor of turkey erythrocyte membranes, showing strong competitive antagonism selective for human $P2Y_1$ over human P2Y2, human P2Y4 or rat P2Y6 receptors (Boyer et al., 1998).

ARL 66096 (2-propylthio-D-β,γ-difluoromethylene ATP) is a selective potent $P2Y_{ADP}$ (P_{2T}) antagonist (pA_2 8.7). Antagonism though potent is not purely competitive and also shows weak antagonism at $P2Y_1$ and P2X receptors (Humphries et al., 1994). It is hoped this compound will be useful in preventing platelet aggregation in the clinic.

1.8 Signal Transduction of P2Y receptors

From hydropathy analysis it was determined that P2Y receptors were seven transmembrane domain containing G protein-coupled receptors. P2Y receptors were first identified as linking to phospholipase C and the production of inositol polyphosphates and raised intracellular Ca^{++}. As further characterisation of P2Y receptor signal transduction advances, more signalling molecules are linked to modulation by P2Y receptors. The following sections discuss the signal transduction mechanisms and molecules involved in P2Y receptor signalling.

1.8.1 Structure of P2Y receptors

The structure of P2Y receptors based on rhodopsin is consigned to class A group V in the G protein linked seven transmembrane domains (7TM) superfamily. P2Y receptors consist of 7TM domains with three intracellular and three extracellular loops with a extracellular N-terminus containing sites for glycosylation and an intracellular COOH-terminus containing sites of phosphorylation. P2Y receptors are amongst the smallest G protein-linked receptors, being around 350 amino acids in length. The areas of highest homology are contained in the transmembrane domains, which is why they are used for generation of degenerate primers for cloning new P2Y receptors (Libert et al., 1989). Different receptor structure is reflected in the pharmacological characteristics of the P2Y receptors. Certain residues are conserved between P2Y receptors as they represent a necessary function of the receptor. There are conserved cysteine residues in the first and second extracellular loops that may represent a site for a disulphide bridge. Site-directed mutagenesis studies have shown that the human $P2Y_1$ receptor contains two disulphide bridges linking residues Cys124 in extracellular loop 1 (EL1) to Cys202 in EL2 and Cys42 in the N-terminal section to Cys296 in EL3 (Moro et al., 1999). Mutational studies have been carried out for the mouse $P2Y_2$ receptor (Erb et al., 1995) and for the human $P2Y_1$ receptor (Jiang et al., 1997; Moro et al., 1998; 1999). Each site-directed mutagenesis pin-points the importance of that amino acid in receptor stability or ligand binding.

Receptor and ligand interactions were studied with the site-directed mutagenesis with the idea that locating the amino acids of interaction will help design better agonists and antagonists. Erb et al., (1995) set out to map the mouse $P2Y_2$ receptor and concluded that amino acids with a positive charge would be the

likely targets as the fully ionised forms of the nucleotides are the most active. In the mouse $P2Y_2$ receptor four positively charged residues, His262 and Arg265 in TM 6 and Lys289 and Arg292 in TM 7 were mutated. His262Leu, Arg265Leu and Arg292Leu all caused a 100-850 fold decrease in the potency of ATP and UTP and rendered ADP and UDP ineffective. The mutation Lys289Arg led to an increase in the activity of ADP and UDP 100 fold above ATP and UTP. It was concluded that His262, Arg265 and Arg292 all have interactions with the phosphate groups of nucleotides.

Mutational analysis at the human $P2Y_1$ receptor by Jiang et al., (1997) uncovered several different areas of ligand interactions. Using 2MeSATP as the agonist, mutation Ser317Ala did not alter agonist potency. Replacement of alanines at residues His132, Tyr136, Phe226 or His277 all resulted in a 7-18-fold decrease in 2MeSATP potency showing residues slightly modulating ligand binding. Mutations Lys280Ala and Gln307Ala both greatly reduced ligand binding with a large reduction in potency. Mutations Arg128Ala, Arg310Ala and Ser314Ala however showed no activity of 2MeSATP. These mutations all point to TM3 and TM7 as critical components of the ligand-binding pocket. It was noted that mutations that decreased 2MeSATP activity also decreased 2MeSADP and 2-(hexylthio)-AMP potencies. Interestingly mutations in TM5 of Thr221Ala and Thr222Ala both confer a larger inhibition of triphosphates than diphosphates possibly implicating a role for these residues in γ-phosphate recognition. Moro et al., (1999) also investigated human $P2Y_1$ mutation analysis of ligand binding. Using Glu209 (EL2) and Arg287 (EL3) they mutated Glu209Arg which selectively reduced the potency of 3'NH_2-ATP, consistent with the hypothesis of direct contact between EL2 and nucleotide ligands, and Arg287Lys which greatly reduced the potency of 2-(hexylthio)-AMP,

implicating Arg287 in coordination of the alpha- and beta- phosphates of the nucleotide triphosphate chain.

Further mutational analysis as well as computer modelling of the ligand binding sites will help future development of more specific and potent agonists and antagonists.

1.8.2 G Proteins

G proteins are membrane bound heterotrimeric guanine-nucleotide binding proteins. G proteins are composed of α, β, and γ proteins, each of which has different isoforms. G protein-coupled receptors were first discovered by Rodbell and Gilman in the 1970's. Rodbell and co-workers (Lin et al., 1977) discovered that glucagon activation of rat liver adenylate cyclase was modulated by GTP and glucagon-receptor binding was inhibited by guanine nucleotides. Gilman and co-workers (Maguire et al., 1976) showed similar results with the β-adrenergic receptor and went on to purify the G protein required to reconstitute GTP-sensitive agonists binding and stimulation of adenylate cyclase (Northup et al., 1980). These G proteins were discovered to link to many ligand-receptors creating what is today a vast superfamily of receptors including small biogenic amines e.g. adrenaline, small peptides such as enkephalins and large glycoproteins such as luteinising hormone. These receptor-ligand interactions with G proteins enable coupling to a diverse array of secondary messenger systems, including activation/inhibition of adenylate cyclase and Ca^{++} mobilisation via inositol-1,4,5-triphosphate. After cloning of the β-adrenergic receptor the structure of the G protein-coupled receptors was found to contain seven transmembrane domains. The third intracellular cytoplasmic loop is

thought to be involved in G protein-receptor interaction (Strader et al., 1995). A histidine residue, conserved in P2Y receptors, is found near transmembrane domain three which is proposed to be involved in coupling ligand-binding to G protein activation as seen in other G protein-coupled receptors (Fraser et al., 1988).

The mechanism behind G protein activation and coupling to secondary messenger systems is that G proteins are activated by exchange between the GDP-bound inactive form and the GTP-bound active form (Figure 1.4). On binding GTP the G protein dissociates into the GTP bound G_α subunit and the $\beta\gamma$ subunit. The G_α subunit hydrolyses GTP to GDP enabling the G_α subunit to reassociate with the $\beta\gamma$ subunit. This inactive complex can then associate with inactive receptor to begin the cycle again.

G protein α subunits are divided into four subfamilies G_s, G_i, G_q, and $G_{12/13}$ (Hepler and Gilman, 1992) on the basis of sequence and sensitivity to bacterial toxins.

G protein α_s subunits are sensitive to cholera toxin. Cholera toxin causes ADP-ribosylation of α_s subunit preventing GTPase activity and binding to subunit $\beta\gamma$. The α_s subunit was recognised by Gilman, (1987) by its ability to activate adenylate cyclase.

α_i subunit class has expanded to include α_{i-1}, α_{i-2}, α_{i-3}, α_o, α_t, α_{t2}, and α_z. α_i subunits were first named by the ability to inhibit adenylate cyclase (Neer and Clapham, 1988). G protein α_i and α_o are sensitive to pertussis toxin which has been shown to ADP-ribosylate a cysteine residue preventing activity. Only α_z is resistant to pertussis toxin as the consensus site for ADP-ribosylation is missing (Fong et al., 1988).

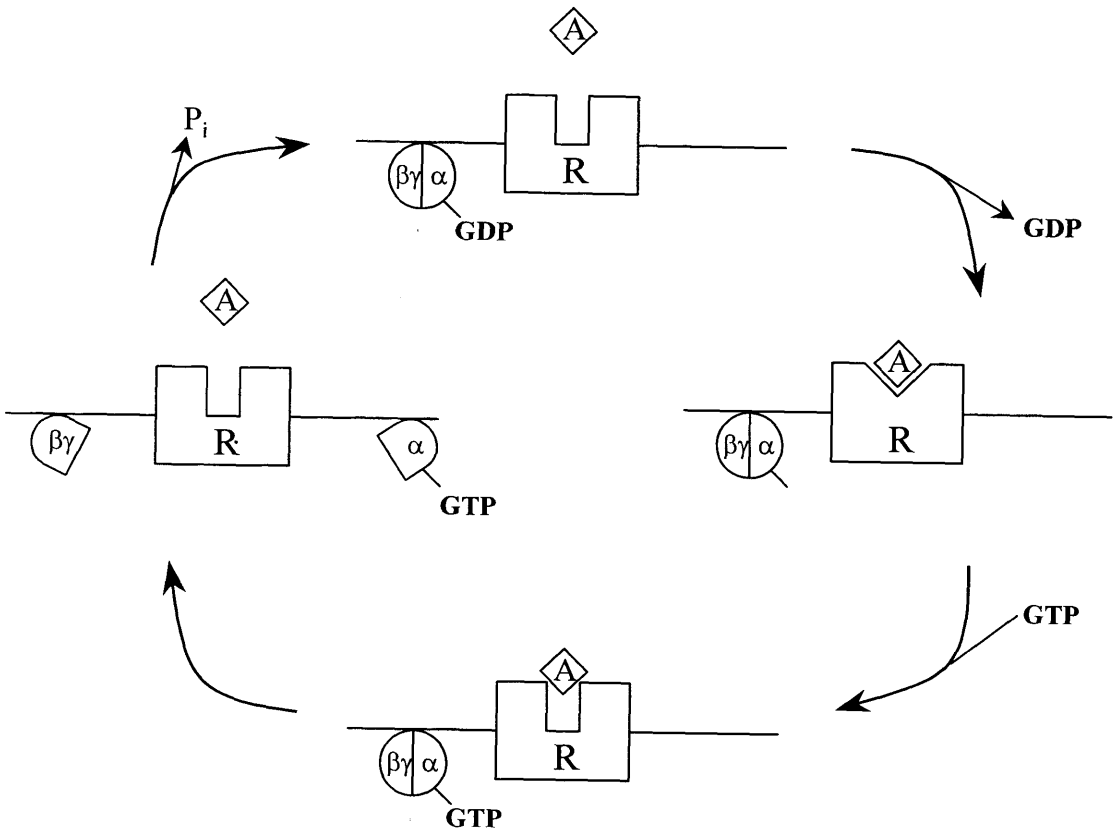


Figure 1.4
Flow diagram representing the ternary complex model of the G protein cycle.

G protein subunit α_q class includes members α_q, α_{11}, and α_{16}. All link to activation of phospholipase C with a rank order of PLCβ1 > PLCβ2 > PLCβ3 (Nakamura et al., 1995). Stimulation of $G_{q/11}$ via receptor activation causes the phosphorylation of tyrosine residue (Tyr356) which was essential for activation (Umemori et al., 1997).

G protein α_{12} and α_{13}, which are pertussis toxin insensitive, have no well defined targets of action (see Offermanns and Schultz, 1994). They have been implicated in mitogenesis (Aragay et al., 1995; Collins et al., 1996), apoptosis (Berestetskaya et al., 1998), signalling to PLD, which is dependent on Rho GTPase (Plonk et al., 1998), couple to the angiotensin 1A receptor (Macrez-Lepretre et al., 1997) and thromboxane A2 receptors (Harhammer et al., 1996), and are phosphorylated on platelet activation (Offermanns et al., 1996).

There are currently twelve G protein γ subunits and six G protein β subunits that have been identified (Ray et al., 1995; Simon et al., 1991; Watson et al., 1996). Therefore there are many combinations of βγ units that can be made. G protein βγ-subunits once formed are essentially a monomer as they are only separated by denaturation. While G protein α-subunits are implicated in activation or inactivation of various enzymes, the role of the βγ-subunits is now established also as a signalling molecule (Crespo et al., 1994; Koch et al., 1994; Faure et al., 1994; van Biesen et al., 1995). A dual role has been observed for activation of β–adrenergic receptors leading to modulation of mitogen activated protein kinase (MAPK) activity. While activating G_s increases adenylate cyclase and activation of PKA leading to an inhibition of MAPK activity, the βγ-subunits lead to an activation of MAPK via a Ras-dependent mechanism (Crespo et al., 1995). A recent review discusses the roles of βγ-subunits in signalling (Clapham and Neer, 1997) showing a wide diversity of

signalling pathways including ion channels, PLA_2, PLCβ1–3, adenylate cyclase II and IV, G protein-coupled receptor kinases, PI3K, MAPK and tyrosine kinase cascade, secretion and plasma membrane Ca^{++} pump. Further discussion of MAPK and tyrosine kinase activation is found in section 1.8.5 and 1.8.6.

1.8.2.1 P2Y receptors and G proteins

The level of promiscuity compared to fidelity that exists between G proteins and receptors is a complex issue. It is possible that receptors, when expressed at similar levels regardless of cell type, will link to a defined set of G protein subunits. Different tissue types may express receptors at different levels, however, causing differential linkage to signalling pathways. Dependent on the cell type they are expressed in, receptors may be able to link to more than one set of G proteins, causing a redundancy in receptor G protein linkage, helping to account for the differing types of signalling responses observed from a particular receptor. Another possibility is that single G protein subtype activation may cause activation of multiple signalling pathways via the different G protein subunits e.g. βγ subunit signalling.

P2Y receptors have been linked to many types of G protein subunits. Pertussis toxin, which affects the $G_{i/0}$ protein family, has been useful in determining signalling pathways. Studies on both native cells and transfected systems have aided identification of the G proteins involved. Problems exist however in native cells which may express multiple receptor subtypes and new uncharacterised receptors. It can also become complicated under certain circumstances involving receptor cross-talk. An example of this was observed in MDCK cells by Post et al., (1996) where

ATP and UTP caused activation of G_s and adenylyl cyclase indirectly by release of PGE_2.

The $P2Y_1$ receptor, being pertussis toxin insensitive and activating PLC, is thought to couple to G_q protein in native cells, e.g. in bovine aortic endothelial cells (Purkiss et al., 1994; Motte et al., 1993; Pirotton et al., 1996) and rat aortic smooth muscle cells (Pediani et al., 1999). This has also been shown for the cloned turkey $P2Y_1$ receptor by Filtz et al., (1994) and turkey and bovine $P2Y_1$ receptors (Roberts et al., 1999).

The $P2Y_2$ receptor has been shown to link to $G_{i/0}$ protein, being pertussis toxin sensitive, in BAEC's (Purkiss et al., 1994; Erb et al., 1993; Pirotton et al., 1996). In rat aortic smooth muscle cells Pediani et al., (1999) observed activation of Ca^{++} signalling by UTP that had both a pertussis toxin sensitive and insensitive component. This could be due to: 1. Different G protein signalling that could be via one receptor to two different G proteins; 2. Co-expression of $P2Y_2$ and $P2Y_4$ receptors linking to different G proteins; 3. Only partially effective disabling of $G_{i/o}$ by pertussis toxin. Other native cells that link $P2Y_2$ to a pertussis toxin sensitive G protein include bovine ciliary epithelium cells (Shahidullah and Wilson, 1997), MDCK-D1 cells (Firestein et al., 1996), PC12 cells (Soltoff et al., 1998a) and CHO cells (Dickenson et al., 1998), although the inhibition is only partial in some cases possibly implying multiple G protein coupling. Human EAhy 926 endothelial cells express a UTP and ATP responsive receptor likely to be $P2Y_2$ that is linked to a pertussis toxin insensitive G protein (Graham et al., 1996). Work on isolated receptors has shown the $P2Y_2$ receptor to link to a pertussis toxin sensitive G proteins and insensitive G proteins. In *Xenopus* oocytes $P2Y_2$ exhibits linkage to two different G proteins (pertussis sensitive and non-sensitive) and two different ion

channels, Ca^{++} dependent chloride channel and inward-rectifier potassium channels (Mosbacher et al., 1998). Filippov et al., (1997) using cRNA for rat $P2Y_2$ injected into rat superior cervical sympathetic (SCG) neurons showed multiple coupling to G proteins. A pertussis toxin sensitive G protein linking to inhibition of N-type Ca^{++} channel current and an insensitive G protein linking to inhibition of M-type K^+ channel current was observed. Roberts et al., (1999) show a pertussis sensitive component to PLC activation by $P2Y_2$ receptors transfected into 1321N1 cells exhibiting a 27.5 ± 0.6 % inhibition at 20 min. The partial inhibition observed can be explained by coupling of the $P2Y_2$ receptor to both $G_{i/0}$ and $G_{q/11}$.

The $P2Y_4$ receptor has been suggested to couple to multiple G proteins, including the G_i protein, in transfected 1321N1 cells. A partial inhibition with pertussis toxin was observed which altered with time, inhibiting the response to UTP at early times (62± 5% inhibition at 30 s), but its effects were no longer observed at 5 or 20 min (Communi et al., 1995; 1996a). Another report has shown however that human $P2Y_4$ activation in 1321N1 cells leads to time or dose dependent PLC activation which is pertussis toxin insensitive (Roberts et al., 1999). Degeneracy in linkage may also be true for the native cell, but may reflect the nature of high receptor expression in a transfected system. In a lung submucosal derived cell line $P2Y_4$ receptor activation was shown to inhibited by pertussis toxin, suggesting $G_{i/o}$ involvement (Communi et al., 1999)

The $P2Y_6$ receptor is thought to link to G_q proteins as an insensitivity to pertussis toxin has been demonstrated (Chang et al., 1995; Robaye et al., 1997) as well as coupling to PLC. Filippov et al., (1999) using cRNA for $P2Y_6$ injected into neurons observed activation of the $P2Y_6$ receptor led to inhibition of N-type Ca^{++}

currents and M-type K^+ currents that was pertussis toxin sensitive and insensitive respectively showing multiple coupling.

The $P2Y_{11}$ receptor, when expressed in CHO-K1 cells, links to activation of adenylyl cyclase and also activation of PLC. In 1321N1 cells $P2Y_{11}$ activates adenylyl cyclase much less potently while still activating PLC (Communi et al., 1997b; Kennedy et al., 1999b). Since activation of adenylyl cyclase may occur via the G_s protein the $P2Y_{11}$ receptor is probably linked directly to G_s as well as possibly another G protein linked to PLC. This though has yet to be proven experimentally as activation of adenylyl cyclase could be independent of G_s involving Ca^{++} or PKC (see 1.8.8).

1.8.3 Phospholipases

Phospholipases play important roles in cellular metabolism involving biosynthesis and degradation of membrane lipids. Certain phospholipases generate lipid second messengers that are implicated in the signal transduction process. Among these phospholipases are PLA_2, PLC and PLD, which will be discussed further in the following sections.

1.8.3.1 PLA_2

The phospholipases are named by the position at which they attack the phospholipid backbone. PLA_2 catalyses the production of arachidonic acid and lysophospholipids by attacking the middle group on membrane phospholipids. These by-products of PLA_2 activity are implicated downstream of PLA_2 as they are

precursors for other lipid mediators such as prostaglandins, leukotrienes and platelet activating factor (Dennis, 1987). Two types of PLA_2 exist: secreted PLA_2 ($sPLA_2$) and cytosolic PLA_2 ($cPLA_2$). Secreted PLA_2s are small, water-soluble proteins that have been identified in snake and bee venoms, and in the mammalian pancreas. After secretion $sPLA_2$ are thought to bind to the exterior of cells and carry out hydrolysis of phospholipid substrates, in some cases producing arachidonic acid, which is free to act on cells in an autocrine/paracrine fashion or be re-uptaken as a precursor molecule. PLA_2s are grouped into numbered subsets with $cPLA_2$ being in group IV. This phospholipase shows preference to arachidonic acid containing phospholipids and its translocation to the membrane is dependent on calcium (Clark et al., 1991; Sharp et al., 1991). Activation of PLA_2 often involves secondary messengers such as G proteins and protein tyrosine kinases. Group IV $cPLA_2$ is regulated by phosphorylation at ser505 by the MAP kinase cascade (Lin et al., 1993). The production of prostacyclin (PGI_2) via PLA_2 has been shown to be regulated by tyrosine kinases and MAPK (Patel et al., 1996a).

P2Y receptor regulation of PLA_2 has been demonstrated in many cell types including astrocytes (Bolego et al., 1997), MDCK kidney epithelial cells (Firestein et al., 1996), BAEC's (Patel et al., 1996a), RAW 264.7 macrophage cells (Lin and Lee, 1996), rat thyroid cells (Smallridge and Gist, 1994) and HL60 cells (Xing et al., 1994). PLC-mediated Ca^{++} elevation and PKC have both been reported to modulate $cPLA_2$ activity (Carter et al., 1988; 1989).

1.8.3.2 PLC

Phospholipase C catalyses the hydrolysis of the membrane phospholipid phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P_2 or PIP_2] to produce two intracellular messengers, inositol trisphosphate [Ins(1,4,5)P_3] and diacylglycerol (DAG). Ins(1,4,5)P_3 induces the release of Ca^{++} from intracellular stores (Berridge, 1993) by binding to specific Ins(1,4,5)P_3 receptors which act as calcium channels which when activated open to release the calcium. DAG is bound in the membrane where it activates protein kinase C (Rhee and Choi, 1992). PLC exists as three isoforms, β, γ and δ. All isoforms have a pleckstrin homology (PH) domain near the N-terminus and two regions known as X and Y, which are involved in catalysis. The region between X and Y domains is short in PLC-β and PLC-δ but longer in the PLC-γ isoform containing SH2 and SH3 domains, enabling interaction with tyrosine phosphorylated proteins.

The PLC-β isoform can also be subdivided into four different subtypes, named PLC-β1-4. Activation of PLC-β can be via GTP bound $G_q\alpha$ proteins and also by Gβγ subunits. G_i protein subtypes can therefore activate PLC-β by release of Gβγ subunits. These G protein subunits activate the isoforms of PLC-β differently. $G_q\alpha$ activates with a rank order of PLCβ1> PLCβ2> PLCβ4> PLCβ3 and Gβγ with a rank order of PLCβ3> PLCβ2> PLCβ1 (Lee and Rhee, 1995). PLCβ-4 is not activated by Gβγ subunits (Lee et al., 1994a). The binding of $G_q\alpha$ and Gβγ subunits occurs separately on PLC-β, allowing activation by G_i and G_q proteins to have additive effect.

Many G protein coupled receptors activate PLC-β, including angiotensin II, histamine and muscarinic acetylcholine m1 and m3 receptors. P2Y receptors were

linked to PLC early in P2 receptor research on tissues including vascular endothelial cells (Pirotton et al., 1987) and turkey erythrocytes (Boyer et al., 1989). The cloned mammalian P2Y receptors $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ are all coupled to PLC-β activation when cloned into 1321N1 cells (Schachter et al., 1996; Parr et al., 1994; Communi et al., 1995; Chang et al., 1995; Communi et al., 1997a).

PLC-γ, of which there are two isoforms, are known to be activated by growth factor tyrosine kinase receptors as well as non-receptor tyrosine kinases. Platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF) and nerve growth factor (NGF) all activate PLCγ1 (Rhee and Choi, 1992). Growth factor association with its receptor leads to dimerisation and activation of tyrosine kinase. PLCγ1 is also tyrosine phosphorylated and is able, via its SH domains, to associate with tyrosine residues on the activated receptor. Activation of PLCγ1 leads to hydrolysis of $PtdInsP_2$ to $Ins(1,4,5)P_3$ and DAG.

Although it seems that G protein receptors activate PLC-β and receptor tyrosine kinases activate PLC-γ (Rhee and Bae, 1997) there are now exceptions to this as G protein receptors have been associated with tyrosine phosphorylation events and PLC-γ activation. Angiotensin II, which was already known to activate PLC-β, has been shown to activate PLC-γ1 in rat aortic smooth muscle cells (Marrero et al., 1994). However, in human aortic vascular smooth muscle cells (VSMC), Schelling et al., (1997) reported coupling of AT_1 receptors to PLC-β but not to PLC-γ, which may reflect species differences. A report by Ushio-Fukai et al., (1998) investigated rat VSMC further reporting coupling of AT_1 receptors to both PLC-β and PLC-γ with early events in $InsP_3$ production controlled by G protein-linked PLC-β and later stages controlled by tyrosine kinase dependent PLC-γ. Vassort and Puceat (1997)

also reported increased PLC-γ activity via tyrosine kinases due to P2 receptor activation leading to increased $InsP_3$ levels in cardiac cells.

1.8.3.3 PLD

Phospholipases D catalyses the production of phosphatidic acid (PA) and free polar head groups e.g. free choline from membrane phospholipids containing phosphatidylcholine. PA acts as a secondary messenger and can also be metabolised to form DAG, activating PKC, by PA phosphohydrolase. Conversion back to PA from DAG is possible by the enzyme DAG kinase. So far only one isoform of PLD has been cloned (Hammond et al., 1995) though evidence exists for further subtypes. Activation of PLD can occur by two main mechanisms being PLC dependent or independent leading to various cellular responses including mitogenesis (see Boarder, 1994). PKC activation can occur via production of elevated Ca^{++} or production of DAG by PLC (Pfeilschifter and Merriweather, 1993). PDGF receptor activation of PLD was shown to be dependent on PLCγ, tyrosine phosphorylation and PKC activation (Lee et al., 1994b; Yeo et al., 1994). Angiotensin II a G protein-linked receptor activates PLD in rat VSMC's increasing production of DAG. Evidence has been shown correlating PLC activation, PLD activation and partially dependent activation of mitogenesis (Morton et al., 1995; Wilkie et al., 1996). Evidence for PLC independent pathways exists including low-molecular-mass G proteins (Bowman et al., 1993), ADP-ribosylation factor (ARF) a small GTP dependent regulatory protein (Brown et al., 1993; Cockcroft et al., 1994) and tyrosine kinase dependent PDGF and EGF receptor activation (Fukami and Takenawa, 1992; Cook and Wakelam, 1992).

P2Y receptor activation of PLD has been demonstrated in a number of studies. Bovine adrenal medullary endothelial cells displayed $P2Y_2$ receptor activation of PLD that correlated with PLC activation (Purkiss et al., 1993). Malcolm et al., (1995) observed P2 receptor activation of PLD in rat liver plasma membranes that did not correlate with PLC activation observed. In BAEC cells Purkiss et al., (1992), demonstrated that production of PA can not only be produced from PLD conversion of phosphatidylcholine to free choline and PA but also from the action of DAG kinase on DAG produced from PLC catalysis of PIP_2. Detection of increased PA levels therefore does not always correlate with increased PLD activity.

1.8.4 Tyrosine kinase-linked receptors

Growth factor receptors are members of a large family of receptors called receptor tyrosine-kinases (RTK) that consist of transmembrane polypeptides with a protein tyrosine-kinase domain in their intracellular portion. It is important to understand the mechanics of growth factor receptor signal transduction as current research implicates a large role for tyrosine phosphorylation not only in growth factor signalling but also in P2Y G protein linked receptor signalling (Bowden et al., 1995; Graham et al., 1996; Soltoff et al., 1998a;b). Growth factors, such as EGF and PDGF, bind to their receptor causing dimerisation and receptor tyrosine autophosphorylation (Heldin, 1995) followed by initiation of a complex series of intracellular reactions (Malarkey et al., 1995). The result of these interactions can be diverse including proliferation, differentiation, cell motility, production of extracellular matrix and altered gene expression (Fantl et al., 1992; Van der Geer et

al., 1994). Growth factors themselves are able to promote dimerisation. For example, PDGF is itself a dimer enabling binding to two receptor units simultaneously. The ability of growth factor receptors to phosphorylate themselves on tyrosine residues is key to their mechanism of signalling. These tyrosine residues when phosphorylated become targets for association with proteins containing Src-homology 2 (SH2), SH3, protein tyrosine binding domains (PTB) or pleckstrin homology (PH) domains (Koch et al., 1991; Kavanaugh and Williams, 1994; Macias et al., 1994). Many cellular active proteins contain these binding motifs such as PLCγ (Ronnstrand et al., 1992), Ras GTPase activating protein (Ras-GAP), SH-protein tyrosine phosphatase SH-PTP1 and 2 and tyrosine kinase, Src. Other proteins containing these domains appear to act only as adapter molecules bringing together tyrosine phosphorylated enzymes such as growth factor receptor binding protein 2 (Grb2) (Arvidsson et al., 1994), and the p85 subunit of phosphatidylinositol 3'-kinase (PI3K) (Coughlin et al., 1989). Signalling of growth factor receptors causing increased mitogenesis has been well studied and shown to link to a protein called mitogen activated protein kinase or MAPK. This protein will now be discussed in more detail in the following section.

1.8.5 MAPK

Mitogen activated protein kinase (MAPK) was identified as a serine/ threonine kinase based on its ability to phosphorylate microtubule associated protein 2 after insulin stimulation (Ray and Sturgill, 1987). MAPK activity was first associated with two proteins, which have since been cloned and identified as p42 (ERK2) and p44 (ERK1) (Boulton et al., 1990). The MAPK family comprises of several different parallel protein kinase cascades (see Figure 1.5). Seger and Krebs

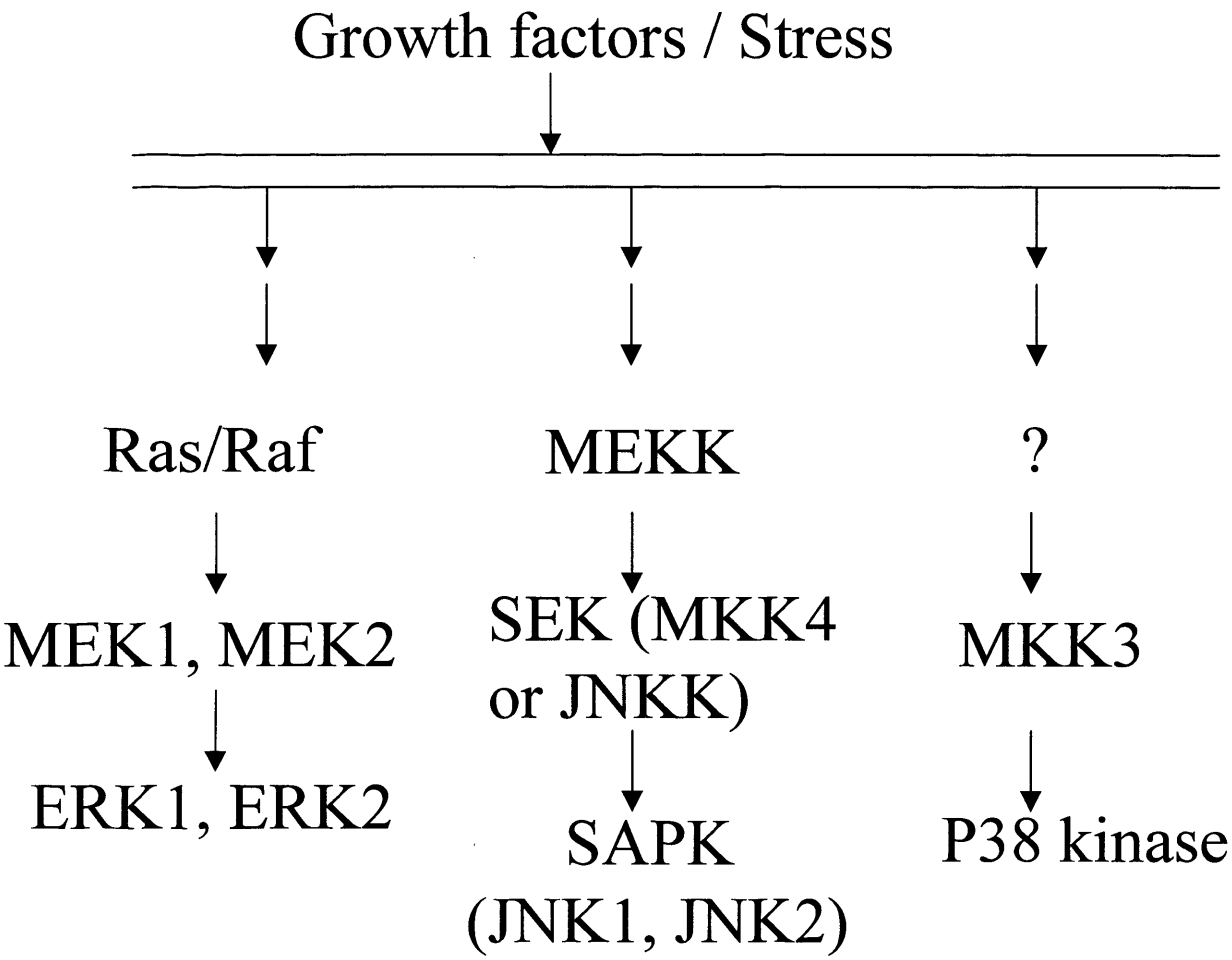


Figure 1.5
Parallel signalling pathways to MAPK activation.

(1995) suggested the use of the terms ERK1, for p44, and ERK2, for p42, to help distinguish between the different MAPK isoforms. ERK is activated by dual phosphorylation of a T-X-Y motif on tyrosine residue 185 followed by threonine residue 183 for rat ERK2 (Anderson et al., 1990; Zhang et al., 1994), threonine 188 and tyrosine 190 in *Xenopus* isoforms (Nishida and Gotoh, 1993) and threonine 202 and tyrosine 204 for human ERK1 (Sturgill et al., 1988; Payne, 1991). Phosphorylation is catalysed by a protein that was identified as MAPK or ERK kinase (MEK) (Crews and Erikson et al., 1992). Phosphorylation of both residues is important for enzyme activity as point mutation of either threonine or tyrosine causes inactivity (Robbins et al., 1993).

The signalling cascade activating MAPK has been investigated thoroughly by activation of growth factors. This will now be discussed in the next section.

1.8.5.1 Growth Factor activation of MAPK

As was discussed in earlier in section 1.8.4, growth factor receptors activate tyrosine kinase activity. In signalling to MAPK receptor dimerisation leads to association of the receptor with Shc (SH2 domain-containing α2 collagen) via its SH2 domain. Shc consists of three isoforms (Pelicci et al., 1992) $p46^{Shc}$, $p52^{Shc}$ and $p66^{Shc}$. Shc is tyrosine phosphorylated providing a site for Grb2, which can also directly associate with growth factor receptors (Lowenstein et al., 1992). Grb2 contains both SH2 and SH3 domains and acts as an adapter protein between Shc and mSos (mammalian homologue of *Drosophila* son-of-sevenless). The protein mSos is a guanine nucleotide exchange factor (GNEF) which activates Ras by increasing the

exchange of GTP for GDP (Yokote et al., 1994; Pruett et al., 1995; Chook et al., 1996).

Activation of p21 Ras has been established as a link between tyrosine kinase receptors and Raf (Pronk and Bos, 1994; Avruch et al., 1994). Active Ras, being a protooncogene, has been shown to be important in cell growth and transformation (Trahey and McCormick, 1987). Mutations in Ras have been shown to block growth factor mediated activation of both Raf and MAPK (Troppmair et al., 1992; de Vries-Smits et al., 1992). Association of Raf to Ras causes translocation of Raf to the cell membrane. Binding, however, is not sufficient for activation. Movement of Raf to the cell membrane is the key (Stokoe et al., 1994) with activation suggested to occur possibly via another protein of the 14-3-3 family (Morrison, 1994). Interaction of Ras and Raf is dependent on cAMP and PKA activation (Kikuchi and Williams, 1996). Activated Raf causes activation of MEK by phosphorylation on two serine residues in its catalytic domain (Huang et al., 1993; Zheng and Guan, 1994). MEK in turn dual phosphorylates MAP kinase on tyrosine and threonine residues causing activation (Crews and Erikson, 1992). A summary of this pathway can be seen in Figure 1.6. Activation of MAPK leads to activation of proteins such as Rsk and translocation from the cytoplasm into the nucleus with further activation of proteins such as Elk-1, c-Fos and c-Myc leads to cell proliferation.

This thesis is particularly interested in the role of ERK in P2Y receptor signalling and this will now be discussed further in the following section.

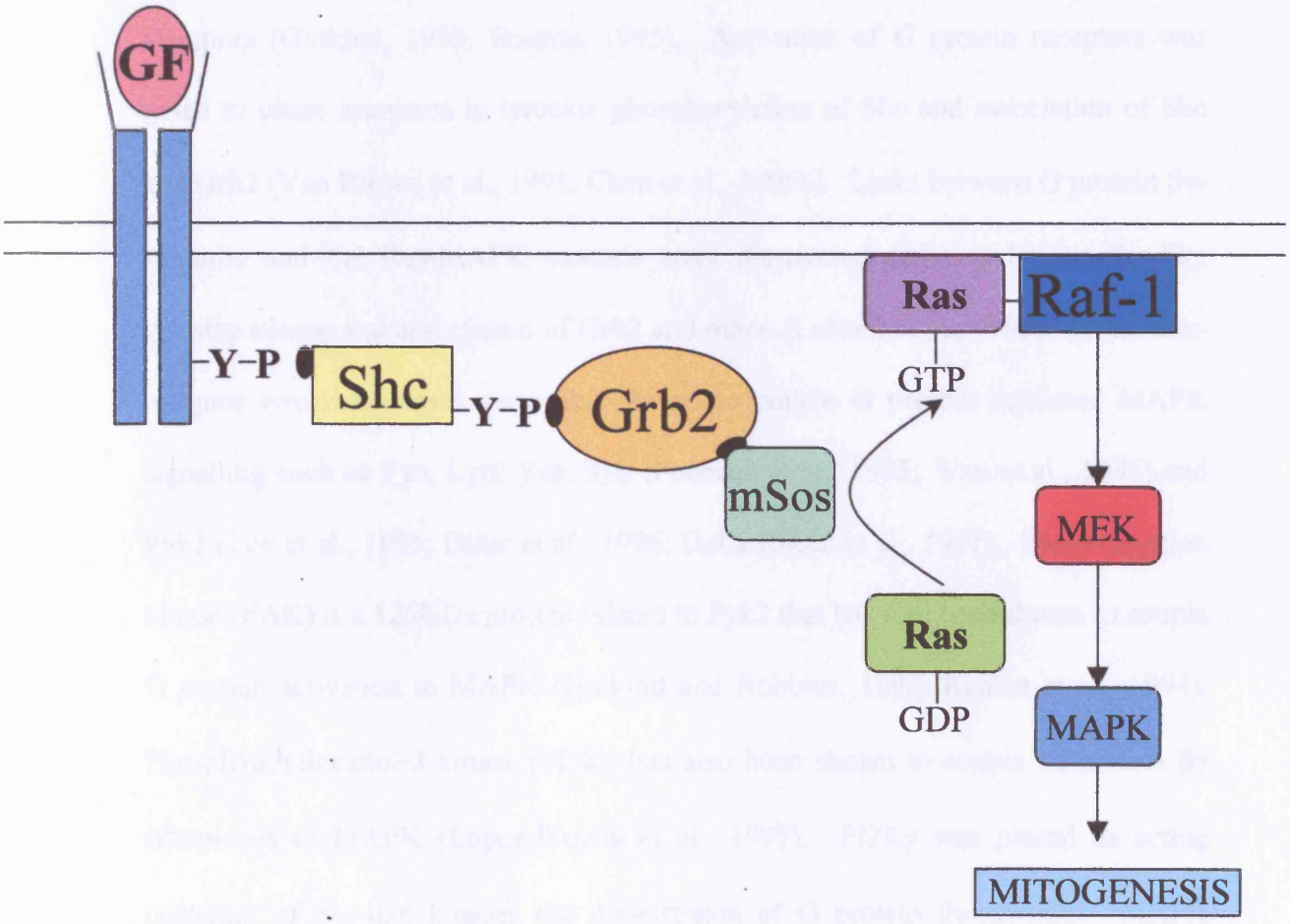


Figure 1.6
The growth factor receptor signalling pathway to MAPK.

1.8.5.2 P2Y receptor activation of MAPK

Apart from tyrosine kinase receptor activation of MAPK, evidence is now emerging of control of tyrosine kinases and the MAPK cascade by G protein-linked receptors (Gutkind, 1998; Bourne, 1995). Activation of G protein receptors was noted to cause increases in tyrosine phosphorylation of Shc and association of Shc and Grb2 (Van Biesen et al., 1995; Chen et al., 1996b). Links between G protein βγ-subunits and the Ras-MAPK cascade were discovered utilising Src or Src-like tyrosine kinase and association of Grb2 and mSos (Luttrell et al., 1996). Other non-receptor tyrosine kinases were also shown to couple G protein mediated MAPK signalling such as Fyn, Lyn, Yes, Syk (Ptasznik et al., 1995; Wan et al., 1996) and Pyk2 (Lev et al., 1995; Dikic et al., 1996; Della Rocca et al., 1997). Focal adhesion kinase (FAK) is a 125KDa protein related to Pyk2 that has also been shown to couple G protein activation to MAPK (Gutkind and Robbins, 1992; Rankin et al., 1994). Phosphatidylinositol-3 kinase (PI3K) has also been shown to couple G_i protein βγ complexes to MAPK (Lopez-Ilasaca et al., 1997). PI3Kγ was placed as acting upstream of Src-like kinases but downstream of G protein βγ-subunits. MAPK activation may also be Ras independent in certain circumstances (Pace et al., 1995; Takahashi et al., 1997) while PKC may be involved in both Ras-dependent and independent pathways (Thomas et al., 1992; Hawes et al., 1995). MAPK activation that is dependent on PKC by G_q coupled receptors may not require Ras activation at all. G_q coupled receptor activation has been shown to be PKC-dependent (Hawes et al., 1995), partially PKC-dependent (Crespo et al., 1994) or completely PKC-independent (Charlesworth and Rozengurt, 1997).

ATP was first shown in 1994 to activate MAPK or ERK in primary cultures of rat cerebral cortical astrocytes (Neary and Zhu, 1994) and rat renal mesangial cells (Huwiler and Pfeilschifter, 1994). Stimulation of rat cerebral cortical astrocytes with 100μM ATP for 15 minutes caused a three to four fold increase in MAPK activity, which was inhibited by suramin. Inhibition was not observed with increased cAMP levels, leading to the conclusion that Raf was not involved in P2 stimulated MAPK. No responses were observed for P1 receptor agonists. Astrocytes have been extensively studied to discover the pathway linking P2Y receptor activation and increased mitogenesis observed. King et al., (1996a) established the presence of both $P2Y_1$ and $P2Y_2$ receptors on primary cultured rat astrocytes linked to increased Ca^{++} release and MAPK activation. Neary et al., (1998) investigated human foetal astrocytes observing MAPK responses to P1 receptor agonists, 2-chloroadenosine and 5'-N- ethylcarboxyamidoadenosine, which were not observed in rat astrocytes. MAPK activation via P2Y and P1 receptors in human foetal astrocytes is downstream of both PKC and MEK as shown by inhibition with Ro 31-8220 and PD 98059 respectively. More recently the P2Y signalling pathway to MAPK was further characterised by Neary and co-workers (1999) in astrocytes showing that P2Y receptors stimulated by ATP cause MAPK activation that is not dependent on PI-PLC activity or associated Ca^{++} mobilisation but is dependent on phosphatidylcholine hydrolysis by PLD and PKC activity. Further investigation established a possible role for calcium independent PKC-δ in mediating P2Y signalling to MAPK in human astrocytes (Neary et al., 1999). Working on cultured astrocytes Chen and Chen (1998) showed MAPK activation by $P2Y_1$ and $P2Y_2$ receptors with no P1 receptor activity detected. MAPK activation was upstream of $cPLA_2$ and arachidonic acid release but downstream of PKC. The pathway was

sensitive to extracellular calcium influx and may indicate a role of calcium sensitive PKC-α. Munsch et al., (1998) describe astroglial cells which also show expression of $P2Y_1$ and $P2Y_2$ receptors that are coupled to MAPK activation. No responses were observed for P1 agonist activation of MAPK while P2Y receptor mediated activation is dependent on PKC and extracellular calcium but unaffected by pertussis toxin or PI3K inhibition. Endothelial cells have also been studied for P2Y activation of MAPK. Graham et al., (1996) investigated EAhy 926 human endothelial cell line showing activation by UTP and ATP (EC_{50} values 5.1 +/- 0.2 μM for UTP; 2.9 +/- 0.8 μM for ATP) but not by 2MeSATP, indicating $P2Y_2$ receptor activation of MAPK. $P2Y_2$ mediated MAPK activation in EAhy 926 cells was dependent on PKC and extracellular Ca^{++} but was not dependent on pertussis toxin. Bovine aortic endothelial cells also studied exhibited activation by 2MeSATP indicating the presence of $P2Y_1$ receptor activation of MAPK. Further work by Patel et al., (1996a) established activation of both $P2Y_1$ and $P2Y_2$ leads to MAPK activation, which is required for PGI_2 release stimulated by P2Y receptors previously shown to require PKC, which is likely to be PKC-ε isoform (Patel et al., 1996b). Rat brain microvascular endothelial cells (RBEC) have also been reported to activate MAPK in response to UTP and ATP action at P2Y receptors (Albert et al., 1997). This is discussed further in chapter five as it is part of this thesis. Dickenson et al., (1998) investigated $P2Y_2$ receptor activation in Chinese hamster ovary (CHO) cells. Activation of the endogenous $P2Y_2$ receptor with UTP stimulated time and concentration-dependent increases in MAP kinase activity (EC_{50} 1.6 +/- 0.3 μM). The MAP kinase response to UTP was partially blocked by pertussis toxin (67 +/- 3% inhibition) and by the PKC inhibitor Ro 31-8220 (10 μM; 45 +/- 5% inhibition),

indicating the possible involvement of both G(i)/G(o) protein and G(q) protein-dependent pathways in the overall response to UTP.

Vascular smooth muscle proliferation is important in disease states and ATP has been shown to cause VSM proliferation that involves MAPK activation (Erlinge, 1998). Yu et al., (1996) showed in rat aortic VSM that P2Y receptor activation was coupled to a pertussis toxin insensitive G protein and triggered phosphoinositide hydrolysis with subsequent activation of protein kinase C (PKC), Raf-1, and MAPK. PKC down-regulation studies using TPA imply a role for PKC isoforms α and δ in MAPK activation. ATP also caused accumulation of c-fos and c-myc mRNAs, increased DNA synthesis and cell proliferation. Harper et al., (1998) showed activation of MAPK and increased [^{3}H]thymidine incorporation by UTP at proposed $P2Y_4$ receptors. An RT-PCR study on rat cultured aortic VSM showed expression of $P2Y_2$, $P2Y_4$ and $P2Y_6$ mRNA's but no $P2Y_1$ was detected. Using coronary artery smooth muscle cells, Wilden and colleagues (1998) showed that activation of a UTP preferring P2Y receptor led to activation of MAPK that was dependent on MEK activation but not on PI3K activity. They also showed that activation of both PI3K and MAPK was required for cell proliferation implying independent activation of these two pathways is required for proliferation in these cells. ATP induced cell proliferation has also been shown in renal inner medullary collecting duct cells (Ishikawa et al., 1997) via the $P2Y_2$ receptor but not $P2Y_1$ receptor, though both activate PLC activity.

Different receptor expression and tissue type differences appear to create a wide number of responses and signalling pathways. What was not clear however was how P2Y receptors were linking to the MAPK cascade. Soltoff and co-workers (1998a;b) have established activation of a $P2Y_2$ receptor in PC12 cells. UTP and

ATP stimulation of MAPK (EC_{50} ~ 25 μM) were pertussis toxin sensitive implying signalling via G_i protein and were sensitive to inhibition of Ca^{++} release. Increased tyrosine phosphorylation was observed in multiple proteins, including p42 MAP (ERK2) kinase, related adhesion focal tyrosine kinase (RAFTK also known as Pyk2 and CAKβ), focal adhesion kinase (FAK), Shc, and PKC-δ. Downregulation of PKC caused only partial reduction in MAPK, which may mean there are PKC-dependent and independent pathways involved. P2Y receptor stimulation also increased the association of Shc and Grb2. The results suggest that the $P2Y_2$ receptor-initiated activation of MAP kinase was dependent on the elevation of [Ca2+](i), involved the recruitment of Shc and Grb2, and was mediated by Pyk2 and PKC. Further work established a requirement for the epidermal growth factor receptor activation downstream of Pyk2 (Soltoff, 1998b). This type of transactivation of the EGF receptor has also been observed for angiotensin II in rat liver epithelial cells (Li et al., 1998) and also for PDGF receptor (Linseman et al., 1995; Daub et al., 1996). P2Y mediated MAPK activation was recently shown to be modulated by both PKC and PKA in human embryonic kidney (HEK) 293 cells (Gao et al., 1999). $P2Y_2$ receptor activation in HEK-293 cells leads to MAPK activation via the $G_{q/11}$ protein. MAPK activation is unaffected by the tyrosine kinase inhibitor genistein, attenuated by a phospholipase C inhibitor (U73122), and is abolished by either the MEK inhibitor (PD98059), dominant negative Ras or Ro 31-8220. Increased cAMP levels are thought to activate MAPK via PKA acting at B-Raf and inhibit MAPK by action of PKA at c-Raf-1 (Daaka et al., 1997; Vossler et al.,1997). In HEK 293 cells activation of A_{2B} adenosine receptors via G_s or the presence of forskolin can activate MAPK via action of PKA at B-Raf. This may also be counter-balanced by PKA inhibition of c-Raf-1 also present or possibly by activation of PKC-ζ, via $G_{q/11}$,

which can inhibit B-Raf (Gao et al., 1999). $P2Y_2$ receptor activation of MAPK can therefore be modulated by cAMP-dependent PKA in both an inhibitory and stimulatory manner in HEK 293 cells.

Further work still needs to be carried out for P2Y receptor activation of MAPK, in particular the role of the different P2Y subtypes. Though some theoretical pathways have been established these need to be tested more completely and across tissue types to establish the role that P2Y receptors have in modulating MAPK activity. Also further research needs to investigate the role P2Y receptors may have in regulating other MAPK/ERK related pathways. Hamada et al., (1998) showed activation of JNK by ATP implying ATP may have a role to play in stress related MAPK signalling pathways.

1.8.6 Protein Kinase C

1.8.6.1 Subtypes and structure

Protein Kinase C (PKC) consists presently of a family of 11 isotypes (α, β_I, β_{II}, γ, δ, ε, ζ, θ, ι, λ and η) of serine/threonine kinases requiring threonine phosphorylation for activation. Some PKC isoforms can be activated by the cell permeable lipid diacylglycerol (DAG) produced by phosphatidylinositol 4,5 bisphosphate (PIP_2) hydrolysis by Phospholipase C. On the basis of activators PKC isoforms can be separated into several subgroups: C- conventional isoforms (α, β, γ) activated by Ca^{++}, phosphatidyl serine, phorbol esters and DAG, N- novel isoforms (δ, ε, η, θ) activated by phosphatidyl serine, phorbol esters and DAG but not Ca^{++} and A – atypical (ζ, λ, ι) activated by phosphatidyl serine but not Ca^{++}, phorbol esters or DAG. Phorbol esters strongly activate PKC, which is linked to

their tumour promoting properties, for example, TPA (12-0-tetradecanoyl phorbol 13-acetate) is a compound used extensively to study PKC activation. PKD previously termed PKC μ, is also activated by phorbol esters and DAG. Closely related to the PKC family is the PKC Related Kinases (PRK) which may be involved in cytoskeletal changes and bind phospholipids, arachidonic acid and RhoA GTPase. All PKC isoforms are constructed with a kinase catalytic site and variable regulatory domains. Subgroups C, N and A all have pseudosubstrate binding domains that are involved in the inactive state of PKC.

1.8.6.2 PKC inhibitors

Ro 31-8220 is a competitive general PKC inhibitor based on bisindolylmaleimide (Davis et al., 1992). Ro 31-8220 is selective for protein kinase C (IC_{50}=10nM) over CaM kinase II (IC_{50}=17μM) and PKA (IC_{50}=900nM) and inhibits PKC in intact platelets and T cells (IC_{50}=200nM). Ro 31-8220 has been reported to inhibit MAP kinase phosphatase-1 expression and activate the stress activated protein kinase, JNK-1 (Beltman et al., 1996)

Go 6976 is an indolocarbazole identified as a novel inhibitor of PKC (Martiny-Baron et al., 1993). The Go 6976 compound is a selective inhibitor of Ca^{++} sensitive PKC isoforms inhibiting PKC α (IC_{50}=2.3nM) and β1(IC_{50}=6.2nM) whereas micromolar concentrations do not inhibit PKC δ, ε and ζ isoforms which are Ca^{++} insensitive.

Go 6850, also known as GF 109203X, is based on bisindolylmaleimide and is a general PKC inhibitor. Go 6850 acts as a competitive inhibitor for the ATP

binding site of PKC. PKC isoforms α, β_I, β_{II}, γ, δ and ε are inhibited by Go 6850 with a rank order of α >β1 >ε >δ >ζ.

1.8.6.3 PKC and P2Y cellular signalling

PKC is a key enzyme in many signalling pathways due to its ability to serine and threonine phosphorylate protein substrates. Regulation of PKC activity is important therefore when investigating signal transduction mechanisms. Modulation of signalling from P2Y receptors via PKC has been shown to be important in many signalling pathways. PKC has been shown by Gobran et al., (1998) to be important in development. In developing lung type II cells $P2Y_2$ receptor signalling increases with age due to lack of certain PKC isoform expression early in cell development.

PKC is known to regulate P2Y induced PLC activity. Purkiss et al., (1994) investigated endogenous $P2Y_1$ and $P2Y_2$ receptors expressed on BAEC's which were differentially controlled by PKC. $P2Y_1$ receptor activation leads to production of $InsP_3$ that was inhibited by TPA activation of PKC but potentiated by Ro 31-8220, a PKC inhibitor. $P2Y_2$ receptor activation of PLC was however unaffected. TPA is a known activator of PKC when used for short-term stimulations. Long-term stimulation of PKC with TPA leads to down-regulation of PKC. Variation of the time of TPA stimulation affects each of the PKC isoforms in a different manner (Oliver and Parker, 1992). A 6 hour exposure to TPA in BAEC's led to a 90% downregulation of PKC-α but not PKC ε and ζ which remained unaffected (Patel et al., 1996a). Chen and Chen (1997) studied Neuro 2A mouse neuroblastoma cells in which a P2Y receptor responding to UTP and UDP caused activation of PLC that was dependent on PKC-ε. Inhibition of PLC activity occurred with short-term

application of TPA which was not observed on long-term exposure to TPA. This pattern of PLC modulation mirrored the down-regulation of membrane-bound active PKC-ε.

PKC has also been shown to play a role in P2Y mediated mitogenesis, prostaglandin and nitric oxide (NO) release. P2Y studies on rat aortic VSMC proliferation have established a positive role for PKC signalling to the p42/p44 MAPK cascade involving PKC-α and/or PKC-δ acting upstream of Raf kinase (Yu et al., 1996). In porcine aortic VSMC's Wang and co-workers (1992) also observed ATP induced increases in mitogenesis that were only partially inhibited by PKC downregulation. The other pathway accounting for this discrepancy in ATP induced mitogenesis involved activation of arachidonic acid metabolism, PGE_2 release and cAMP accumulation. On EAhy 926 human endothelial cells, Graham et al., (1996) established that $P2Y_2$ receptors functionally couple to p42/p44 MAP kinase that is dependent on PKC, as TPA down regulated PKC-α and PKC-ε isoforms causing a 90% inhibition of MAP kinase activity. In another endothelial cell line, Patel et al., (1996b) established that $P2Y_1$ and $P2Y_2$ receptor activation of p42/p44 MAP kinase was required for BAEC production of PGI_2, which previously had been shown to be PKC dependent (Patel et al., 1996a). Extracellular ATP was linked to p42/p44 MAP kinase and PKC-ε and PKC-δ in cardiac cells (Vassort and Puceat, 1997). In human foetal astrocytes Neary and colleagues (1998) reported that both P2 and P1 receptor activation lead to p42/p44 MAP kinase activation, involved in human foetal astrocyte proliferation, that were both blocked by Ro 31-8220, a general PKC inhibitor. In CHO cells, Dickenson et al., (1998) also describe endogenous $P2Y_2$ receptor activation of MAP kinase that is partially inhibited by Ro 31-8220. The partial inhibition of MAP kinase suggests a role for PKC independent activation of MAP

kinase, which was discussed in section 1.1.3. Recently Soltoff and colleagues (1998a; 1998b) reported that in PC12 cells, $P2Y_2$ receptors link to p42/p44 MAP kinase in a PKC dependent manner that is proposed to be via PKC-δ.

1.8.7 Adenylate cyclase

Adenylate cyclases are a family of enzymes that produce 3',5'-cyclic adenosine monophosphate (cAMP). There are currently nine cloned mammalian isoforms numbered I-IX all having a similar structure of two transmembrane domains each consisting of six transmembrane helices and two cytoplasmic regions (Hurley, 1999). Each adenylate cyclase isoform is regulated differently by various signalling molecules. Forskolin, a proposed structural homologue of a natural activator, positively regulates all isoforms except isoform IX. G protein sub-unit $G\alpha_s$ activates all adenylate cyclases and is synergistic with forskolin. $G\alpha_i$ inhibits isoforms V and VI by binding at the catalytic site of adenylate cyclase (Dessauer et al., 1998). $G\beta\gamma$ sub-units also regulate adenylate cyclase inhibiting types I, III and VIII but in the presence of $G\alpha_s$ type II is activated by $G\beta\gamma$ sub-units. Ca^{++}/calmodulin regulates type I adenylate cyclase binding in cytoplasmic region one. Protein phosphorylation of adenylate cyclase can stimulate or inhibit cAMP production. PKC activates type II adenylate cyclase by threonine phosphorylation (Bol et al., 1997) whereas CaM kinase II inhibits type III by serine phosphorylation (Wei et al., 1996).

1.8.7.1 P2Y regulation of adenylate cyclase

Modulation of cAMP production by P2Y receptors has been investigated for sometime. Both inhibition and activation of adenylate cyclase have been observed. Native cell experiments have shown many different tissue-types express P2Y receptors that modulate adenylate cyclase. P2Y receptors inhibit adenylate cyclase production of cAMP in various cell types including renal epithelial cells (Anderson et al., 1991), Schwann cells (Berti-Mattera et al., 1996), rat hepatocytes (Okajima et al., 1987), smooth muscle cells (Murthy et al., 1998), C6 glioma cells (Boyer et al., 1993) and platelets (Cristalli and Mills, 1993). Inhibition of adenylate cyclase has been attributed to direct action of the $G\alpha_i$ sub-unit rather than accumulation of Ca^{++} as sensitivity to pertussis toxin has been observed. Subtypes of P2Y receptor associated with cAMP accumulation inhibition include $P2Y_1$ (Boyer et al., 1993).

P2Y receptors promote adenylate cyclase production of cAMP in HL-60 cells (Choi et al., 1997; Jiang et al., 1997), BAEC cells (Tada et al., 1992), myotubules (Henning et al., 1993) and NG108-15 cells (Matsuoka et al., 1995). However, in some cases activation of adenylate cyclase has been attributed to breakdown of ATP to adenosine and subsequent activation of A_2 adenosine receptors. More recently a P2Y receptor was cloned named $P2Y_{11}$ (Communi et al., 1997b) which is positively linked to adenylate cyclase in CHO-K1 cells. Adenosine was ruled out as a mediator of activation of adenylate cyclase showing direct activation by ATP acting at the $P2Y_{11}$ receptor. It was also noted that the pharmacological profile showed similarities with the HL-60 cell line. The $P2Y_{11}$ receptor also exhibits activation of PLC together with cAMP modulation showing multiple signalling pathways activated by a single receptor. Matsuoka et al., (1995) had shown that in NG108-15

cells, separate P2Y receptors modulated the PLC and cAMP modulation events. The existence of complex cAMP modulation by action of ATP at P2Y receptors and P1 receptors prompted further work on isolated receptors. The cloned receptors in 1321N1 cell-line, like the $P2Y_{11}$ receptor, were characterised for cAMP modulation. None of the receptors tested altered basal levels of cyclic AMP, but forskolin elevated levels of cyclic AMP were reduced on stimulation of $P2Y_1$ receptors, increased by $P2Y_4$ and $P2Y_6$ receptors and unchanged by $P2Y_2$ receptors (Roberts et al., 1999).

1.9 P2Y receptors : physiology

Due to the widespread nature of P2 receptor expression, physiological responses to ATP are observed in many tissue types (Dubyak and El-Moatassim, 1993). Defects in P2Y receptor signalling are involved in pathological disease states. P2 receptors also represent, due their signalling abilities, targets for therapeutic intervention under diseased conditions (further discussed in section 1.10). This section will discuss the various roles that P2Y receptors have in different tissues.

1.9.1 Nervous system

There is a large amount of evidence for the functional presence of P2 receptors on neurons, glial cells and endothelial cells of the nervous system (see Barnard et al., 1997; Dubyak and El-moatassim, 1993; Zimmermann, 1994). P2X and P2Y receptors are both present in the brain and may be co-expressed in certain regions. ATP is known to act as a fast neurotransmitter in the CNS (Bean et al.,

1990, 1992; Evans et al., 1992; Edwards et al., 1992) being mediated by the fast acting P2X ATP-gated cation-selective channels. ATP is known to play a role in nociceptive nerves (Cook et al., 1997). $P2X_1$ and $P2X_2$ receptors are involved in the pain response via the spinal cord (Kennedy and Leff, 1995).

Stimulation of sympathetic nerves causes smooth muscle contraction in the periphery showing evidence of non-adrenergic, non-cholinergic (NANC) transmission (Von Kugelen and Starke, 1991). Nerve stimulation causes release of ATP and contraction in smooth muscle from various tissue types including guinea pig submucosal arterioles (Evans and Suprenant, 1992) and rabbit ear artery (Kennedy et al., 1986).

ATP is known to modulate release of neurotransmitters such as noradrenaline (Allgaier et al., 1995) and acetylcholine (Cunha et al., 1994). In rat superior cervical ganglion neurons, ATP elicits noradrenaline release in an entirely Ca2+-dependent manner. This modulation was attributed to $P2X_2$ receptors that promote release of noradrenaline and P2Y receptor subtypes that inhibit release (Boehm, 1999). The dual role of ATP may therefore be to promote release and also inhibit further release by a negative feedback mechanism.

1.9.2 Role of P2Y receptors in the vasculature

The role of P2Y receptors in the vasculature can be split into three main parts based on tissue location of P2Y expression. The cell types involved are endothelial cells, smooth muscle cells and platelets.

Bovine aortic endothelial cells represent an example of endothelial cells which have been extensively studied. BAEC's express $P2Y_1$ and $P2Y_2$ receptors that

are both linked to activation of PLC and increased Ca^{++} (Wilkinson et al., 1993; Motte et al., 1993). P2Y receptors in BAEC's exhibit differential coupling to Gα proteins as $P2Y_1$ is linked to pertussis-insensitive G_q-α and $P2Y_2$ is coupled to pertussis-sensitive G_i-α and also differential response to PKC activation which inhibits $P2Y_1$ but not $P2Y_2$ receptor signalling (Purkiss et al., 1994). In blood vessels the endothelial cells form a layer between the lumen and a smooth muscle layer. Action of nucleotides on the endothelial layer causes production and release of nitric oxide (endothelial derived relaxation factor) and prostacylin (PGI_2). These active compounds act at the smooth muscle layer causing relaxation (Boeynaems and Pearson, 1990; Wilkinson et al., 1994b). This mechanism of dilation could be used when the intact blood vessel comes under increased stress and pressure causing possible release of ATP (see section 1.2.4). The endothelial layer also acts as a barrier preventing the direct action of ATP on smooth muscle causing vasoconstriction, proliferation and other associated pathologies (Erlinge, 1998; also discussed in section 1.10.3)

As well as relaxation, smooth muscle can also be contracted by sympathetic nerve stimulation via P2X receptors. Vascular smooth muscle cells express $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors. Characterisation of these receptors need to be clarified as UTP can cause vasoconstriction in a variety of tissues (Saiag et al., 1990; Matsumoto et al., 1997; Rubino and Burnstock, 1996) and also activates PLC, MAPK and proliferation (Harper et al., 1998).

Platelet function in the vasculature is extremely important. Not only do platelets contain a large source of ATP and other inflammatory mediators but also express the $P2Y_1$ and P_{2T} receptors. Platelet aggregation is induced by P2Y receptor activation of platelets, and once aggregated the inflammatory mediators are released

(Hourani and Hall, 1994). These may act on the endothelial layer causing relaxation, as previously discussed, but also platelet inhibition. Under normal circumstances vessel dilation and inhibitory feedback limit the aggregation of platelets. However if the endothelial layer is damaged due to trauma, platelet aggregation will be amplified leading to action of nucleotides directly at smooth muscle cells causing constriction and proliferation (Hourani and Hall, 1996; Boarder and Hourani, 1998).

1.9.3 Urogenital

P2 receptors have been studied extensively in the rat and guinea-pig bladder. ATP is released in the rat bladder as a consequence of purinergic nerve stimulation leading to P2X receptor activation which is responsible for the non-adrenergic non cholinergic (NANC) contractile response observed (Tong et al., 1997). NANC contractions were estimated to account for about 50% of the maximum contractile response in rat bladder. ATP was reported by Boland et al., (1993) to mediate relaxation by mouse bladder smooth muscle cells after a short contraction period. This was further investigated in the rat bladder by Bolego et al., (1995) attributing the biphasic response to expression of P2X receptors in detrusor smooth muscle mediating the short-term contraction and G protein-linked P2Y receptors the long-term relaxation possibly via factors released from the epithelium acting on smooth muscle. UTP was also investigated, causing a rapid and concentration dependent contraction of rat bladder that was not desensitised by α,β-meATP, suggesting that this response was not linked to P2X receptors. Another report, however, by Hashimoto and Kokubun, (1995) found α,β-meATP to desensitise UTP induced contraction in rat bladder smooth muscle. Both P1 and P2 receptors exist in the

bladder and were investigated by King et al., (1997) in rat urinary bladder detrusor smooth muscle. ATP mediated contraction via P2X receptors whereas adenosine acting at P1 receptors caused relaxation. ATP and adenosine can therefore modulate detrusor muscle responses. McMurray et al., (1998) have also shown that P2Y mediated relaxation in marmoset urinary bladder smooth muscle is inhibited by tosphechloromethylketone, suggesting a role for the activation of cyclic AMP-dependent PKA in the relaxation response.

The vas deferens has also been studied for response to P2Y agonists. Bland et al., (1992) showed, similarly to rat bladder, that in mouse vas deferens ATP may cause contraction at P2X receptors but relaxation afterward via P2Y receptor activation.

1.9.4 Gastrointestinal

Gastro-intestinal roles for nucleotides have been complicated by the presence of multiple receptor subtypes responding to ATP, UTP and adenosine. Both sympathetic and parasympathetic nerves are present in the gut and control of relaxation and contraction is intricate involving multiple muscle layers and multiple receptor expression. In the rat colon muscularis mucosae Hourani et al., (1993) demonstrated contraction after application of ATP, UTP or adenosine acting via a P2Y receptor or an A1 adenosine receptor. Rat duodenum exhibits different activity being relaxed on addition of adenosine or ATP while UTP and ATPγS cause contraction. Separation of the duodenum to longitudinal muscle and the muscularis mucosae showed that a P2Y receptor on longitudinal muscle mediates relaxation with no nucleotide causing contraction and the muscularis mucosae expresses P2X

and a proposed P2U receptor both mediating contraction with no nucleotides causing relaxation (Johnson et al., 1996). Cat colon circular muscle contains P2X receptors which mediate contractions and P2Y and P1-purinoceptors which mediate relaxation (Venkova et al., 1994).

1.10 Therapeutic role of P2Y agonists / antagonists

The wide distribution of P2 receptors and physiological functions as stated previously mean that modulation of P2 receptor function may have therapeutic advantages (Appleman and Erion, 1998). The use of P2Y receptor modulation as therapy is discussed in the following sections.

1.10.1 Diabetes

The presence of P2Y receptors on insulin-secreting pancreatic β-cells is well documented (Li et al., 1991; Hillaire-Buys et al., 1992,1993). Activation of the P2Y receptor leads to PLC activation and Ca^{++} release, increased insulin secretion and improved glucose tolerance. Ectonucleotidase resistant ADPβS has been shown to stimulate insulin release in diabetic rats remaining effective after oral administration (Hillaire-Buys et al., 1992,1993). Petit et al., (1996) discuss the role P2 receptors play in glucose homeostasis. While P2Y receptors are involved in insulin secretion via pancreatic β-cells, A1 adenosine receptors are implicated in antilipolytic action on adipocytes. There is therefore a role for P2Y and A1 agonist to play in control of diabetes.

1.10.2 Cystic Fibrosis

Cystic fibrosis is a result of defective epithelial cell chloride transport via the cystic fibrosis transmembrane conductance regulator (CFTR). The result of defective chloride transport is dehydration and thick mucus secretions in the lung that are not removed easily (Boucher et al., 1986; Noone and Knowles, 1993). Activation of the $P2Y_2$ receptor present via UTP or ATP activates calcium dependent outward rectifying chloride channels (ORCC) and release of chloride ions (Mason et al., 1991). The CFTR is thought to be associated with ORCC and provides, in normal conditions, a channel for ATP release, which subsequently activates $P2Y_2$ and chloride release via ORCC (Schwiebert et al., 1995). The defective CFTR therefore cannot supply the ATP leading to defective chloride transport. Therapy involves aerosol addition of UTP that would then directly activate ORCC chloride release. UTPγS would be a good candidate as it would be resistant to any ectonucleotidases present (Lazarowski et al., 1996). Most studies have implicated $P2Y_2$ as the main receptor involved but some reports have shown UDP (Lazarowski et al., 1997c) or adenosine (O' Reilly et al., 1998; Stutts et al., 1995) as also having a modulatory effect. Although activation of $P2Y_2$ helps in chloride secretion via ORCC and alleviation of cystic fibrosis conditions, it has been questioned if under normal conditions CFTR causes release of ATP. Schwiebert et al., (1995) demonstrated CFTR release of ATP using path-clamp channel conductance measurements. Watt et al., (1998) have demonstrated via four different methods ATP is not released from cells via CFTR but due to a shear stress response to media displacement. Further investigations have yet to be carried out to understand the mechanisms behind CFTR involvement in ATP release.

1.10.3 Hypertension

Normally vasculature is composed of an endothelial layer that acts as a barrier between the lumen and a smooth muscle layer. ATP produced from platelets activates P2Y receptors on the endothelial layer inducing production of prostacyclin (PGI_2) and nitric oxide (NO). These in turn inhibit platelet activation and relaxant effects on the smooth muscle layer, thus reducing the potential for development of hypertension and maintaining a balanced system. However with endothelial layer damage (mechanical or diseased-induced) the smooth muscle layer is exposed to nucleotides leading to contraction and proliferation. With a reduced endothelial layer there is a smaller production of PGI_2 or NO leading to increased contraction and platelet activation. Overgrowth of vascular smooth muscle due to hyper-proliferation leads to further vessel constriction and formation of atherosclerosis and hypertension. Therefore potential therapy targets for hypertension and atherosclerosis are for anti-proliferative drugs targeted at smooth muscle growth. Activation of remaining endothelial layer P2Y receptors to induce relaxation would also be advantageous.

1.10.4 Thrombosis

Endothelial cells that are damaged can release large localised concentrations of ATP and ADP. ADP activates platelets by binding to the $P2Y_{ADP}$ (P_{2T}) receptor. Platelet activation at sites of injury leads to an accumulation of platelets and fibrinogen and formation of a clot. Though a necessary part of wound healing under certain conditions over activation of platelets leads to thrombosis. Antagonist action

of compound ARL 67085 at $P2Y_{ADP}$ (P_{2T}) receptors acts as a potent anti-thrombotic agent (Humphries et al., 1995) that is currently undergoing trials. More selective than other anti-thrombotic agents it has a wide dose seperation between inhibition of thrombosis and excess bleed time (Williams et al., 1996).

1.10.5 Urinary incontinence

As was discussed earlier in section 1.9.3 the contractile response of the detrusor muscle is mediated by the activation of P2X receptors. Control of micturition (urination) involves contraction of the detrusor muscle after voluntary relaxation of the external urethral sphincter. Antagonists selective for P2X receptors may help in the treatment of chronic bladder disorders.

1.10.6 Gastro-intestinal

ATP is co-stored and co-released with noradrenaline in sympathetic nerves supplying the intestine (see Hoyle and Burnstock, 1991). P2Y receptors mediating relaxation are located on stomach and intestinal smooth muscle and in endothelial and smooth muscle cells of vessels supplying the intestine. There is therefore potential for agonists or antagonists of these receptors to be developed for use in gastro-intestinal disease.

1.11 Thesis Objectives

The primary aims of the thesis were to use cloned G protein-coupled P2Y receptors transfected into 1321N1 human astrocytoma cells to study the pattern of second messenger production and mechanisms by which cell signalling pathways are activated in a common host cell. The secondary objectives were to try to expand the knowledge of P2Y receptor subtype activation and signal transduction in primary rat brain endothelial cells.

Chapter 2

Materials and Methods

2.1 Cell culture

2.1.1 Production of 1321N1 transfectants

1321N1 cells are an adherent astrocytoma based human cell line, which exhibit no P2 agonist responses detected in PLC, Ca^{++} or MAPK second messenger systems. It was therefore chosen as an ideal candidate host cell for transfection of P2Y receptors. Cloning efforts from various groups have produced many P2Y subtypes that have subsequently been transfected to produce stable 1321N1 cell lines. 1321N1 cells transfected with turkey $P2Y_1$ ($tP2Y_1$), human $P2Y_2$ ($hP2Y_2$) or human $P2Y_4$ receptors were donated by Dr. G.A. Weisman, University of Missouri-Columbia, U.S.A. 1321N1 cells transfected with rat $P2Y_6$ receptors were a gift from Dr T.K. Harden, University of North Carolina at Chapel Hill, U.S.A.

The $tP2Y_1$ receptor cDNA was subcloned into pcDNA3 vector containing a strong cytomegalovirus promoter for high expression and a gene for neomycin/geneticin resistance. Using a calcium precipitation method (Chen and Okayama, 1987) the vector containing the $tP2Y_1$ receptor was transfected into wild type 1321N1 and a stable cell line selectively grown in media containing geneticin (G418) antibiotic (Filtz et al, 1994).

The human $P2Y_2$, $P2Y_4$ and rat $P2Y_6$ receptors were all initially subcloned into a retroviral vector pLXSN. This receptor:vector construct was transfected into PA317, an amphotrophic packaging cell line. The packaged virus was then used to transfect the 1321N1 cells and using G418 antibiotic, a stable cell line was selected by dilution cloning.

2.1.2 Maintenance of transfected 1321N1 cells

1321N1 cells were cultured in Dulbecco's modified Eagles medium (DMEM) with 4.5g/L of glucose supplemented with 10% Foetal Calf Serum (FCS), 25 i.u. ml^{-1} penicillin, 25 μg ml^{-1} streptomycin and 25 μg ml^{-1} glutamine and kept in a sterile incubator at 37^0C with 5% CO_2. Cells grown in $175cm^2$ tissue culture flasks were split when confluent by washing three times with 10 ml of Earle's balanced salt solution without Mg^{++}/Ca^{++} (EBSS) after which 3ml of trypsin/EDTA solution was added to the flask. After cell detachment with trypsin, 10ml of 1321N1 media was added to inhibit any further trypsin digestion. The detached cells in media were then pelleted in a 30ml sterile tube at 200g for 5 minutes. The cell pellet was resuspended in 5ml of 1321N1 media through an 18-gauge needle and an appropriate dilution ratio (between 1:3 and 1:8) used to plate out further flasks and plates. Cells were used near confluence and cultured in 24 well plates for total [^{3}H]-inositol (poly) phosphates and [^{3}H]-thymidine experiments and in either 35mm tissue culture dishes or 6 well plates for western blotting and MAPK assay.

2.1.3 Storage of transfected 1321N1 cells

When not in use, 1321N1 cells were frozen down and stored under liquid nitrogen. The cells were grown to confluence in a $175cm^2$ flask washed 3 times with 10ml of EBSS (without Mg^{++}/Ca^{++}) and 3 mls of trypsin/EDTA solution added to detach the cells from the flask. Cells were then pelleted in a centrifuge and resuspended in 2mls of freezing medium [45% 1321N1 medium, 45% FCS and 10% Dimethylsulphoxide (DMSO)] through an 18 gauge needle. 1ml was stored per vial

in liquid nitrogen. Cells were grown up from storage by adding one vial to 50mls of warmed 1321N1 media in a 175cm^2 flask.

2.1.4 Primary preparation of rat brain microvascular endothelial cells

Rat Brain Endothelial Cells (RBEC's) were prepared from adult Wistar rats by an adaptation of the method described by Rubin (1991) for porcine brain. Cerebral cortices from freshly killed rats were cleaned of meninges, chopped and homogenised in ice-cold buffer (HEPES-buffered M199, 10% FCS, 1% penicillin-streptomycin) with a glass on glass Wheaton homogeniser, with 89-127 μm clearance followed by 25-76 μm clearance. The resulting slurry was centrifuged in 30% bovine serum albumin (BSA) and the capillaries collected in the pellet. These were then sequentially pipetted through a 150 μm and two 60 μm nylon mesh filters, and the filtrate collected on the two 60 μm mesh filters was digested for 1 hour in enzyme solution consisting of homogenising buffer with 1 mg ml^{-1} collagenase/ dispase and 20 U ml^{-1} DNase. Capillaries were then plated into 96 well plates for the cyclic AMP assays, 3.5 cm dishes or 6 well plates for western blotting or the MAPK assay. The endothelial cells grown from the capillaries formed a confluent monolayer with diffuse immunoreactivity for Factor VIII.

2.1.5 Maintenance of RBEC's

Rat brain microvascular endothelial cells were maintained in DMEM with D-Val supplemented with 20% FCS, 25 i.u. ml^{-1} penicillin, 25 μg ml^{-1} streptomycin, 25 μg ml^{-1} glutamine, 2 i.u. ml^{-1} heparin and 60μg ml^{-1} endothelial cell growth supplement. Media was replaced on the cells every third day and cells used when a confluent monolayer was observed.

2.2 Bradford protein assay

Bradford protein estimation technique (Bradford M.M. 1976) was used to quantify the amount of protein in a cell lysate. Bradford reagent was made by dissolving 100mg of Coomasie Brilliant Blue G-250 in 50ml of 95% ethanol and 100ml of 85% w/v phosphoric acid, making the solution up to a final volume of 1 litre. The solution was mixed, filtered and stored at 4^0C in the dark. The mechanism behind the Bradford assay is the absorbance maximum of the dye shifts from 465nm to 595nm due to stabilisation of the anionic form of the dye by hydrophobic and ionic interactions with proteins. A standard curve was generated by creating a dilution series made up of BSA, from 0 to 80μg / ml, and addition of 2ml of Bradford reagent with 1 ml of standard solution. The solution was vortexed and left to stand for 5 minutes. The absorbance of the solutions was read at 595nm in glass or polystyrene cuvettes on a spectrophotometer. Unknowns were diluted to 1 ml and following addition of 2 ml of Bradford reagent, read on the spectrophotometer. The standard absorbances produced a linear plot from which unknowns were calculated.

2.3 SDS PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using Biorad minigel apparatus. A 10% running gel was mixed using 10% acrylamide: bisacrylamide, 375 mM Tris-HCl (pH 8.8), 1% SDS, 0.004% tetramethylenediamine (TEMED) and 0.1% ammonium persulphate. This was cast between two glass plates, 200µl of ethanol laid on top to aid polymerisation and provide a horizontally set gel. After 15 minutes the ethanol was removed and excess washed away with a 0.5% SDS solution. The stacking gel consisting of 5% acrylamide: bisacrylamide, 125 mM Tris-HCl (pH 6.8), 0.1% SDS, 0.001% TEMED and 0.1% ammonium persulphate was then poured on top and the comb inserted 0.5cm from the running gel. This was then allowed to set for 15 minutes and the comb removed. The wells were then washed with running buffer (25 mM Tris, 190 mM glycine and 0.66 mM SDS) through a needle and syringe. The apparatus was then assembled and upper and lower reservoirs filled with running buffer ready for loading and running the gel at approximately 25mA (constant) per gel. Samples were boiled at 100^0C for 5 minutes, prior to loading, with an equal volume of double concentration sample buffer [100mM Tris-HCl (pH6.8), 4% SDS, 20% glycerol, 5% 2-mercaptoethanol, and 0.01% bromophenol blue]. Protein standards (205 kDa, 116 kDa, 97.4 kDa, 66 kDa, 45 kDa and 29 kDa) were boiled for 1 minute at 100^0C and loaded onto the gel in an outer lane.

2.4 Western blotting

Cells were maintained in 1 ml of serum-free media per well of a 6 well plate 24hr prior to stimulation. When stimulating 100 µl of an 11X concentration of agonist was added to the serum-free media in the culture dishes and left for the required time in a 37^{0}C water bath. Media and agonist were removed by quickly inverting onto tissue and the reaction stopped by addition of liquid nitrogen. Cells were lysed and scraped into 500µl of ice-cold homogenising buffer. Homogenising buffer consisted of 20 mM Tris-HCl pH 7.4; 2 mM ethylenediamine tetra-acetic acid (EDTA); various protease and phosphatase inhibitors: 10 µg ml^{-1} leupeptin, 20 µM E-64, 2 mg ml^{-1} aprotinin, 1 mM pepstatin A, 50 mM sodium fluoride, 2.5 mM sodium orthovanadate, 1 mM phenylmethyl sulphonylfluoride (PMSF); a source of excess phosphate: 62.5 mM β - glycerophosphate and 0.1% Triton X-100 which is a detergent. After scraping, cells were vortexed and centrifuged for 10 minutes at 14,000 r.p.m. at 4°C. A sample of the supernatant was kept for a Bradford assay (protein concentration determination) and an equal volume of a double concentration of Laemmli's sample buffer added to the remainder.

Equal amounts of protein sample were run on 10% SDS PAGE as described previously. Proteins were then wet blotted to nitrocellulose paper for detection with antibody. Using Biorad apparatus, a sandwich consisting of the gel against a sheet of nitrocellulose with 4 pieces of 3mm blotting paper and a final fibre pad on each side was placed into the blotting cassette. The blotting cassette was placed into the blotting tank which was filled with buffer consisting of 48mM Tris, 39mM glycine, 0.037% SDS and 20% methanol. The gel was positioned next to the cathode and the

nitrocellulose towards the anode. The proteins were then transferred at 100 constant volts for 1 hour.

The blots were washed with TBS-T (pH 7.6; 20 mM Tris-HCl, 137 mM sodium chloride and 0.1% Tween 20) or PBS-T (pH 7.5; 80 mM di-sodium hydrogen orthophosphate, 20 mM Sodium di-hydrogen orthophosphate, 100 mM sodium chloride and 0.05% Tween 20) depending on antibody manufacturing recommendations. Blots were then blocked overnight in appropriate blocking agents made up in the recommended buffer. Excess blocking agent was removed by washing for 30 minutes (5 minute washing change) before primary antibodies were added to the blots. To save on antibody, immunoblotting was carried out on a glass plate by inverting the blot onto a 1ml pool of antibody in buffer, saving approximately 80% of the antibody over other methods. Table 2.1 describes the various conditions used for western blotting with the antibodies used in this study. After the primary antibody blots were washed for 30 minutes (5 minute washing change) and the appropriate secondary antibody linked to horseradish peroxidase was then added for 1 hour at 1:5000 followed by washing for 30 minutes (5 minute washing change). Resultant blots were developed using enhanced chemi-luminescence (ECL) reagents and exposed to autoradiograph film. Densitometry analysis was carried out on the produced autoradiograph.

ANTIBODY	BLOCK	Ab DILUTION	Ab EXPOSURE TIME	WASH BUFFER
PY20 Phospho tyrosine mAb	5% BSA	1:1000	1.5Hrs	TBS-T
Phospho-MAPK pAb	10% Marvel	1:1000	1.5Hrs	PBS-T
SHC pAb	10% Marvel	1:250	1.5Hrs	TBS-T
SHC mAb	10% Marvel	1:750	1.5Hrs	TBS-T
PYK2 mAb	10% Marvel	1:1000	1.5Hrs	TBS-T
PKC α	10% Marvel	1:1000	1.5Hrs	TBS-T
PKC β	10% Marvel	1:250	1.5Hrs	TBS-T
PKC ε	10% Marvel	1:500	1.5Hrs	TBS-T
PKC θ	10% Marvel	1:250	1.5Hrs	TBS-T
PKC λ	10% Marvel	1:1000	1.5Hrs	TBS-T
PKC ζ	10% Marvel	1:1000	1.5Hrs	TBS-T

Table 2.1 Western blotting conditions used for various antibodies described in this thesis.

2.5 Shc Immunoprecipitation

Cells were grown to confluence in 6 well plates and stimulated as previously described for western blotting. Cells were lysed into 500µl / well of homogenising buffer and scraped using a 1ml syringe plunger barrel and lysates pipetted into screw cap eppendorfs. Lysed cells were vortexed and spun for 10 minutes at 14000 rpm and then 200µl of the supernatant taken for the immuno-precipitation procedure. 2µl of Shc polyclonal Ab (Transduction Laboratories) was added to 200µl of the cell lysate, vortexed and left on ice for 90 minutes. After this 70µl of 15% Protein A sepharose was added and left on roller at 4^0C for 90 minutes. The immunoprecipitation lysate was then spun for 1 minute at 14000 rpm and Protein A sepharose beads:Shc pAb:Shc proteins were washed four times in lysis buffer with 1 minute spins between. After final spin lysis buffer was decanted off the sepharose beads and 30µl of double concentrated SDS sample buffer was added. Beads were boiled for 5 minutes releasing antibody and Shc proteins and following a short spin used for western blotting.

2.6 Cell stimulation

This section will cover the methods adapted for cell stimulation and a brief description of the different compounds used in this thesis and their background in signal transduction.

As was discussed in the introduction section 1.2.4, extracellular nucleotides are released when cells become stressed. This is particularly important to account

for when stimulating transfected 1321N1 cells. Purkiss et al., (1994) and Lazarowski and colleagues (1995; 1997) addressed this problem after observing release of ATP and UTP from cells and adapted the stimulation protocol to minimise such a release. The normal protocol for stimulating cells involves an overnight incubation in serum free medium which is aspirated and replaced with a concentration of agonist for the required time followed by aspiration and termination of cell stimulation. These media changes stress the cells causing the endogenous release of nucleotides activating P2 receptors present causing high basal levels. To circumvent this problem a protocol was devised to prevent unnecessary disturbance of the cells. The serum free media is added for 24 hours as previously but a small volume of concentrated agonist is added to begin the stimulation, which is aspirated as the stimulation is stopped. In a direct comparison of altered protocols between 1321N1 cells transfected with turkey $P2Y_1$ or human $P2Y_2$ receptors, Charlton et al., (1998) showed that stimulation levels increased when a stress-free method was employed. The altered protocols changed fold over basal levels from 1.5 to 5.22 for turkey $P2Y_1$ and 2.32 to 7.26 for human $P2Y_2$. This was due to a reduction in basal levels increasing the overall fold stimulation observed. In this thesis all protocols for cell stimulation have been adjusted to minimise endogenous release by addition of agonists to the overnight serum-free media (see table 2.2).

Pervanadate is a compound created by the addition of equal concentrations of H_20_2 and sodium orthovanadate (Gordon, 1991). Sodium orthovanadate alone is a tyrosine phosphatase inhibitor but pervanadate is a more potent inhibitor on whole cells. Sodium orthovanadate was observed by us to only be effective in broken cell preparations (Purkiss and Boarder, unpublished). This was thought to be due to its

Protocol	Plate type	Serum-free volume	Stimulating volume	Pre-incubation /stimulation volumes
Western blotting/ MAPK assay	6 well or 35 mm well	1ml	100µl of 11 times concentration agonist	100µl of 11 times pre-incubation followed by 110µl of 11 times agonist
cAMP assay	96 well	100µl*	20µl of 6 times concentration agonist	-
Total inositol (poly)phosphates assay	24 well	0.5 ml	50µl of 11 times concentration agonist	-

* Left on cells for 40 minutes prior to stimulation

Table 2.2 Stimulation protocols used in this thesis

inability to cross the cell membrane. Pervanadate is therefore more potent on whole cells possibly due to its ability to cross the cell membrane. Protein tyrosine phosphorylation plays a key role in signal transduction and the balance of tyrosine phosphorylated proteins is kept between tyrosine kinases and phosphatases. Pervanadate is of use in signal transduction investigations when considering the role that phosphatases play.

The PKC inhibitors used in this thesis are also described in chapter 1 section 1.8.6.2. Ro 31-8220 (for full name description see abbreviations) is a general PKC inhibitor based on the bisindolylmaleimide compound (Beltman et al., 1996). Also based on bisindolylmaleimide, Go 6850 is a general inhibitor of PKC. Go 6976 however is an indolocarbazole and only inhibits the calcium-sensitive PKC isoforms i.e. α and β isoforms.

PD 98059 acts as a selective inhibitor of the phosphorylation and activation of MAPK kinase or MEK, which phosphorylates MAPK or ERK on threonine and tyrosine. PD98059 acts by binding to MEK such that access is blocked to activating enzymes. PD98059 does prevent activation occurring from already phosphorylated MEK or other serine/threonine protein kinases including Raf-1, MAPK, JNK, cyclin A/cdk2, PKA and PKC (Alessi et al., 1995; Dudley et al., 1995).

2.7 cAMP assay

Cyclic AMP was measured in trichloroacetic acid extracts of cells, after neutralisation with ether extraction and addition of $NaHCO_3$ using the protein binding assay of Brown et al (1971). Cells were used at confluency in 96 well plates and prior to stimulation placed in a 37^0C water bath. Existing media was aspirated

and replaced with 100µl / well Krebs-Ringer-Henseleit buffer (119mM NaCl, 10mM HEPES, 4.78 mM KCl, 1.24mM $MgSO_4$, 25mM $NaHCO_3$, 1.32mM CaCl and 10mM glucose) and left for 40 minutes. Cells were then stimulated with 20µl / well of a 6 times concentrated agonist solution. Reactions were left for 5 minutes and stopped with addition of 60µl / well 1.5M TCA. Plates were removed from the water bath and placed on ice for 90 minutes to extract. 150µl of TCA extract was taken and added to polypropylene tubes. TCA was removed by washing three times with 2ml of ether, vortexing between each wash, and the upper phase removed. Tubes were left for residual ether to evaporate and 100µl taken to a new tube. This was neutralised by addition of 25µl of 60mM $NaHCO_3$ and 25µl of 30mM EDTA pH 7.0 to each tube. Samples were then stable for storage at 4^0C.

A standard curve (Figure 2.1) was generated by dilution of 5µM cAMP standard to achieve 10 standards from 0 - 10 pmole / 50µl. Standards were diluted in blank buffer consisting of KRH buffer that had been treated with TCA, ether washed, and neutralised. [^{3}H]cAMP solution was made up from 2µl of [^{3}H]cAMP (1mCi/ml) and 8 ml of 50mM Tris/4mM EDTA buffer. 50µl of standard or sample was added to 100µl of [^{3}H]cAMP solution (approx 50-100 X 10^3 d.p.m.) and 150µl of binding protein (isolated from bovine adrenal glands). This was vortexed and left on ice for 90 minutes. After this incubation tubes were vortexed, 250µl of charcoal suspension added, then vortexed again, and left on ice for 8 minutes. Tubes were then vortexed and spun for 4 minutes at 13000 rpm. 450µl of the supernatant was removed without disturbing the charcoal pellet and pipetted into a scintillation vial. 5ml of emulsifier safe scintillant was added and the vials vortexed and counted for [^{3}H].

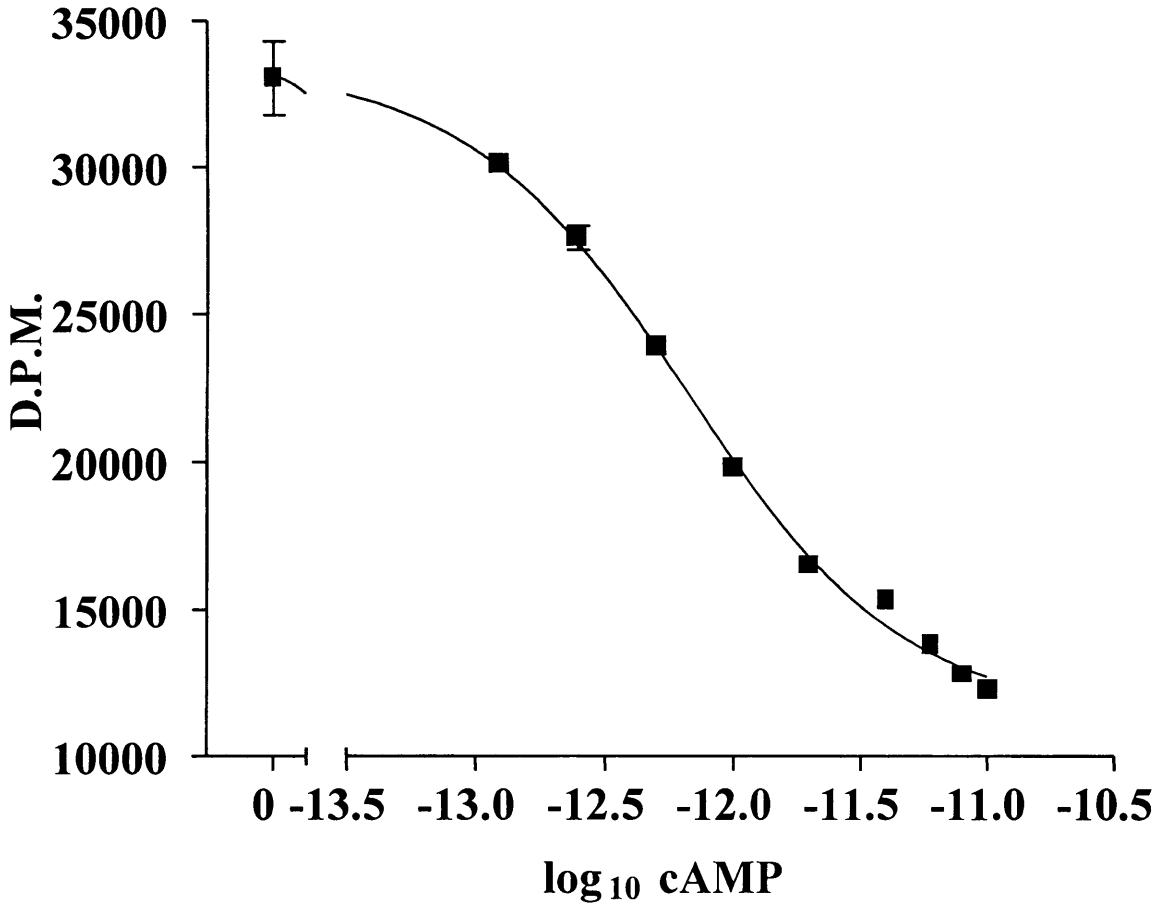


Figure 2.1 Standard curve for cyclic AMP assay showing log scale of amount of cAMP against [^{3}H]cAMP (disintegration's per minute).

2.8 p44/p42 MAPK assay

To detect the activity of p44 and p42 MAP kinase, an assay was developed in the laboratory based on the peptide APRTPGGRR, which is phosphorylated by MAPK at the threonine residue (T) (Clark-Lewis et al 1991; Wilkie et al., 1996). This nonapeptide substrate was chosen as it shows a higher specificity to MAPK than other peptides used previously e.g. myelin basic protein or MBP (Wilkie, 1997). Increased specificity of APRTPGGRR for MAPK rather than MBP, which shows phosphorylation by kinases other than MAPK, produced lower basal levels and a larger fold over basal increase when stimulated. The increased phosphorylation of the nonapeptide substrate is paralleled by an increase in tyrosine phosphorylation of MAPK, shown using a phospho-MAPK antibody, in stimulated cell extracts. FPLC analysis from activated cells revealed that only fractions containing phosphorylated p42 and p44 isoforms of MAPK, phosphorylated the nonapeptide substrate (Wilkie, 1997; Wilkie et al., 1996; Patel et al., 1996). The compound PD98059, a specific MEK inhibitor, was also able to inhibit both agonist activated tyrosine phosphorylation of p42 and p44 MAPK and increased phosphorylation of the nonapeptide (Wilkie, 1997). Tyrosine phosphorylation of MAPK only indicates that MAPK may be activated, as it requires dual phosphorylation at tyrosine and threonine, but from the evidence presented it can be concluded that increased phosphorylation of the nonapeptide is due to increased MAPK activity.

Previous protocols had used renatured SDS-PAGE containing MBP to detect ^{32}P phosphorylation in cell extracts run on the gel. This method was expensive and offered limited results using semi-quantitative densitometry of autoradiographs. Therefore a method was developed using a homogenising buffer (described in

section 2.4), containing protease and phosphatase inhibitors, that reduced the dephosphorylation of p44/p42 MAPK enabling stable production of activated enzyme from stimulated cell extracts. The precise method is outlined in the following paragraph. Assay conditions were optimised on the basis of linear kinetics, strong inducible signals and economy to give the protocol outlined below (Wilkie, 1997).

Cells were stimulated, as for western blotting (section 2.4), and reactions stopped with liquid nitrogen. After thawing on ice, 250µl of ice cold homogenising buffer (described in 2.4) was added per well of a 6 well plate, after which cells were scraped, vortexed and centrifuged for 15 minutes. Supernatants were taken and assayed for protein content. Samples were protein equalised by addition of lysis buffer. On ice a 10µl supernatant sample (approx. 10µg of protein) was added to 15µl of assay buffer containing 25mM $MgCl_2$, 1mM of the nonamer MAPK peptide substrate, APRTPGGRR, and 50µM ATP / ^{32}P γ ATP (1µCi) in a 500µl eppendorf tube. The mixture was incubated at 30^0C for 20 minutes on a hot block after which the reaction was stopped with addition of 20µl of ice cold 20% TCA. The stopped reactions were spotted onto P81 phospho-cellulose squares and excess ^{32}P γ ATP removed by washing with 75mM phosphoric acid. The P81 squares were then added to scintillation vials with 5ml of emulsifier safe scintillant and counted for [^{32}P].

2.9 Total [^3H] inositol polyphosphates

Measurement of inositol phosphate accumulation was essentially as described in Charlton et al., (1996a). Cells were sub-cultured into 24 well multiwell plates and grown to near confluence. Cells were then labelled with D-myo-[2-^3H] inositol by

removal of the growth medium and addition of 1μCi / ml (0.074 MBq / ml) of D-myo-[2-^{3}H] inositol in 0.5 ml of serum free media [M199 (low inositol) supplemented with 1% penicillin / streptomycin and 1% glutamine] for 24 hours. The plates were then placed in a 37^{0}C water bath and 50μl per well of 110mM LiCl was added for 10 minutes. After this 50μl of a 12 times concentration of stimulating media containing 10mM LiCl was added for 15 minutes. Aspiration of the stimulating media and addition of 0.5ml of 0.5M trichloroacetic acid (TCA) stopped the reaction. The plates were then placed on ice and left to extract for 1 hour. Samples were pipetted from the wells into polypropylene vials and washed with 2ml of diethyl ether to remove the TCA. The tubes were then neutralised by addition of 125μl of 60mM $NaHCO_3$ to each tube at which point they could be stored at 4^{0}C. Using Dowex-I Cl ion exchange resin, labelled inositol phosphates were separated. Columns of Dowex resin were charged with formate ions using 1M formic acid. Samples were added to the columns and unbound inositol and contaminants washed away with 20 ml / column of water. Next 10ml 60 mM ammonium formate was run through the columns to elute contaminating glycerophospholipids. The inositol phosphates were then eluted with 5 ml of 2M ammonium formate into scint vials and 10ml of FLO-SCINT IV scintillation cocktail added. Vials were vortexed well to ensure mixing and counted for [^{3}H].

2.10 Incorporation of [^{3}H] thymidine

Cells were plated into 24 well plates and grown to near confluence. Media were aspirated from the wells and replaced with 0.5ml serum free medium (DMEM containing 4.5g/L glucose supplemented with 25 i.u. ml^{-1} penicillin, 25 μg ml^{-1}

streptomycin and 25 μg ml^{-1} glutamine) for 24 hours at 37^0C. The cells were then incubated with the required agonists for 1 hour by addition of 100μl of 6 times concentration agonist. After 1 hour the stimulating media was replaced with 0.5ml of fresh serum free media and left for a further 18 hours. [^{3}H]-thymidine in serum free media was then added to each of the wells (0.5μCi / well final concentration) and left for a further 4 hours. After this final incubation cells were removed from the incubator and placed on ice. Media was aspirated and cells washed sequentially for 5 minutes with two 1ml washes with BSS, two 500μl washes with 5% TCA and two 500μl washes with ethanol. The last ethanol wash was aspirated and the plates left to dry after which 500μl of 0.5M NaOH was added and the plates frozen and thawed. 400μl of sample was then taken and added to scintillation vials and vortexed with 5mls of emulsifier safe scintillation cocktail and counted for [^{3}H].

2.11 Materials

Tissue culture plasticware and media were supplied by Gibco, Paisley, UK. Nucleotides were supplied by Sigma, Poole, UK for UTP, UDP, ATP and ATPγS and RBI-Sigma for 2MeSATP. PKC inhibitors Ro 31-8220, Go 6976 and Go 6850 and MEK inhibitor PD98059 were supplied by Calbiochem, Nottingham, UK. Radioactive isotopes [^{3}H] thymidine, [^{3}H] inositol, [^{3}H] cyclic AMP and γ [^{32}P]-ATP were supplied from Amersham Life Sciences. Equipment for SDS PAGE, blotting and densitometry were supplied by Biorad, Hert., U.K. Polyacrylamide (ratio 37.5:1) was supplied by Anachem. Primary antibodies monoclonal PY20, monoclonal Pyk2 and polyclonal Shc were supplied by Transduction Laboratories, Lexington, USA.

Monoclonal Shc antibody was supplied from Santa Cruz, USA. New England Biolabs, USA, supplied phospho-MAPK p44/p42 primary polyclonal antibody. PKC antibodies were supplied from Promega, USA. Anti-mouse IgG HRP secondary antibody, Anti-rabbit IgG HRP secondary antibody and ECL reagent were all supplied by Amersham Life Sciences. Most other chemicals and general reagents were supplied by Sigma or Fisher Scientific, Loughborough, U.K.

2.12 Statistical analysis

Results are expressed, unless otherwise stated, as means $\pm$ standard error for n values of 3-5. Comparisons were carried out using the appropriate statistical tests being Students t test, one way ANOVA with in some cases a Dunnet's or Bonferroni's post-test. P values of less than 0.05 were considered to be significant. Figures containing p value data are represented as $p<0.05$ = *, $p<0.01$ = ** and $p<0.001$ = ***. Concentration-response curves were fitted using non-linear regression sigmoidal dose-response (variable slope) and log EC_{50} calculated using Graph Pad Prism 2.0 (Graph Pad Software Incorporated).

Chapter 3

Effect of purinergic agonists on tyrosine phosphorylation of 1321N1 cells transfected with P2Y receptors

3.1 Introduction

Single transmembrane receptors for growth factors have intrinsic tyrosine kinase activity and are linked to regulation of tyrosine protein phosphorylations. It is now well established that 7-transmembrane G protein receptors can also be linked to tyrosine phosphorylation of cellular proteins (Malarkey et al., 1995; Bourne, 1995), including activation of p42/p44 MAPK, which requires both tyrosine and threonine phosphorylation. The pathway to tyrosine kinase activation by G-protein linked receptors can occur via PLC-dependent or PLC-independent pathways and also PKC-dependent or PKC-independent pathways (see section 1.8.5.2). Tyrosine phosphorylation of intracellular proteins has been shown to be important in cell division, growth and proliferation stimulated by G-protein linked receptors (Anderson et al., 1990; Van Daele et al., 1992).

It was proposed to investigate transfected P2Y receptor signalling in 1321N1 cells using the turkey $P2Y_1$ and human $P2Y_2$ receptors initially due to transfected subtype availability. Initial investigations showed coupling of turkey $P2Y_1$ and human $P2Y_2$ receptors to PLC activity, by total inositol phosphate accumulation, also shown in 1321N1 cells by Filtz et al., (1994) and Parr et al., (1994), respectively. Data by Charlton, (1998) is presented for turkey $P2Y_1$, human $P2Y_2$ and human $P2Y_4$ receptors as well as original data for rat $P2Y_6$ (see figure legends and corresponding text) providing confirmation that transfected receptors characteristically link to PLC activation. This provides the functional background evidence for the transfected P2Y receptors necessary when considering further signalling pathways.

In this thesis chapter, 1321N1 cells transfected with turkey $P2Y_1$ and human $P2Y_2$ receptors were both investigated for coupling to tyrosine phosphorylation

events, continuing native cell work already carried out in the lab on coexisting $P2Y_1$ and $P2Y_2$ receptors in BAEC. It is important that we examine the role that different P2Y receptors may have in regulation of important tyrosine phosphorylation events and their subsequent effect on cellular processes.

3.2 Effect of P2Y agonists on total inositol (poly)phosphate accumulation in P2Y transfected 1321N1 cells

As a background to signalling work it was important to establish that transfected P2Y receptors were functioning in a manner that was characteristic for that particular subtype of P2Y receptor. Native cell studies have all shown coupling of the different subtypes to phospholipase C activity. Therefore each subtype used in this thesis was investigated to show coupling to PLC and that the correct agonist profiles were shown using an assay detecting total inositol (poly)phosphates. Figure 3.1 represents data collected by S.J. Charlton (Charlton, 1998), reproduced with kind permission, for transfected turkey $P2Y_1$, human $P2Y_2$ and human $P2Y_4$ receptors. All data are pooled from three separate experiments each performed in triplicate. Data points are mean $\pm$ standard error of the mean.

Figure 3.1A shows the results for total inositol (poly)phosphates assay on 1321N1 cells transfected with turkey $P2Y_1$ receptors (Charlton, 1998). Cells, pre-labelled 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 (●). Rank order of potency was 2MeSATP>ADP>ATPγS=ATP, with UTP being ineffective, consistent with the reported pharmacological profile of $P2Y_1$. The EC_{50} values for 2MeSATP,

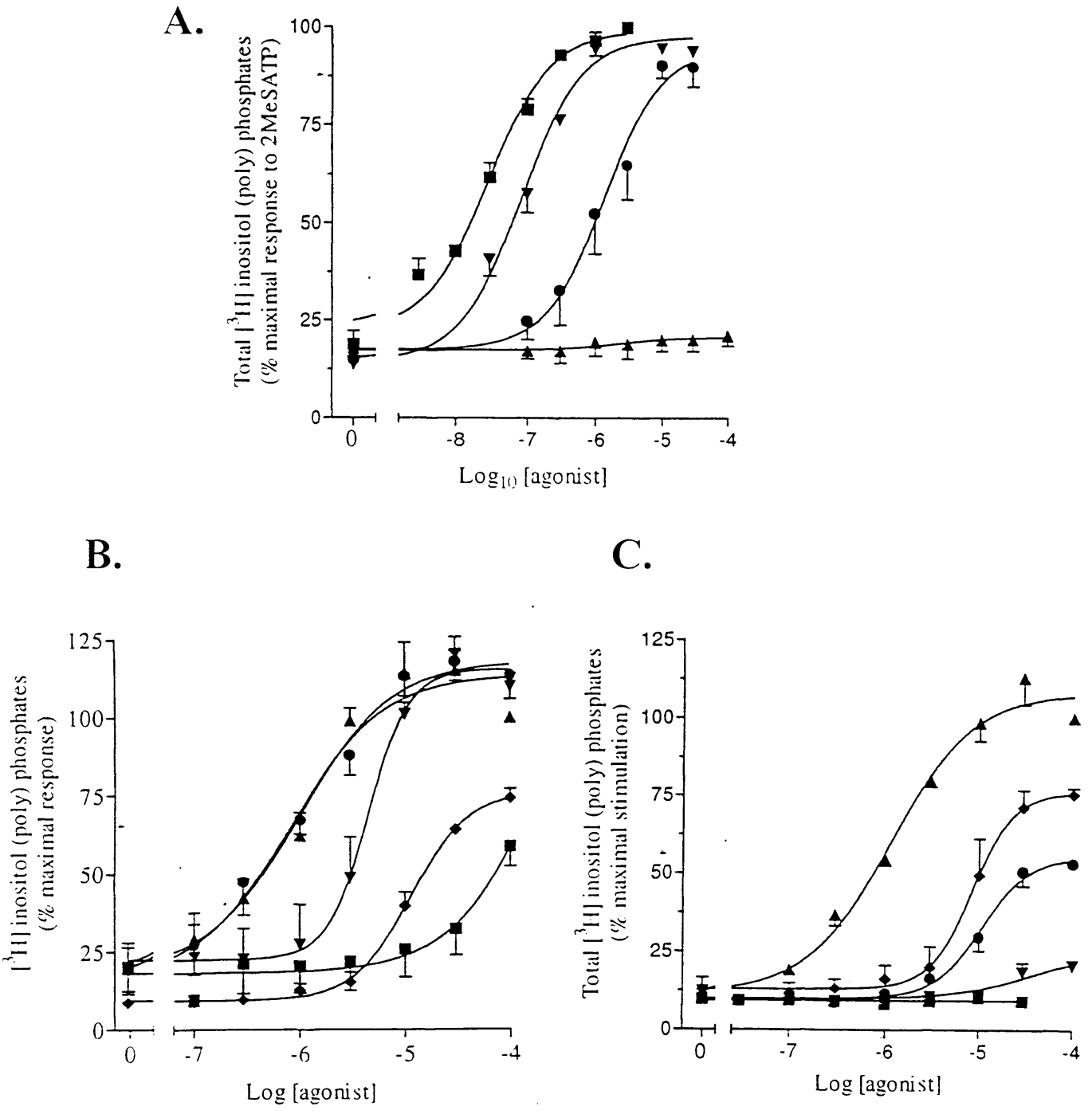


Figure 3.1

Agonist dose response curves for $[^3H]InsP_x$ accumulation in 1321N1 cells transfected with turkey $P2Y_1$ (A), human $P2Y_2$ (B) or human $P2Y_4$ (C) (reproduced with kind permission from Charlton, 1998).

Cells were incubated with increasing agonist concentrations of agonists 2MeSATP (■), UTP (▲), ADP (▼), ATP (●) and UDP (◆). Data are mean ± SEM of three separate experiments each performed in triplicate, expressed as a percentage of the stimulation to 3μM 2MeSATP (turkey $P2Y_1$) or 100μM UTP (human $P2Y_2$ or $P2Y_4$).

ADP, ATPγS and ATP were 28.1 ± 3.6nM, 0.18 ± 0.01μM, 0.49 ± 0.7μM and 1.2 ± 0.7μM, respectively.

Figure 3.1B represents data collected by Charlton (1998) for the total inositol (poly)phosphates assay on 1321N1 cells transfected with human $P2Y_2$ receptors and stimulated with agonists 2MeSATP (■), UTP (▲), ADP (▼), ATP (●) and UDP (◆), at increasing doses. UTP, ATP and ADP all acted as full agonists with UDP a partial agonist and 2MeSATP only a weak agonist. UTP and ATP had similar EC_{50} values of 0.94 ± 0.23μM and 1.50 ± 0.55μM, respectively. ADP and UDP had EC_{50} values of 8.22 ± 3.0μM and 15.8 ± 0.51μM, respectively giving a rank order of potency of UTP=ATP>ADP>UDP characteristic of $P2Y_2$ receptor activation.

Figure 3.1C depicts the effect of an increasing dose of purinergic agonists on total inositol(poly)phosphate accumulation in 1321N1 cells transfected with human $P2Y_4$ receptors (Charlton, 1998). Agonists used were 2MeSATP (■), UTP (▲), ADP (▼), ATP (●) and UDP (◆). UTP was the only full agonist with an EC_{50} of 1.17 ± 0.19μM. UDP and ATP were both partial agonists with EC_{50} values of 5.68 ± 1.19μM and 16.2 ± 4.76μM, respectively. ADP and 2MeSATP both were ineffective. The rank order of potency for PLC activation at the transfected human $P2Y_4$ receptor was therefore consistent with previously reported $P2Y_4$ receptor activation.

Figure 3.2 shows new data for the effect of increasing dose of purinergic agonists on total inositol (poly)phosphate accumulation in 1321N1 cells transfected with rat $P2Y_6$ receptors. Data are mean ± SEM of three separate experiments each performed in triplicate, shown as raw data collected. Agonists used were UDP (◆), UTP (▲), ADP (▼) and 2MeSATP (■). UDP was the most potent full agonist tested

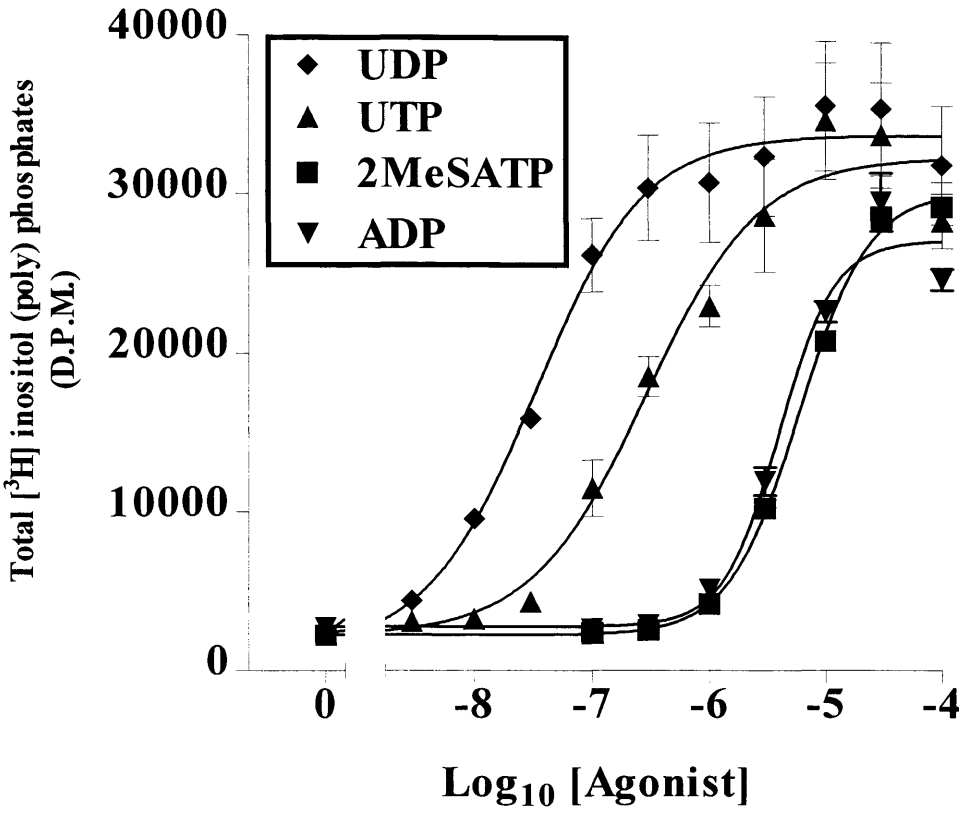


Figure 3.2

Agonist dose response curves for $[^3H]InsP_x$ accumulation in 1321N1 cells transfected with rat $P2Y_6$ receptors.

Cells were incubated with increasing agonist concentrations of UDP (◆), UTP (▲), ADP (▼), and 2MeSATP (■). Data are mean ± SEM of three separate experiments each performed in triplicate, shown as raw data in d.p.m.

with a –log EC_{50} of 7.49 ± 0.17 being more potent than UTP, also a full agonist with a –log EC_{50} of 6.55 ± 0.12. Both 2MeSATP and ADP were potent agonists at the rat $P2Y_6$ receptor, but approximately 100 fold less potent than UDP with –log EC_{50} values of 5.23 ± 0.03 and 5.40 ± 0.05, respectively and reaching ~85% of maximal activation by UDP. This pharmacological profile shows activation of rat $P2Y_6$ transfected in 1321N1 cells characteristic for a $P2Y_6$ receptor.

3.3 Effect of turkey $P2Y_1$ receptor activation on tyrosine phosphorylation in transfected 1321N1 cells

Bovine aortic endothelial cells (BAEC) that express both $P2Y_1$ and $P2Y_2$ receptors (Wilkinson et al., 1993) show tyrosine kinase regulation of PGI_2 release on activation by ATP (Bowden et al., 1995). Both $P2Y_1$ and $P2Y_2$ receptors when activated by 2MeSATP and UTP respectively, stimulate PGI_2 release that is dependent, for both receptors, on tyrosine kinases. The tyrosine phosphorylation important in prostacyclin production occurs downstream of PLC and at different levels along the pathway including PKC, and MAPK controlling $cPLA_2$ activation (Patel et al., 1996a; b). It was proposed that further characterisation of P2Y receptor signalling would be of interest, therefore transfected turkey $P2Y_1$ receptors were investigated for modulation of protein tyrosine phosphorylation.

3.3.1 Effect of 2MeSATP on tyrosine phosphorylation in $tP2Y_1$ transfected cells – Time course

Figure 3.3 depicts the effect of agonist stimulation with time on tyrosine phosphorylated proteins in transfected turkey $P2Y_1$ 1321N1 cells using western

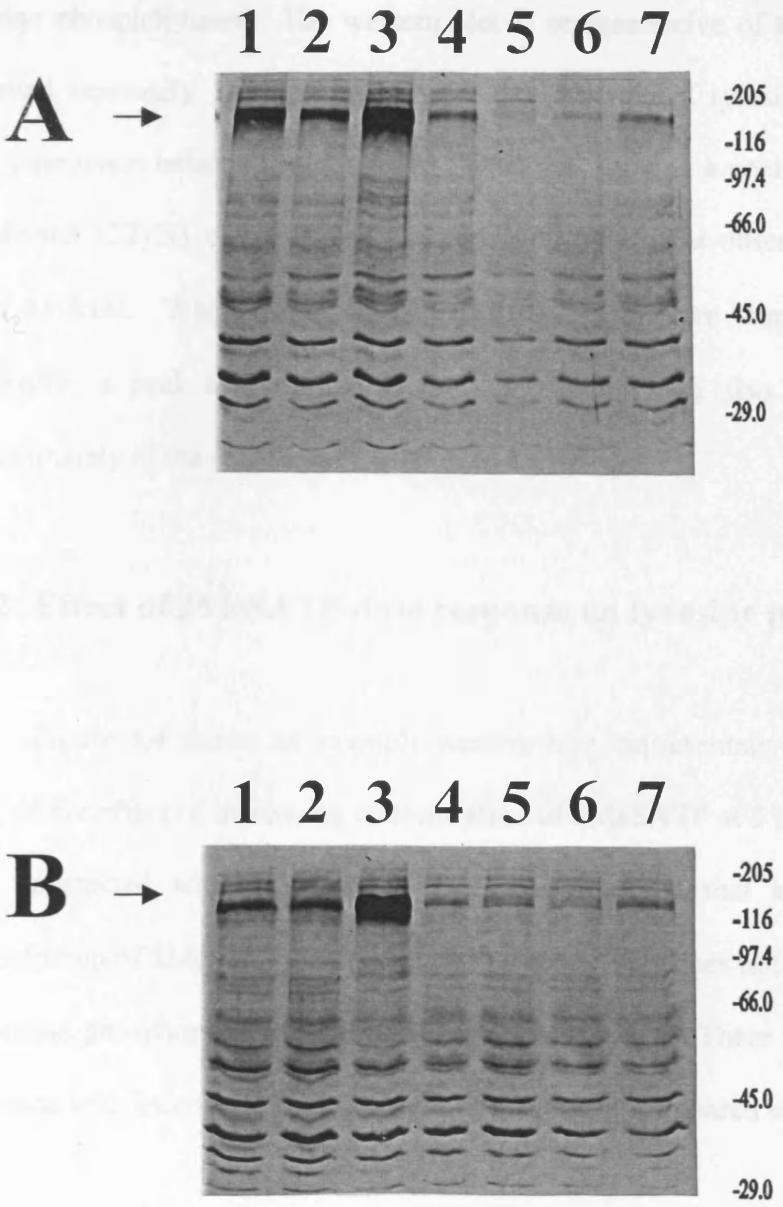


Figure 3.3

Effect of BSS with time on tyrosine phosphorylation in 1321N1 cells transfected with turkey $P2Y_1$ receptors (A) and effect of 30 μM 2MeSATP with time on tyrosine phosphorylation in 1321N1 cells transfected with turkey $P2Y_1$ receptors (B)

Lanes 1 - 7 represent time points 0, 30 sec, 1 min, 2 min, 5 min, 10 min and 20 min respectively for both blots. Blot is representative of three separate similar results. The arrow marks the 180kD region of interest. Other band variations observed on this blot were not observed on other blots.

blotting with PY20 primary antibody that is immuno-reactive to proteins that are tyrosine phosphorylated. The western blot is representative of three similar results achieved separately. Figure 3.3A shows the variation of tyrosine phosphorylation with time when balanced salt solution (BSS) was used as a control on turkey $P2Y_1$ transfected 1321N1 cells. A peak in phosphorylation was observed at 1 minute at about 180kDa. When turkey $P2Y_1$ transfected cells were stimulated with 30μM 2MeSATP, a peak at 1 minute in the same region was also observed that was approximately of the same intensity (Figure 3.3B).

3.3.2 Effect of 2MeSATP dose response on tyrosine phosphorylation

Figure 3.4 shows an example western blot, representative of three separate blots, of the effect of increasing concentration of 2MeSATP at 5 minutes on 1321N1 cells transfected with turkey $P2Y_1$. It was observed that an increase in the concentration of 2MeSATP on turkey $P2Y_1$ transfectants does not lead to an increase in tyrosine phosphorylation of proteins at any band size. There was no observable difference with increasing concentration of 2MeSATP compared with control (BSS).

3.4 Effect of $hP2Y_2$ receptor activation on tyrosine phosphorylation in transfected 1321N1 cells

As was discussed previously bovine $P2Y_2$ receptors have been linked in BAEC to PLC and tyrosine kinases in the pathway to prostacyclin release (Bowden et al., 1995). In human endothelial cells (EAhy 926 cell line) increasing concentrations of UTP cause rises in tyrosine phosphorylation and MAPK activation

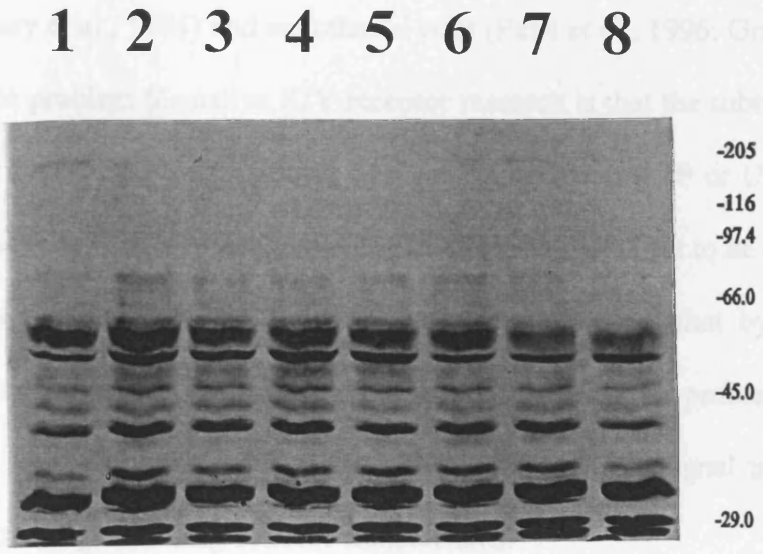


Figure 3.4

Effect of increasing dose of 2MeSATP on general tyrosine phosphorylation in 1321N1 cells transfected with turkey $P2Y_1$ receptors

Cells were stimulated for 5 minutes in each case. Lanes 1 - 8 represent 0, 10nM, 30nM, 50nM, 100nM, 1μM, 3μM, 10μM and 30μM 2MeSATP respectively. Blot is representative of three separate experiments.

(Graham et al., 1996). This is thought to be due to human $P2Y_2$ receptor activation because of the relative potencies of ATP and UTP and no increase was observed with 2MeSATP, a $P2Y_1$ agonist, which is inactive at $P2Y_2$. There is increasing evidence that $P2Y_2$ receptors are linked to activation of MAPK which is tyrosine phosphorylated in renal mesangial cells (Huweiler and Pfeilschifter, 1994), astrocytes (Neary et al., 1994) and endothelial cells (Patel et al., 1996; Graham et al., 1996). A major problem for native P2Y receptor research is that the subtype of P2Y receptor under investigation is unknown because responses to ATP or UTP may be due to more than one P2Y receptor or novel P2Y receptor that is yet to be cloned (see section 1.5 for receptor agonist profiles). It was hypothesised that by using the transfected mammalian receptor, human $P2Y_2$ in 1321N1 cells, this problem could be avoided. The role of tyrosine phosphorylation in human $P2Y_2$ signal transduction was therefore investigated using 1321N1 transfectants.

3.4.1 Effect of UTP on tyrosine phosphorylation in $hP2Y_2$ transfected cells – Time course

Figure 3.5 shows an autoradiographic image of a western blot result for the effect of UTP and BSS with time on 1321N1 cells transfected with the human $P2Y_2$ receptor using the PY20 primary antibody for phosphotyrosine proteins. Figure 3.5A shows at about 180 kDa a protein has increased tyrosine phosphorylation at 1 minute compared with time point zero when human $P2Y_2$ transfects were stimulated with BSS. This is a similar observation to that shown with 1321N1 cells transfected with turkey $P2Y_1$ when stimulated with BSS. Figure 3.5B also shows a similar band, of

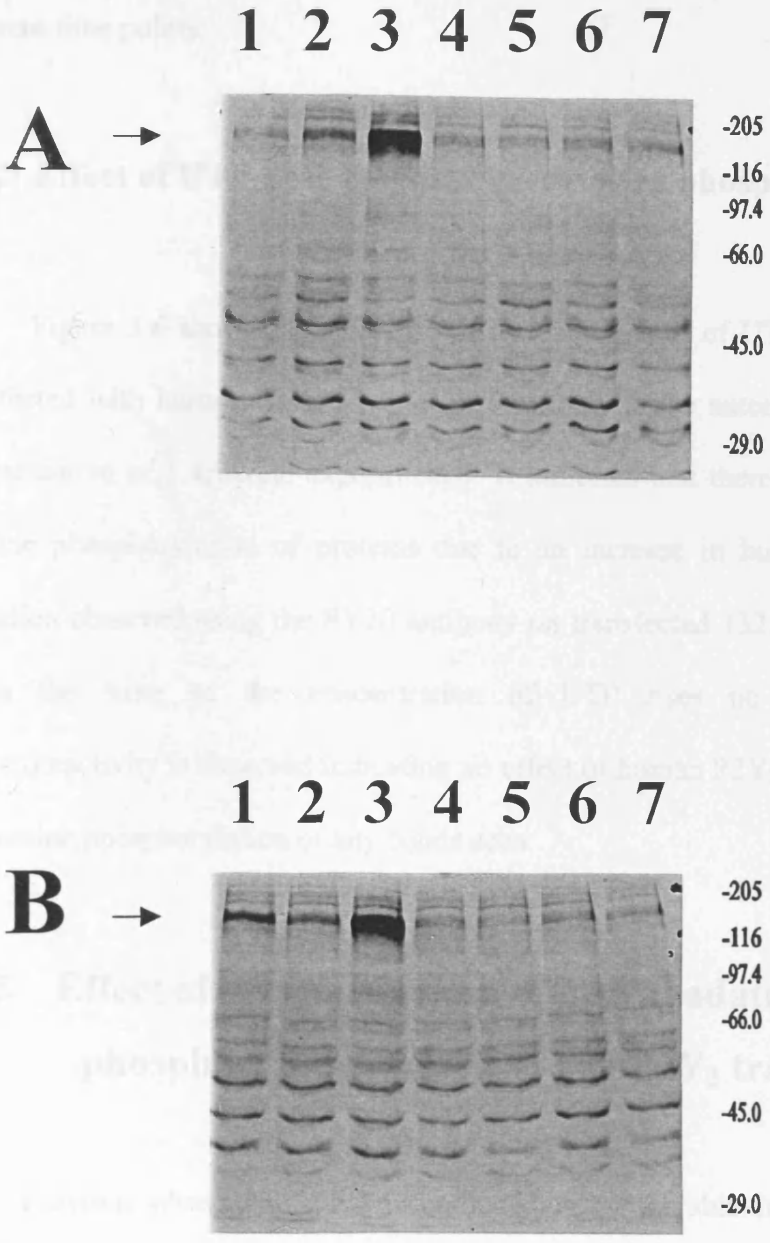


Figure 3.5

Effect of BSS with time on tyrosine phosphorylation in 1321N1 cells transfected with human $P2Y_2$ receptors (A) and effect of 100 μM UTP with time on tyrosine phosphorylation in 1321N1 cells transfected with human $P2Y_2$ receptors (B)

Blots are representative of three separate experiments. Lanes 1 - 7 represent time points 0, 30 sec, 1 min, 2 min, 5 min, 10 min and 20 min respectively for both blots. The arrow marks the 180kD region of interest. Other band variations observed on this blot were not observed on other blots.

similar intensity, when human $P2Y_2$ transfects are stimulated with 100μM UTP at different time points.

3.4.2 Effect of UTP dose response on tyrosine phosphorylation

Figure 3.6 shows the effect of an increasing dose of UTP on 1321N1 cells transfected with human $P2Y_2$ receptor at 5 minutes. The autoradiograph shown is representative of 3 separate experiments. It indicates that there was no increase in tyrosine phosphorylation of proteins due to an increase in human $P2Y_2$ receptor activation observed using the PY20 antibody on transfected 1321N1 cells. Looking across the lanes as the concentration of UTP rises no increase in band immunoreactivity is observed indicating no effect of human $P2Y_2$ receptor activation on tyrosine phosphorylation of any bands seen.

3.5 Effect of increasing dose of pervanadate on tyrosine phosphorylation in $tP2Y_1$ or $hP2Y_2$ transfectants

Previous observations have concluded no observable increase in tyrosine phosphorylation was seen with either turkey $P2Y_1$ or human $P2Y_2$ receptor activation in transfected 1321N1 cells. It was thought that because 1321N1 cells are a transformed cell line either basal levels of tyrosine phosphorylation might be high or a high phosphotyrosine turnover may be masking any effects observable by P2Y receptor activation. Pervanadate is a general protein tyrosine phosphatase inhibitor (Gordon, 1991), which may help show any increase in tyrosine kinase activity not previously observed. Pervanadate was produced prior to use by addition of 250μM

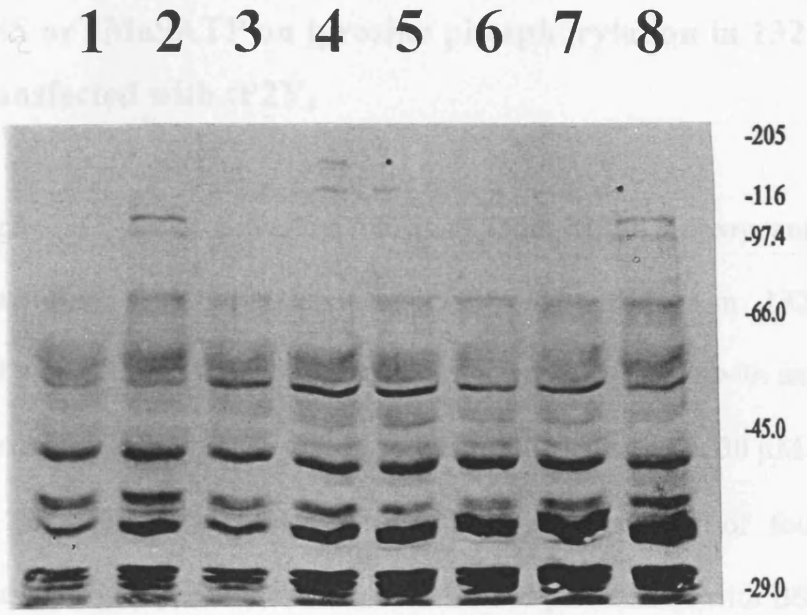


Figure 3.6

Effect of increasing dose of UTP on general tyrosine phosphorylation in 1321N1 cells transfected with human $P2Y_2$ receptors

Cells were stimulated for 5 minutes in each case. Lanes 1 - 8 represent 0, 10nM, 30nM, 50nM, 100nM, 1μM, 3μM, 10μM and 30μM UTP respectively. Blot shown is representative of three separate experiments with similar outcome. Any band variations observed on this blot were not observed on other blots.

H_2O_2 and 250μM sodium orthovanadate and added with either BSS or the P2Y agonists for 5 minutes to see if P2Y receptors under these conditions stimulated tyrosine phosphorylation.

3.5.1 Effect of increasing dose of pervanadate in the presence of BSS or 2MeSATP on tyrosine phosphorylation in 1321N1 cells transfected with $tP2Y_1$

Figure 3.7 shows a western blot using the PY20 primary antibody that detected tyrosine phosphorylation on proteins extracted from 1321N1 cells transfected with the turkey $P2Y_1$ receptor. Cells were stimulated with an increasing dose of pervanadate either in the presence of BSS (Figure 3.7A) or 30 μM 2MeSATP (Figure 3.7B). The autoradiograph shown is representative of four separate experiments each with similar results. The effect of pervanadate with BSS caused a massive increase in phosphotyrosine levels shown at 250 μM pervanadate compared with 5μM. Similarly in the presence of 2MeSATP, pervanadate caused a large increase in tyrosine phosphorylation. No significant difference was seen between the presence of BSS or 2MeSATP.

3.5.2 Effect of increasing dose of pervanadate in the presence of BSS or UTP on tyrosine phosphorylation in 1321N1 cells transfected with $hP2Y_2$

Figure 3.8 shows the effect of increasing dose of pervanadate on 1321N1 cells transfected with human $P2Y_2$ receptors in the presence of BSS or 100μM UTP. The transfected cells showed a large increase in tyrosine phosphorylated proteins on

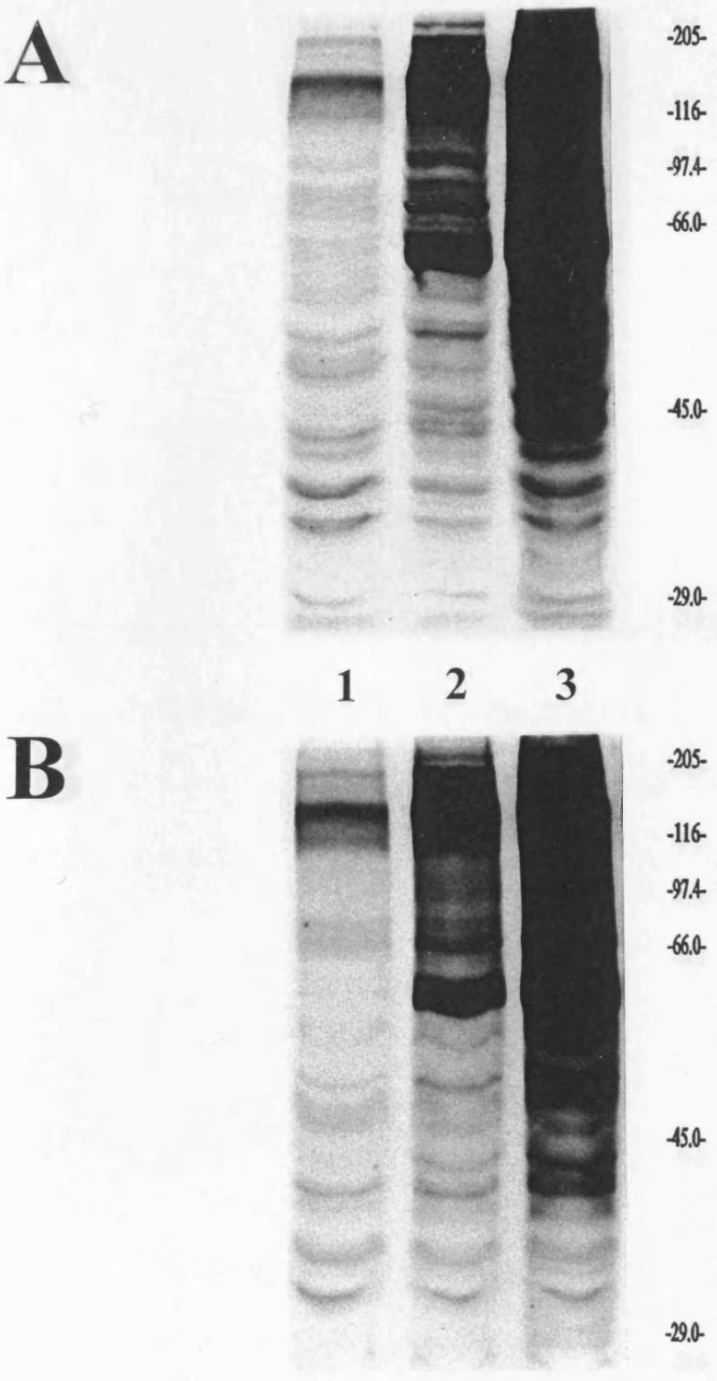


Figure 3.7

Effect of increasing concentration of pervanadate in the presence of BSS on tyrosine phosphorylation in 1321N1 cells transfected with turkey $P2Y_1$ receptors (A) and effect of increasing concentration of pervanadate in the presence of 30μM 2MeSATP on tyrosine phosphorylation in 1321N1 cells transfected with turkey $P2Y_1$ receptors (B)
Lanes 1, 2 and 3 represent pervanadate concentrations of 5μM, 50μM and 250μM respectively. Both experiments A and B were stimulated for 5 minutes (n=4).

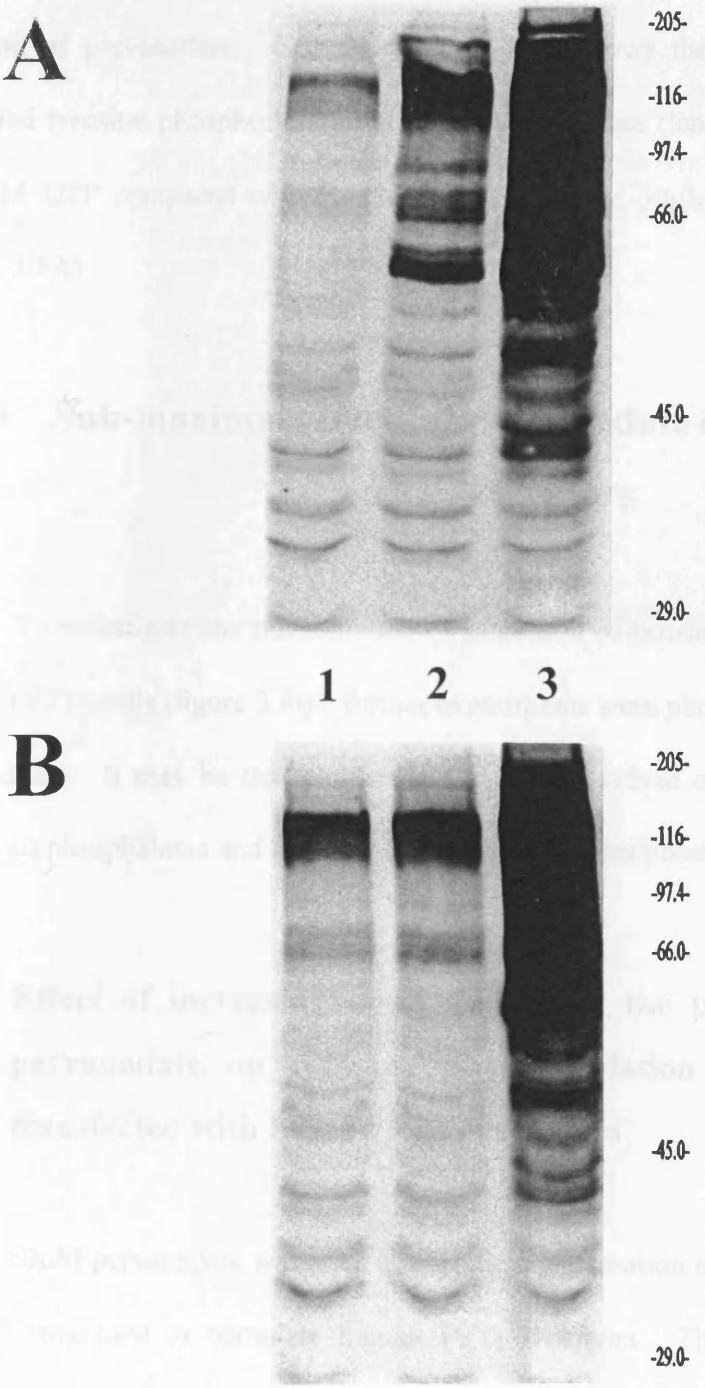


Figure 3.8

Effect of increasing concentration of pervanadate in the presence of BSS on tyrosine phosphorylation in 1321N1 cells transfected with human $P2Y_2$ receptors (A) and effect of increasing concentration of pervanadate in the presence of 100μM UTP on tyrosine phosphorylation in 1321N1 cells transfected with human $P2Y_2$ receptors (B)

Lanes 1, 2 and 3 represent pervanadate concentrations of 5μM, 50μM 250μM respectively. Both experiments A and B were stimulated for 5 minutes and are represented of three similar experimental results.

addition of pervanadate. However, figure 3.8B shows there was a decrease in observed tyrosine phosphorylation at 50μM pervanadate (lane 2) on co-addition of 100 μM UTP compared with co-addition of BSS and 50μM pervanadate (lane 2, Figure 3.8A).

3.6 Sub-maximal effects of pervanadate on transfected $P2Y_2$ receptors

To investigate the phenomenon of inhibition of tyrosine phosphorylation by UTP on $P2Y_2$ cells (figure 3.8B), further experiments were planned using only 50μM pervanadate. It may be that pervanadate at a submaximal concentration does not inhibit all phosphatases and for this reason inhibition was observed.

3.6.1 Effect of increasing dose of UTP in the presence of 50μM pervanadate on tyrosine phosphorylation in 1321N1 cells transfected with human $P2Y_2$ receptors

50μM pervanadate was used at a single concentration and an increasing dose of UTP was used to stimulate human $P2Y_2$ receptors. This would establish a stronger case for linking receptor activation with tyrosine phosphorylation decrease if there was a correlating dose dependant decrease in tyrosine phosphorylation. Figure 3.9A depicts a typical result (representative of three experiments) obtained. A concentration dependant decrease in tyrosine phosphorylation was observed particularly at @65Kd and most noticeable at 30μM UTP and higher.

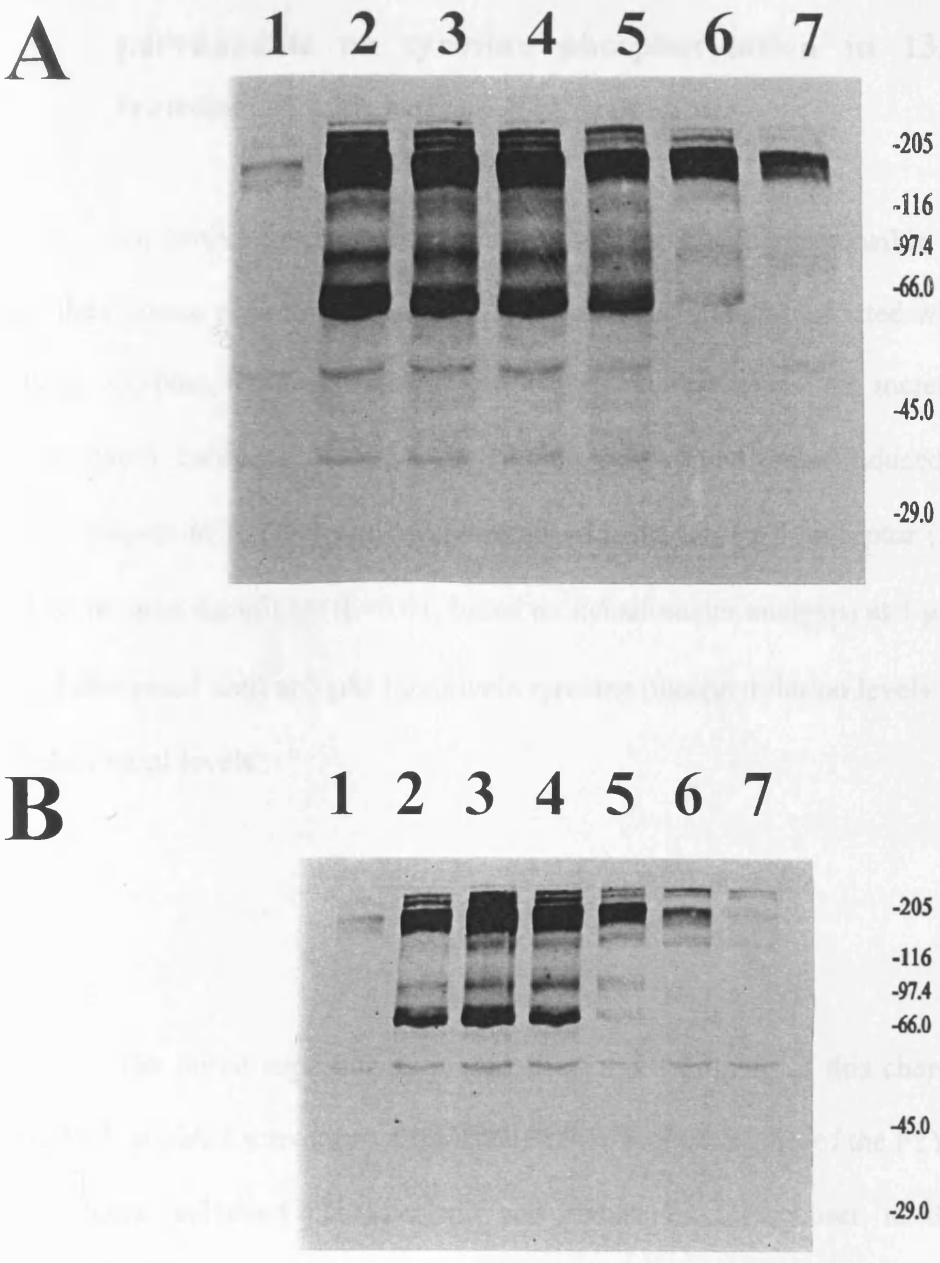


Figure 3.9

Effect of increasing concentration of UTP in the presence of 50μM pervanadate on tyrosine phosphorylation in 1321N1 cells transfected with human $P2Y_2$ receptors (A). Lane 1, BSS only. Lane 2, 50μM pervanadate only. Lane 3-7 50μM pervanadate with an increasing dose of 1μM, 3μM, 30μM, 100μM and 300μM UTP.

Effect of increasing concentration of ionomycin in the presence of 50μM pervanadate on tyrosine phosphorylation in 1321N1 cells transfected with human $P2Y_2$ receptors (B). Lane 1, BSS only. Lane 2, 50μM pervanadate only. Lane 3-7 50μM pervanadate with an increasing dose of 100nM, 300nM, 1μM, 3μM and 5μM ionomycin.

All conditions were stimulated for 5 minutes. Both experiments A and B are representative autoradiographs from three separate experiments.

3.6.2 Effect of increasing dose of ionomycin in the presence of 50μM pervanadate on tyrosine phosphorylation in 1321N1 cells transfected with human $P2Y_2$ receptors

To further investigate the mechanism that may be responsible for inhibition of the tyrosine phosphorylation response in 1321N1 cells transfected with the human $P2Y_2$ receptor, ionomycin, a calcium ionophore, was used. An increasing dose of ionomycin caused a decrease in tyrosine phosphorylation induced by 50 μM pervanadate in 1321N1 cells transfected with human $P2Y_2$ receptor (Figure 3.9B). This became significant ($p<0.01$, based on densitometer analysis) at 1 μM ionomycin and decreased until at 5 μM ionomycin tyrosine phosphorylation levels were equal or below basal levels.

3.7 Discussion

The initial experiments presented at the beginning of this chapter described the PLC coupled activation of transfected P2Y receptors. All of the P2Y receptors in this thesis achieved characteristic and robust PLC responses to their relevant nucleotide agonists. This is an important distinction to make as it shows the transfected receptors to be functional and responsive. This chapter particularly investigates the modulation of cellular tyrosine phosphorylation by transfected P2Y receptors, the results of which will now be discussed.

3.7.1 Effect of turkey $P2Y_1$ receptor activation on tyrosine phosphorylation in transfected 1321N1 cells.

In BAECs stimulation of bovine $P2Y_1$ receptors leads to an increase in many different tyrosine phosphorylated proteins observed by western blot (Bowden et al., 1995). This is not the case for transfected turkey $P2Y_1$ in 1321N1 cells since the single phosphorylated band, observed at 1 minute when stimulated with 30μM 2MeSATP, was also observed on addition of BSS after 1 minute and no other bands of increased immunoreactivity were seen after stimulation of $tP2Y_1$ transfectants with 2MeSATP. The observed stimulation could not therefore be as a consequence of turkey $P2Y_1$ receptor activation unless addition of BSS caused an endogenous release of ATP due to shear stress on 1321N1 cells. This however is unlikely for a few reasons. Firstly, the band intensities for BSS and 2MeSATP stimulated tyrosine phosphorylation are similar when it would be expected that 2MeSATP would have a higher intensity due to higher added agonist concentrations. Shear stress release of ATP with BSS could have equal intensity bands with 2MeSATP only if maximum tyrosine phosphorylation of this band had been achieved. Endogenous release however does not maximally activate turkey $P2Y_1$ receptors as agonist stimulated responses are observed for PLC (section 3.2), though it is possible that a small PLC response could cause maximal activation of tyrosine phosphorylation. Secondly, the time point of 1 minute is shorter than previously observed phosphorylations at 5 minutes (Bowden et al., 1995) no increase in band intensity was observed at 5 minutes. Thirdly, only a single band was observed to be phosphorylated whereas previous reports have indicated several proteins to be tyrosine phosphorylated on $P2Y_1$ stimulation. These observations lead to the most likely conclusion is that the

increased tyrosine phosphorylation was not observed as a consequence of turkey $P2Y_1$ receptor activation but due to stress activation of 1321N1 cells. It is important also to note that the system used in this thesis was a transfected turkey $P2Y_1$ receptor, which was one of the first cloned P2Y receptors. This therefore may account for some differences observed between turkey transfected and native bovine $P2Y_1$ receptors. Structurally they are very similar, with ~85% identity. However, differences are observed at the N-terminal end where the bovine receptor has an 11 a.a. insert, and in transmembrane domains four and five. Data observed by S.J. Charlton (Charlton, 1998), when comparing turkey $P2Y_1$ receptor and bovine $P2Y_1$ receptors, showed the same agonist profiles for the two receptors with a difference in the agonist potencies being reduced 10 fold for bovine $P2Y_1$. This observation can possibly be explained by a difference in the receptor number between turkey and bovine $P2Y_1$, but without accurate receptor number calculations for P2Y receptors this can not be fully answered.

The data collected for 2MeSATP dose response at 5 minutes also reinforces the finding that activation of transfected turkey $P2Y_1$ receptors in 1321N1 does not lead to an observable increase in tyrosine phosphorylation using western blotting with PY20 primary antibody. Dose response stimulation at 5 minutes was chosen based on the observations in BAEC's. Stimulation with a dose increase at 1 minute would have been unlikely to cause an increase in tyrosine phosphorylation based on earlier conclusions that the time course increase in tyrosine phosphorylation was not turkey $P2Y_1$ specific.

3.7.2 Effect of human $P2Y_2$ receptor activation on tyrosine phosphorylation in transfected 1321N1 cells.

In a similar manner to 2MeSATP and BSS effects on the turkey $P2Y_1$ receptor, UTP and BSS cause increases in tyrosine phosphorylation of only a single band at 1 minute on human $P2Y_2$ 1321N1 transfectants. This provides more evidence that the phosphorylation event observed is most likely due to stress activation that is a property of 1321N1 cells and not due to the P2Y receptor transfected into the cells. Endogenous release is not likely to be the cause of the phosphorylation, though ATP or UTP released would activate the human $P2Y_2$ receptor. Neither variation of UTP dose nor stimulation with time causes an increase in tyrosine phosphorylation that can be attributed to the presence of the human $P2Y_2$ receptor. This is at odds with previous reports in endothelial cells (Graham et al., 1996) and astrocytes (Neary et al., 1994) for the human $P2Y_2$ receptor. On human $P2Y_2$ transfection into 1321N1 cells, clones were selected on the basis of strong activation of PLC using total [^{3}H]-inositol polyphosphate accumulation as an indication of PLC activity. It can be concluded that although characteristic PLC activity was observed for human $P2Y_2$ receptors transfected in 1321N1 cells, they were not linked to tyrosine kinase pathways previously observed in native systems.

3.7.3 Effect of increasing dose of pervanadate on tyrosine phosphorylation in $tP2Y_1$ or $hP2Y_2$ transfected 1321N1 cells

Investigations with pervanadate on 1321N1 cells shows that there exists a large turnover of tyrosine phosphorylated proteins, via very active kinases and phosphatases, which can only be seen once a tyrosine phosphatase inhibitor is added.

For turkey $P2Y_1$ transfectants, comparison of figure 3.7A with figure 3.7B shows that there is no significant difference between addition of BSS and addition of 2MeSATP implying that the turkey $P2Y_1$ receptor has no role in modulation of tyrosine phosphorylation in the presence of pervanadate, a tyrosine phosphatase inhibitor.

Addition of UTP and pervanadate caused a reduction in tyrosine phosphorylation when compared to addition of BSS and pervanadate. It can be concluded from this observation that activation of human $P2Y_2$ receptors expressed in 1321N1 cells must activate some kind of phosphatase that is either pervanadate insensitive or active at sub-maximal pervanadate concentrations, or be able to inhibit tyrosine kinase activation by some other mechanism. From the western blots from figures 3.8a and 3.8b, it can be observed that the inhibition most readily occurs at 50μM pervanadate and less so at higher concentrations. Preliminary data have shown that increasing the dose of pervanadate decreases the effect that UTP has on tyrosine phosphorylation. This would support the conclusion that sub-maximal concentrations of pervanadate only partially inhibit certain phosphatases, which on addition of UTP are activated via the human $P2Y_2$ receptor causing the observed decrease in tyrosine phosphorylation.

3.7.4 Sub-maximal effects of pervanadate on transfected $P2Y_2$ receptors

To investigate the submaximal effect hypothesis further, investigations showed that an increase in UTP concentration, in the presence of 50μM pervanadate, achieved a concentration dependant decrease in tyrosine phosphorylation. This result

supports the previous data that activation of human $P2Y_2$ transfectants functionally couples to inhibition of tyrosine phosphorylation by a possible increase in activity of a tyrosine phosphatase not inhibited by 50μM pervanadate. It is unlikely that the response is due to a pervanadate insensitive phosphatase, as UTP would achieve inhibition at all concentration ranges of pervanadate, which it does not. It is also unlikely that inhibition of a tyrosine kinase is involved, as it too would be effective at all pervanadate concentrations. The most plausible explanation therefore is the sub-maximal pervanadate hypothesis.

Ionomycin was also used to see if a calcium release could modulate the tyrosine phosphorylation increase, observed with pervanadate. Ionomycin dose dependently decreased pervanadate increased tyrosine phosphorylation. It could be hypothesised that UTP, acting on human $P2Y_2$ transfectants, is inhibiting the pervanadate induced tyrosine phosphorylation via calcium release, which may be inactivating kinases or activating phosphatases. Preliminary data shows that unlike UTP, ionomycin is able to inhibit tyrosine phosphorylation at all concentrations of pervanadate. Therefore, though ionomycin may be acting at kinases or phosphatases to achieve inhibition of pervanadate induced increases in tyrosine phosphorylation, it is via a separate mechanism to that used by UTP.

3.8 Conclusion

It can be concluded from the data presented in this chapter that, unlike native cell systems, when turkey $P2Y_1$ and human $P2Y_2$ receptors are expressed in 1321N1 cells, they do not couple to an observable increase in tyrosine phosphorylation, either with time or with increasing agonist dose. Although total [^{3}H]-inositol

polyphosphate data shows that both these receptors, when expressed in 1321N1 cells, exhibit characteristic strong PLC activation, they are not linked to an overall observable increase in tyrosine phosphorylation as seen by western blot using the PY20 antibody. However when using pervanadate a decrease in tyrosine phosphorylation was observed when the human $P2Y_2$ receptor was stimulated showing P2Y modulation of tyrosine phosphorylation. No effect was seen with turkey $P2Y_1$ receptors. It may be concluded therefore that although these transfected P2Y receptors do not couple, like growth factor receptors, to a large increase in tyrosine phosphorylation on a number of different proteins, they may be involved in tyrosine phosphorylation at a level unseen by the PY20 antibody. PY20 antibody is fairly sensitive to changes in tyrosine phosphorylation levels within 1321N1 cells as shown by action of pervanadate, but it may have difficulty in detecting small changes in tyrosine phosphorylation. Many seven transmembrane receptors have been shown to link to an increase in tyrosine phosphorylation including P2Y receptors. The reasoning behind why an increase was not observed in 1321N1 transfectants is not clear. One possibility is the sensitivity of the PY20 antibody already mentioned. The chance that receptor transfection has disrupted coupling to tyrosine phosphorylation by P2Y receptors may be considered on several levels. It is unlikely that transfection disrupted the machinery for tyrosine phosphorylation by insertional inactivation of signalling protein genes as lack of coupling is observed in both turkey $P2Y_1$ and human $P2Y_2$ transfectants. High level of receptor expression may alter coupling of the P2Y receptors to, for example, different G proteins altering signalling pathways though this is hard to establish without the means of receptor density detection or control of receptor number. 1321N1 cells also may not possess the signalling proteins necessary for P2Y receptors to couple to tyrosine phosphorylation

increases. It is also possible because of the transformed cell state and the high level of phosphatase activity that increases in tyrosine phosphorylation are only transient. Work with pervanadate however did not uncover any receptor-mediated increases in tyrosine phosphorylation when phosphatases were inhibited and for the human $P2Y_2$ receptor found an interesting decrease. All these possibilities formed ideas for further investigation into modulation of tyrosine phosphorylated proteins by P2Y transfects. If PY20 antibody was not able to detect small increases in tyrosine phosphorylation, would phosphorylation of a single protein be easier to detect? It was partly this hypothesis that lead to work on mitogen activated protein kinases which will be discussed in the next chapter.

Chapter 4

Regulation of p42/p44 Mitogen Activated Protein Kinases by P2Y receptors in 1321N1 human astrocytoma cells transfected with P2Y receptors

4.1 Introduction

In the previous chapter, general tyrosine phosphorylation modulation was investigated in P2Y transfected 1321N1 cells. In this chapter specific tyrosine phosphorylated mitogen activated protein kinases (MAPK's) also termed extracellular signal-regulated kinases (ERK's), are investigated. ERK1 and ERK2, known as $p44^{mapk}$ and $p42^{mapk}$ respectively, have a central critical role to play in cell proliferative signalling events as inhibition of p42/p44 MAPK can prevent cell proliferation (Pages et al., 1993) and constitutive activation can lead to tumorigenesis (Mansour et al., 1994). The pathway by which p42/p44 MAPK is activated may involve a number of different proteins including Pyk2, Grb2, mSOS, Shc, Ras, Raf, MEK and PKC (See section 1.8.5). In native cells MAP kinases are known to be activated by extracellular sources of ATP and UTP (Neary et al., 1994; Huwiler & Pfeilschifter, 1994; Soltoff et al., 1998). Transfected systems allow the isolation of a single P2Y subtype enabling the link between P2Y receptors and activation of these MAP kinases to be more adequately addressed. The aims of this thesis chapter were to determine if transfected P2Y receptors coupled to p42/p44 MAPK in 1321N1 cells, what mechanisms were involved in signal transduction from P2Y receptor to p42/p44 MAPK and whether modulation of mitogenesis shown in transfected 1321N1 cells could be observed.

4.2 Effect of P2Y agonists on p42/p44 MAPK activation in transfected 1321N1 cells

4.2.1 p42/p44 MAPK activity: Effect of P2Y agonists on turkey $P2Y_1$ transfected 1321N1 cells

1321N1 cells, transfected with the turkey $P2Y_1$ receptor, were stimulated for 5 minutes with increasing concentrations of 2MeSATP and UTP and extracts assayed using the peptide kinase assay (Figure 4.1A). An increasing dose of 2MeSATP shows a clear rise in phosphorylation of the nonapeptide substrate indicating an increase in kinase activity. The dose dependant rise reaches a plateau at 1μM 2MeSATP ($p=<0.05$, one way anova with dunnet's post-test vs. control), with a $logEC_{50}$ value of -7.7 ± 0.16, and an approximate 3 fold over basal response. UTP does not show an increase above basal levels of kinase activity.

Figure 4.1B shows a representative western blot of samples taken from 1321N1 cells transfected with turkey $P2Y_1$ cells stimulated with increasing concentrations of 2MeSATP for 5 minutes and probed with a primary antibody for the phosphorylated forms of $p42^{mapk}$ and $p44^{mapk}$. The blot shows, over lanes equally loaded with protein, a rise in immunoreactivity for both forms of MAPK, the p42 band being much more heavily phosphorylated. No increased immunoreactivity was observed on western blot with a similar experiment using UTP (data not shown).

These two methods lead to the same conclusion: that 2MeSATP, an agonist acting at $P2Y_1$ receptors, activates MAPK in a dose dependant manner and that UTP, ineffective at $P2Y_1$ receptors, does not. It also shows that p42 is preferentially activated by turkey $P2Y_1$ receptors transfected into 1321N1 cells.

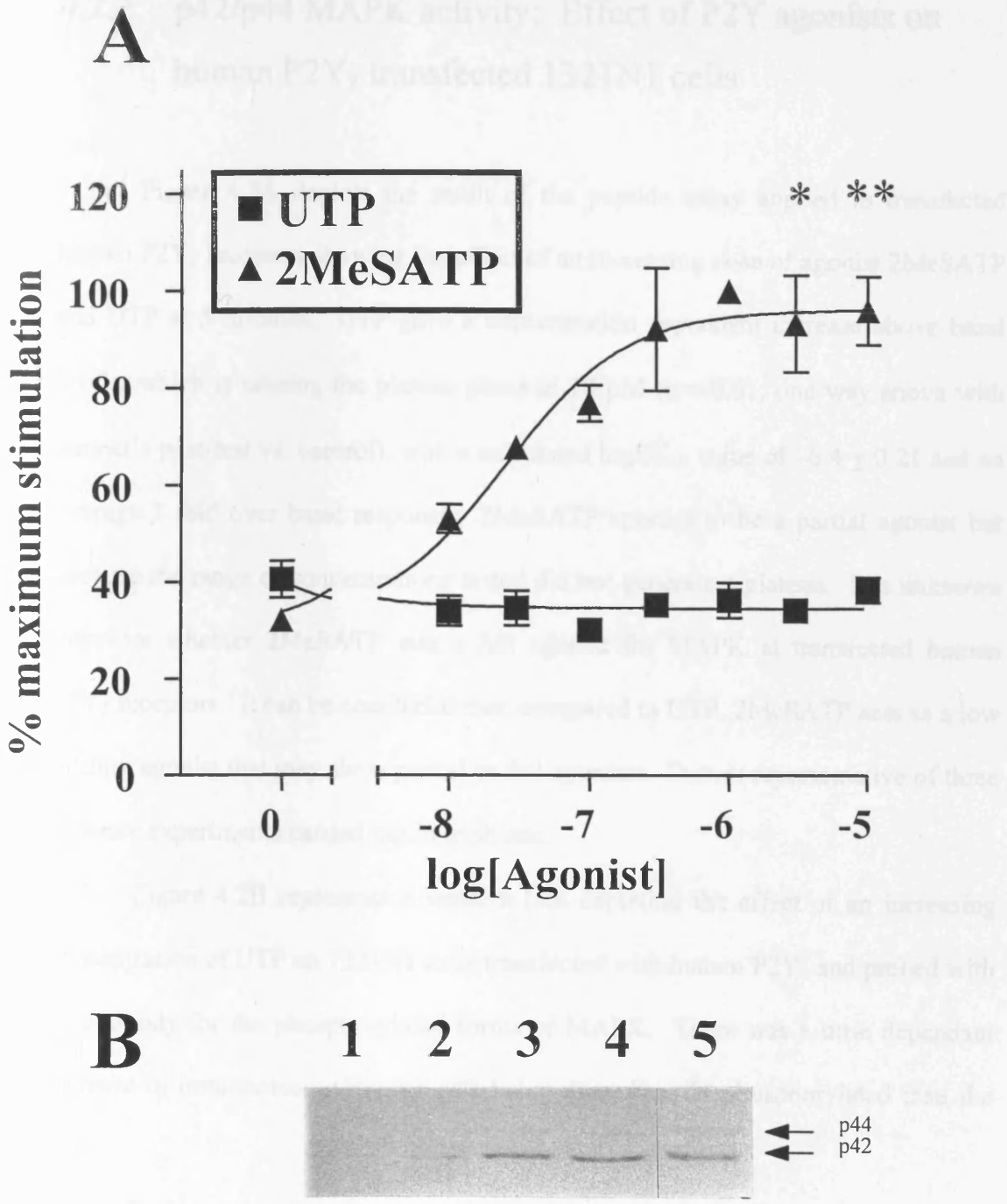


Figure 4.1

Effect of increasing dose of P2Y agonists for five minutes on MAPK phosphorylation in 1321N1 cells transfected with turkey $P2Y_1$ receptors using peptide phosphorylation assay n=3 (A) and effect of increasing dose of purinergic agonists on MAPK phosphorylation in 1321N1 cells transfected with turkey $P2Y_1$ receptors using western blotting with a phospho-specific MAPK antibody n=3 (B) Lanes 1 - 5 represent 0, 30nM, 100nM, 1μM, 3μM 2MeSATP, respectively. * = $p<0.05$ **=$p<0.01$ compared to control (dunnet's post-test).

4.2.2 p42/p44 MAPK activity: Effect of P2Y agonists on human $P2Y_2$ transfected 1321N1 cells

Figure 4.2A depicts the result of the peptide assay applied to transfected human $P2Y_2$ receptors showing the effect of an increasing dose of agonist 2MeSATP and UTP at 5 minutes. UTP gave a concentration dependent increase above basal levels, which is nearing the plateau phase at 10 μM ($p=<0.01$, one way anova with dunnet's post-test vs. control), with a calculated $logEC_{50}$ value of -6.4 ± 0.21 and an average 3 fold over basal response. 2MeSATP appears to be a partial agonist but because the range of concentrations tested did not generate a plateau. It is unknown therefore whether 2MeSATP was a full agonist for MAPK at transfected human $P2Y_2$ receptors. It can be concluded that, compared to UTP, 2MeSATP acts as a low affinity agonist that may show partial or full agonism. Data is representative of three separate experiments carried out in triplicate.

Figure 4.2B represents a western blot depicting the effect of an increasing concentration of UTP on 1321N1 cells transfected with human $P2Y_2$ and probed with an antibody for the phosphorylated forms of MAPK. There was a dose dependant increase in immunoreactivity, the p42 being more heavily phosphorylated than the p44.

Both western blotting and the peptide assay show that activation of the human $P2Y_2$ receptors transfected into 1321N1 cells leads to increased phosphorylation and activation of MAPK.

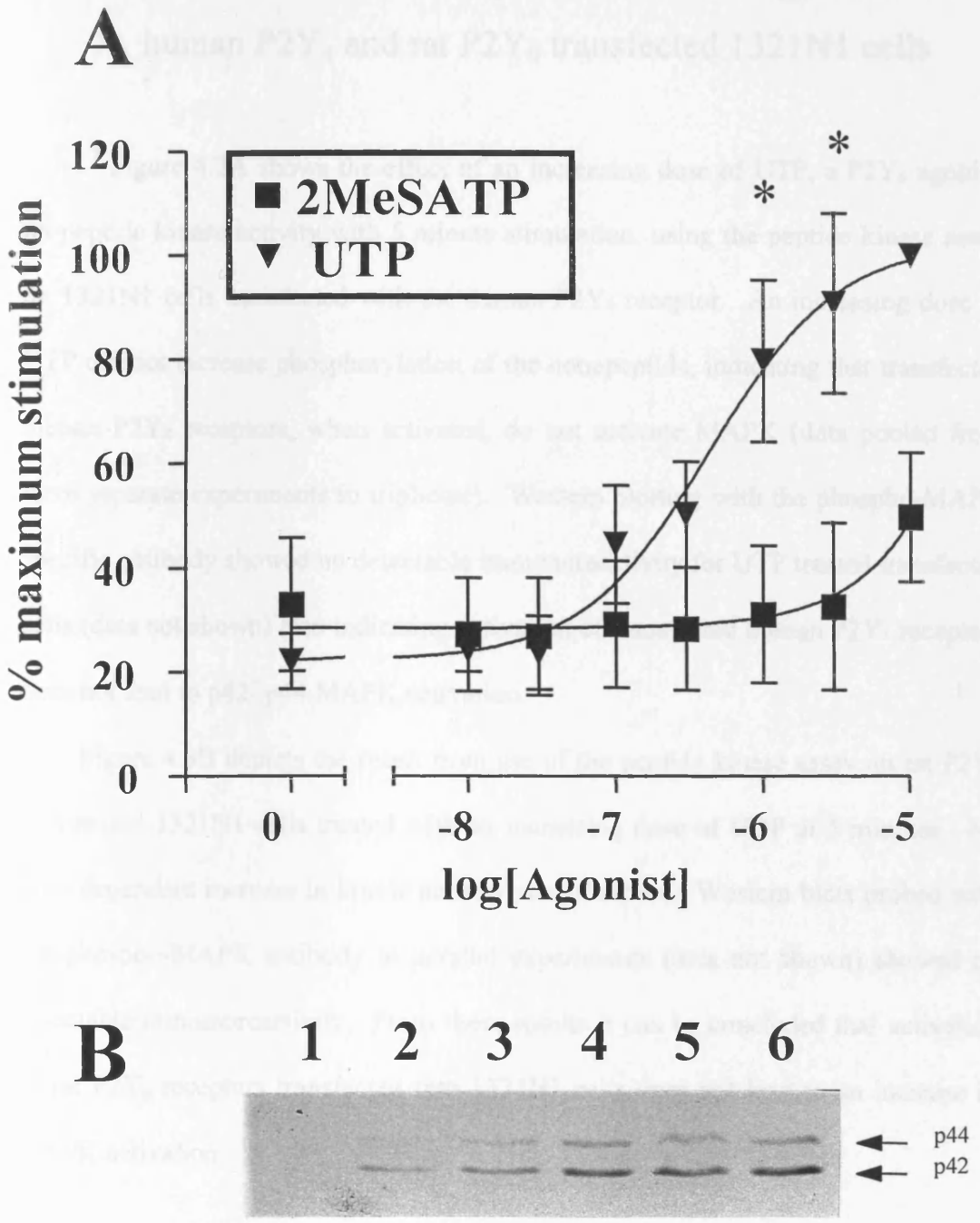


Figure 4.2

Effect of increasing dose of P2Y agonists for five minutes on MAPK phosphorylation in 1321N1 cells transfected with human $P2Y_2$ receptors using peptide phosphorylation assay n=3 (A) and the effect of increasing dose of purinergic agonists on MAPK phosphorylation in 1321N1 cells transfected with human $P2Y_2$ receptors using western blotting with a phospho-specific MAPK antibody n=3 (B) Lanes 1 - 6 represent 0, 30nM, 300nM, 3μM, 100μM, 300μM UTP, respectively. * = $p<0.05$ compared to control (dunnet's post-test).

4.2.3 p42/p44 MAPK activity: Effect of P2Y agonists on human $P2Y_4$ and rat $P2Y_6$ transfected 1321N1 cells

Figure 4.3A shows the effect of an increasing dose of UTP, a $P2Y_4$ agonist, on peptide kinase activity with 5 minute stimulation, using the peptide kinase assay on 1321N1 cells transfected with the human $P2Y_4$ receptor. An increasing dose of UTP did not increase phosphorylation of the nonapeptide, indicating that transfected human $P2Y_4$ receptors, when activated, do not activate MAPK (data pooled from three separate experiments in triplicate). Western blotting with the phospho-MAPK specific antibody showed no detectable immunoreactivity for UTP treated transfected cells (data not shown) also indicating activation of transfected human $P2Y_4$ receptors does not lead to p42/ p44 MAPK activation.

Figure 4.3B depicts the result from use of the peptide kinase assay on rat $P2Y_6$ transfected 1321N1 cells treated with an increasing dose of UDP at 5 minutes. No dose dependant increase in kinase activity was observed. Western blots probed with the phospho-MAPK antibody in parallel experiments (data not shown) showed no detectable immunoreactivity. From these results it can be concluded that activation of rat $P2Y_6$ receptors transfected into 1321N1 cells does not lead to an increase in MAPK activation.

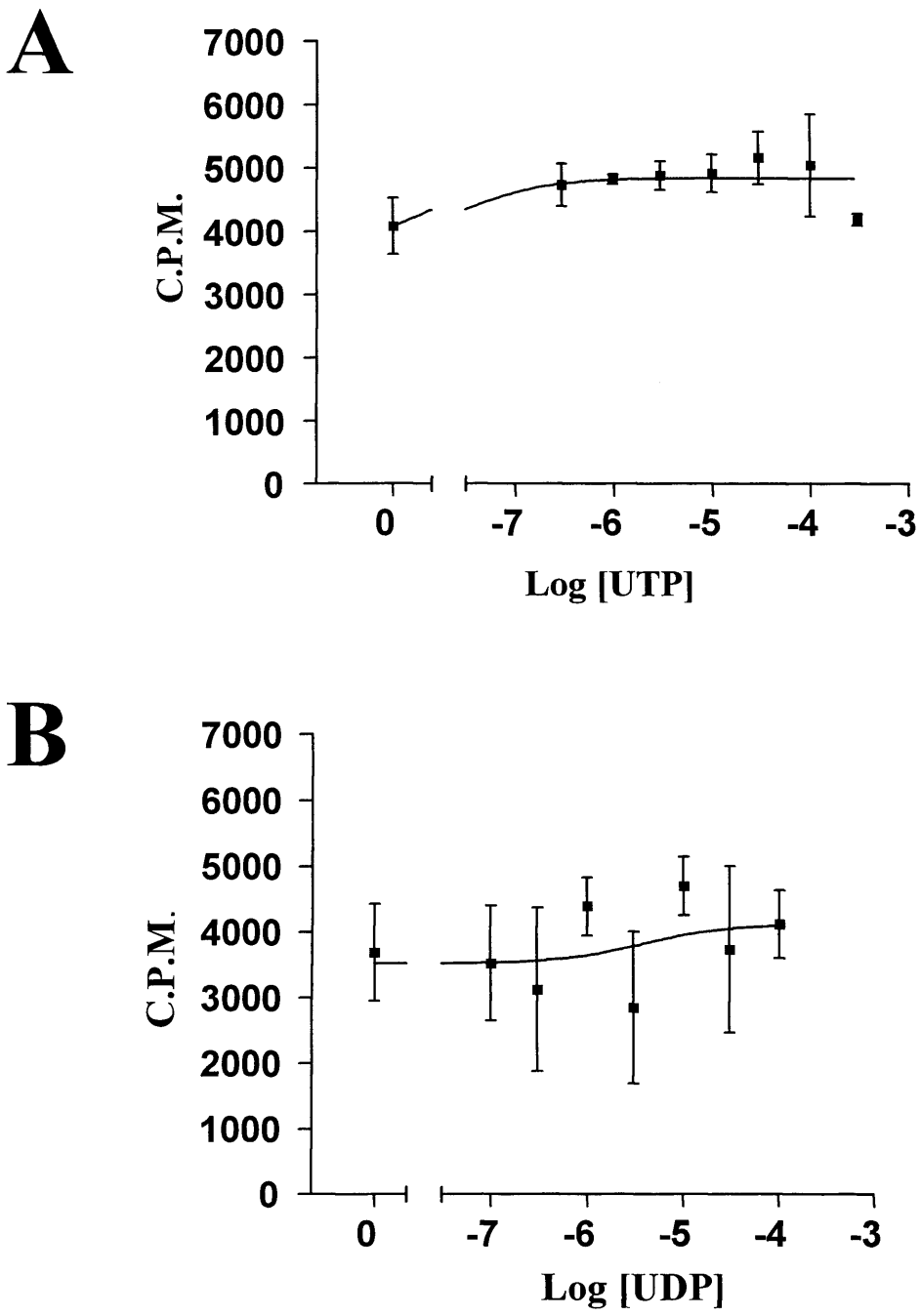


Figure 4.3

Effect of increasing dose of P2Y agonists for five minutes on MAPK phosphorylation in 1321N1 cells transfected with human $P2Y_4$ receptors using peptide phosphorylation assay n=3 (A) Effect of increasing dose of purinergic agonists on MAPK phosphorylation in 1321N1 cells transfected with rat $P2Y_6$ receptors using peptide phosphorylation assay n=3 (B)

4.3 Time course of effect of P2Y agonists on p42/p44 MAPK activation in P2Y transfected 1321N1 cells

4.3.1 Time course of effect of P2Y agonists on turkey $P2Y_1$ transfected cells

1321N1 cells transfected with turkey $P2Y_1$ were stimulated with 30 μM 2MeSATP at various time points and the effect on p42/p44 MAPK activation was observed using the peptide kinase assay (Figure 4.4). As can be seen from the graph, MAPK was activated on receptor stimulation rising significantly at 5 minutes ($p=<0.05$, dunnet's post-test vs. control). There was an apparent drop at 6 minutes which is insignificant due to error. This is followed by a continuing rise at 10 minutes after which activity dropped off slowly with time, though basal levels were not reached after 20 minutes. The overall trend was a rise to a peak at ten minutes though there is no significant difference between 5 minutes and 10 minutes

4.3.2 Time course of effect of P2Y agonists on human $P2Y_2$ transfected cells

Figure 4.5 depicts the effect of 100μM UTP with time on MAPK phosphorylation in 1321N1 cells transfected with human $P2Y_2$ receptors using the peptide phosphorylation assay. No stimulation is seen until after 2 minutes when there is a rapid rise to a peak at 5-6 minutes ($p=<0.05$, dunnet's post-test vs. control). After this point there is a slow decline with basal levels not being reached at 20 minutes.

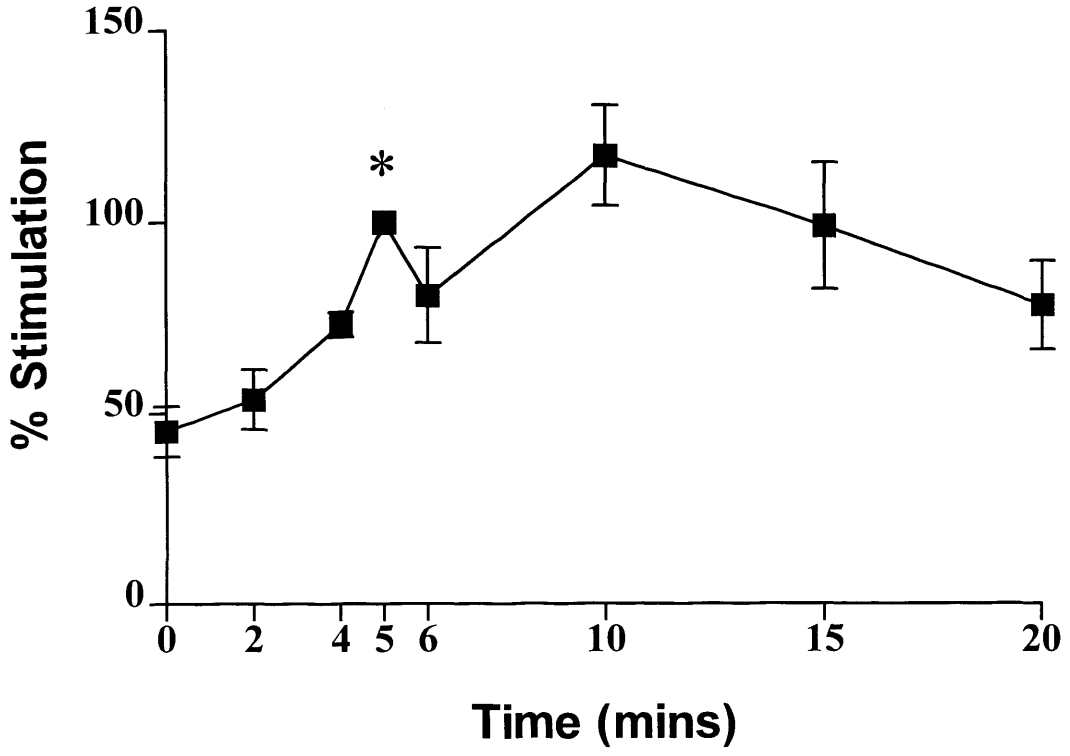


Figure 4.4
Effect of 30μM 2MeSATP with time on MAPK phosphorylation in 1321N1 cells transfected with turkey $P2Y_1$ receptors using peptide phosphorylation assay (n=4). Normalised to 100% at 5 minutes. * = $p<0.05$ compared to control (bonferroni's post-test).

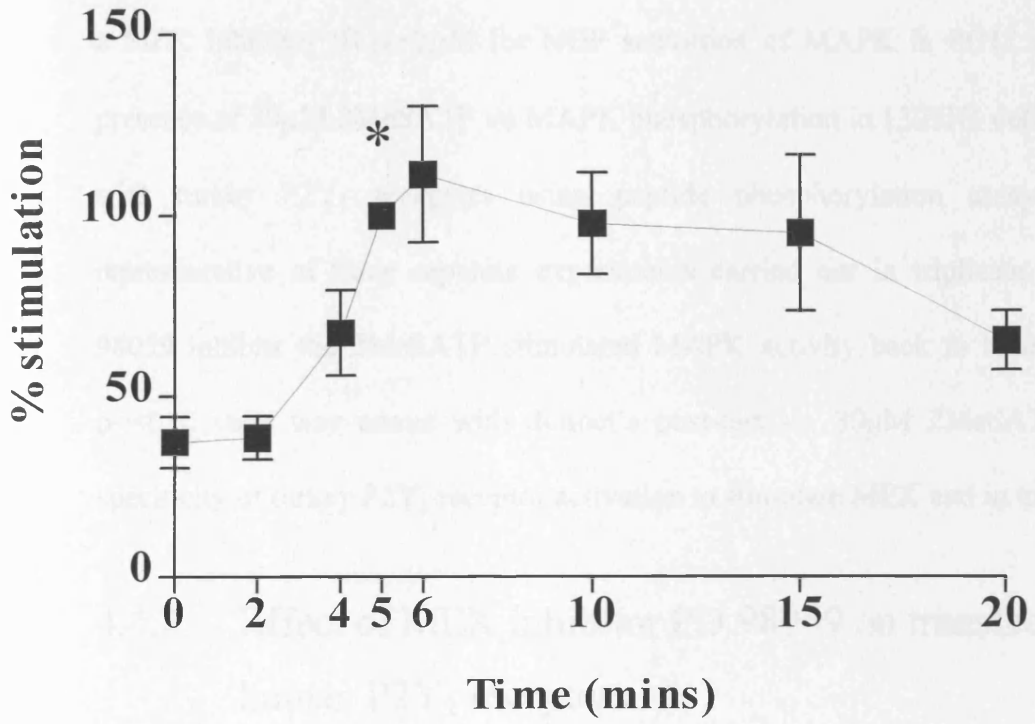


Figure 4.5
Effect of 100μM UTP with time on MAPK phosphorylation in 1321N1 cells transfected with human $P2Y_2$ receptors using peptide phosphorylation assay (n=4). Normalised to 100% at 5 minutes. * = $p<0.05$ compared to control (bonferroni's post-test).

4.4 Effect of the MEK inhibitor PD 98059 on P2Y receptor activated p42/p44 MAPK

4.4.1 Effect of MEK inhibitor PD 98059 on transfected turkey $P2Y_1$ receptors

Figure 4.6 represents the effect of an increasing dose of compound PD 98059, a MEK inhibitor (IC_{50}=2μM for NGF activation of MAPK in PC12 cells), in the presence of 30μM 2MeSATP on MAPK phosphorylation in 1321N1 cells transfected with turkey $P2Y_1$ receptors using peptide phosphorylation assay. Data is representative of three separate experiments carried out in triplicate. 10μM PD 98059 inhibits the 2MeSATP stimulated MAPK activity back to basal levels (**, p=<0.01, one way anova with dunnet's post-test vs. 30μM 2MeSATP) showing specificity of turkey $P2Y_1$ receptor activation to stimulate MEK and in turn MAPK.

4.4.2 Effect of MEK inhibitor PD 98059 on transfected human $P2Y_2$ receptors

The effect of a dose increase of PD 98059 with 100μM UTP on MAPK phosphorylation in 1321N1 cells transfected with human $P2Y_2$ receptors using the peptide phosphorylation assay was also investigated (Figure 4.7). 10μM PD 98059 inhibited peptide phosphorylation due to UTP activated $P2Y_2$ receptors (p=<0.05, one way anova with dunnet's post-test vs. 100μM UTP). This result shows the requirement for MEK activation when UTP activates MAPK. Data is representative of three experiments carried out in triplicate ± S.E.M.

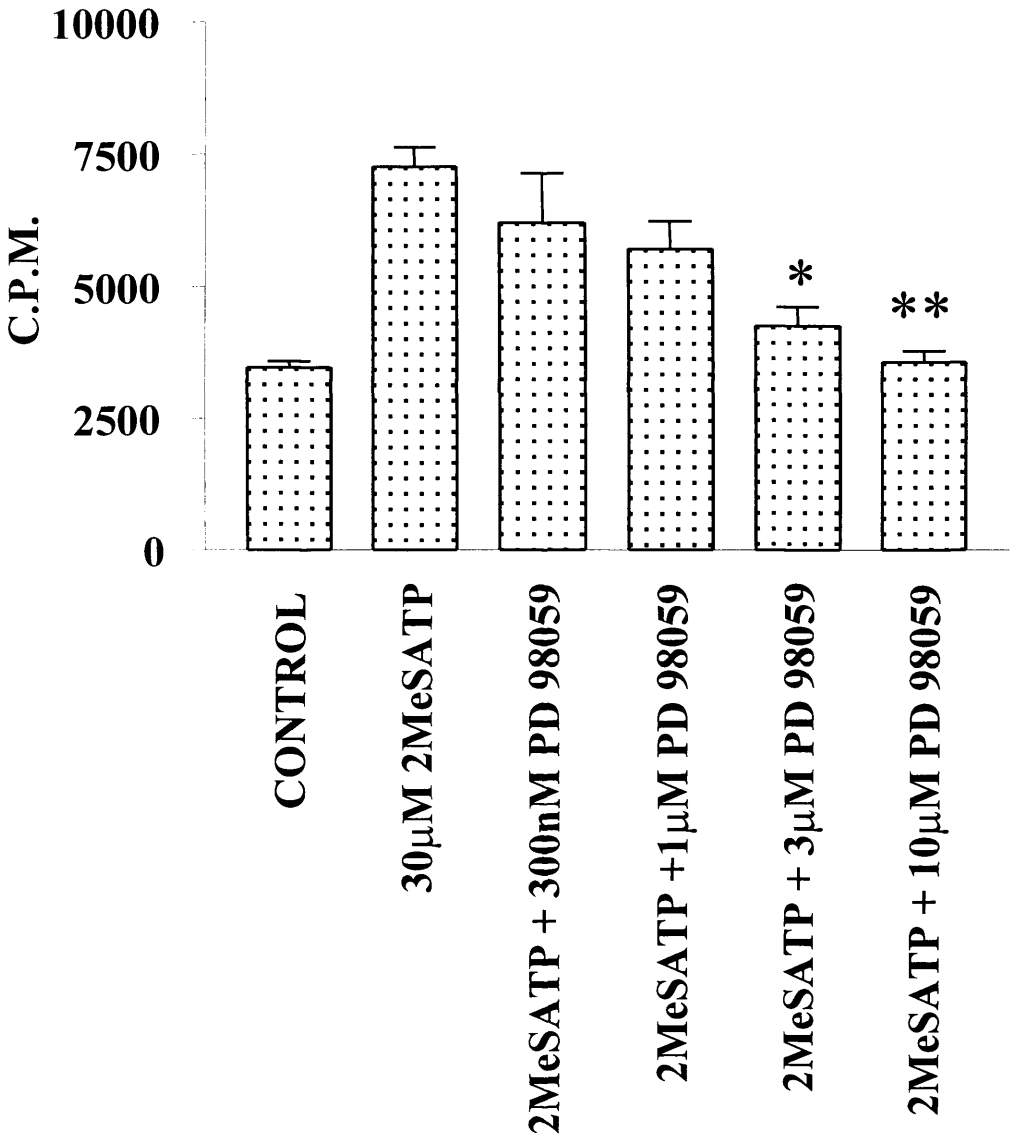


Figure 4.6

Effect of PD 98059 with 30μM 2MeSATP on MAPK phosphorylation in 1321N1 cells transfected with turkey $P2Y_1$ receptors using peptide phosphorylation assay (n=3).

* = $p<0.05$ ** = $p<0.01$ compared to 30μM 2MeSATP (dunnet's post-test).

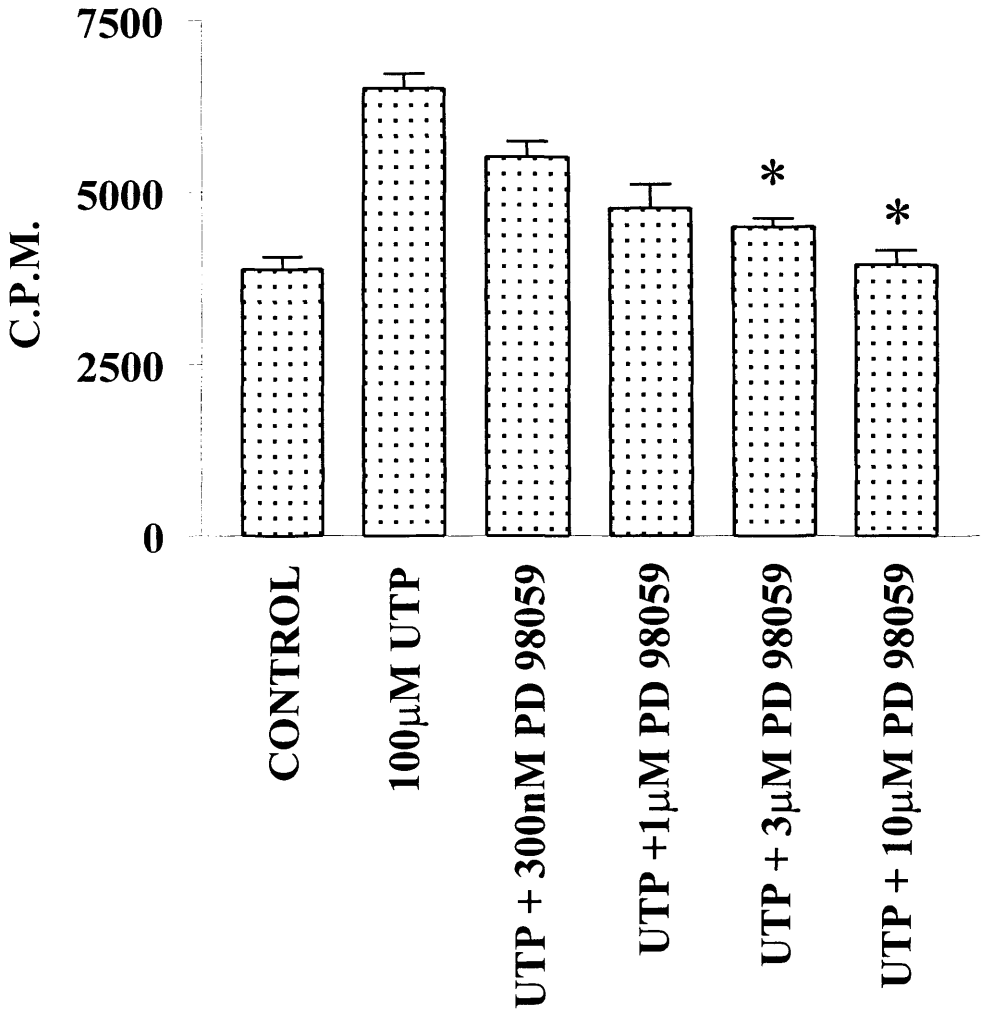


Figure 4.7

Effect of PD 98059 with 100μM UTP on MAPK phosphorylation in 1321N1 cells transfected with human $P2Y_2$ receptors using peptide phosphorylation assay (n=3)

* = $p<0.05$ compared to 30μM 2MeSATP (dunnet's post-test).

4.5 Effect of pervanadate on p42/p44 MAPK phosphorylation by transfected P2Y receptors

4.5.1 Effect of pervanadate on p42/p44 MAPK activation by transfected turkey $P2Y_1$ receptors

Figure 4.8 depicts the effect of 250μM pervanadate in the presence and absence of 30μM 2MeSATP at 5 minutes on p42/p44 MAPK phosphorylation in 1321N1 cells transfected with turkey $P2Y_1$ receptors. Detection of p42/p44 MAPK phosphorylation utilised western blots probed with the phospho-MAPK antibody. Data was collected by scanning the autoradiographs produced using a densitometer, and resultant data graphed. The results show that both 30μM 2MeSATP and 250μM pervanadate alone achieve an increase in MAPK phosphorylation but together show no additive effect or significant decrease compared with pervanadate or 2MeSATP effects alone. Experiments were completed in triplicate and data pooled from three separate experiments and normalised to 250μM pervanadate.

4.5.2 Effect of pervanadate on p42/p44 MAPK activation by transfected human $P2Y_2$ receptors

The effect of pervanadate in the presence of 100μM UTP for 5 minutes on MAPK phosphorylation in 1321N1 cells transfected with human $P2Y_2$ receptors is shown in Figure 4.9. Using phospho-MAPK antibody as a probe, western blots detected p42/p44 MAPK phosphorylation. Scanned densitometer readings show

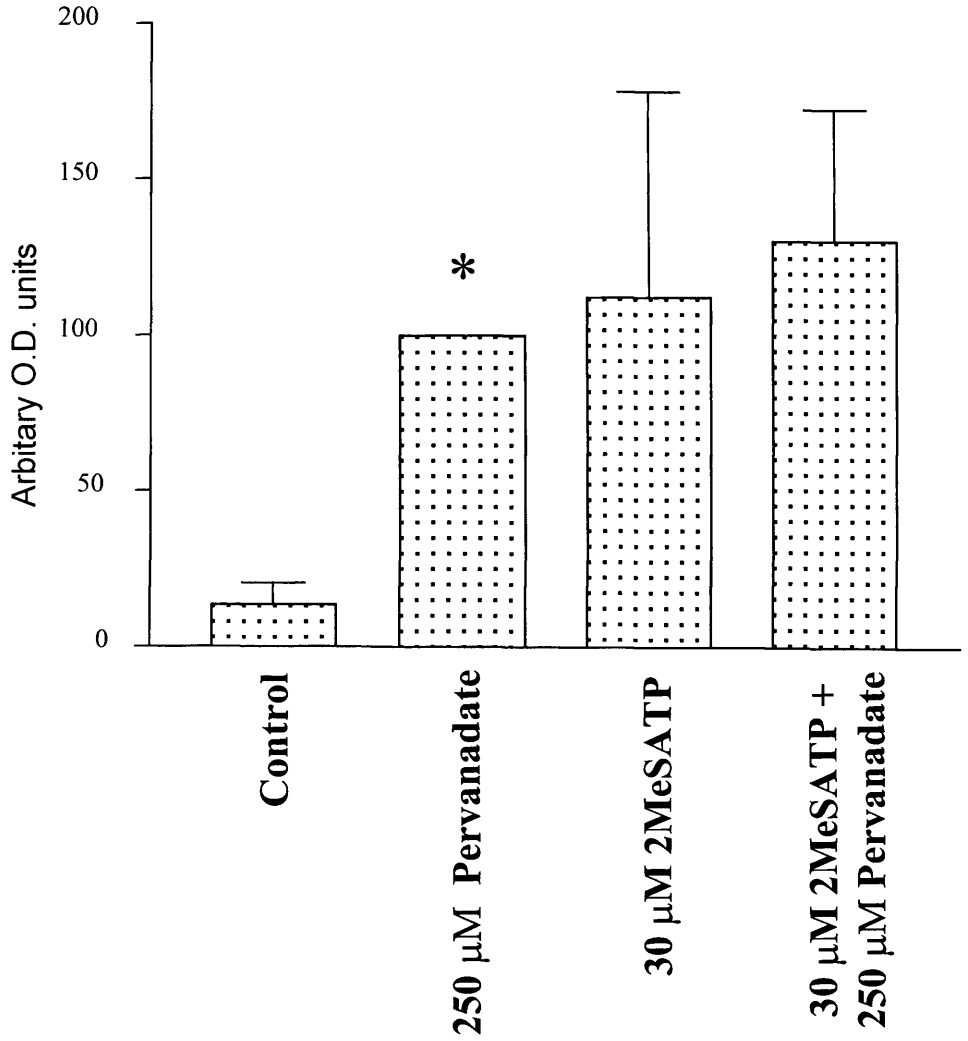


Figure 4.8

Effect of pervanadate with 30μM 2MeSATP for five minutes on MAPK phosphorylation in 1321N1 cells transfected with turkey $P2Y_1$ receptors using densitometry analysis of western blots (n=3) * = $p<0.05$ compared to control (bonferroni's post-test).

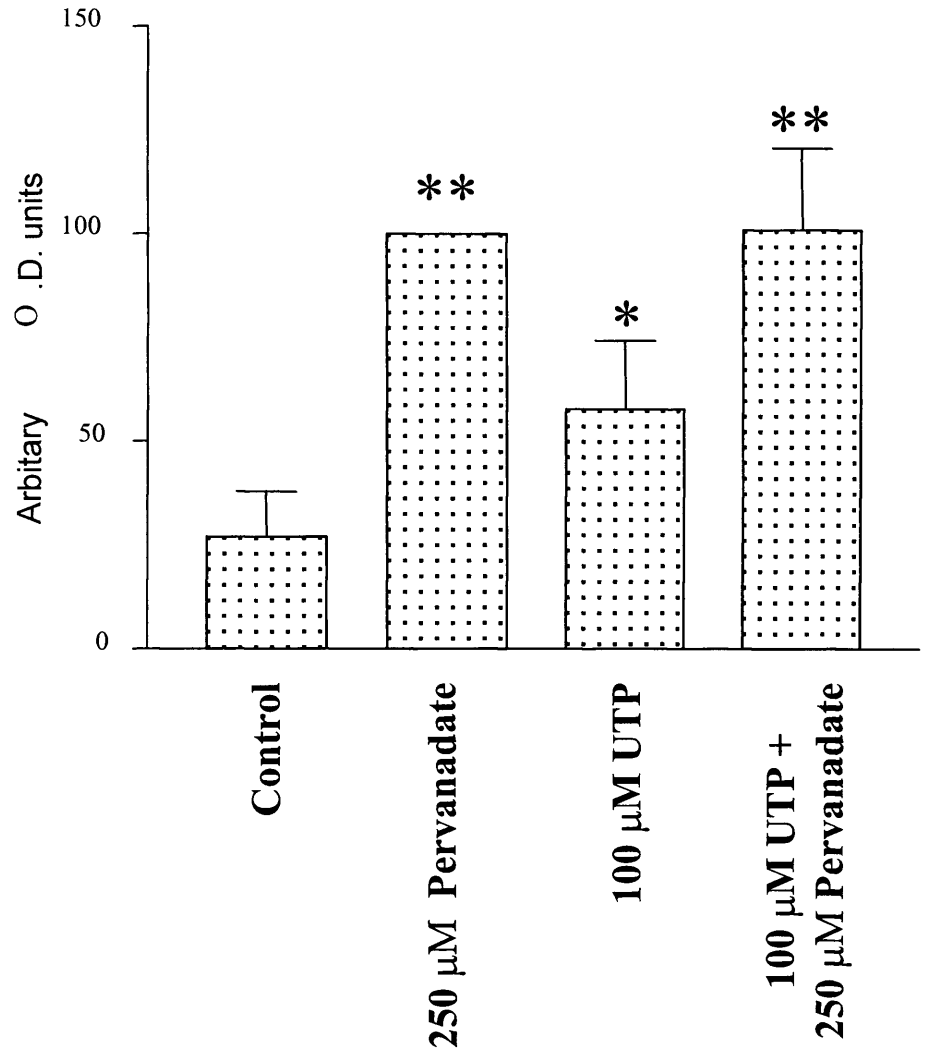


Figure 4.9

Effect of pervanadate with 100μM UTP for five minutes on MAPK phosphorylation in 1321N1 cells transfected with human $P2Y_2$ receptors using densitometry analysis of western blots (n=3) * = $p<0.05$ ** = $p<0.01$ compared to control (bonferroni's post-test).

both 250μM pervanadate and 100μM UTP gave increases above control levels with pervanadate being larger than UTP alone ($p=<0.01$; $p=<0.05$, respectively, one way anova with dunnet's post-test vs. control). When cells were stimulated in the presence of both pervanadate and UTP there was no additive effect and levels equated to pervanadate alone. Experiments were completed in triplicate and data representative of three separate experiments and normalised to 250μM pervanadate.

4.6 Study of PKC isoforms present in 1321N1 cells

Western blotting showing presence of various PKC isoforms in 1321N1 cells is presented in Figure 4.10. Antibodies for PKC isoforms α, β, ε, θ, ζ and λ were used to detect the various isoforms in 1321N1 cell extracts. Western blotting showed the presence of isoforms α (Ca^{++} sensitive), ε and λ (Ca^{++} insensitive isoforms) with no β, ζ or θ being detected.

4.7 p42/p44 MAPK activity: Effect of PKC inhibitors and TPA on transfected 1321N1 cells

4.7.1 Effect of Ro 31-8220 and TPA on p42/p44 MAPK activation by transfected turkey $P2Y_1$ receptors

The effect of Ro 31-8220 and TPA with 30μM 2MeSATP on MAPK phosphorylation in 1321N1 cells transfected with turkey $P2Y_1$ receptors using the peptide phosphorylation assay is shown in figure 4.11. 30μM 2MeSATP showed an increase in MAPK activity (†, $p=<0.05$ bonferroni's post-test vs. control) as seen

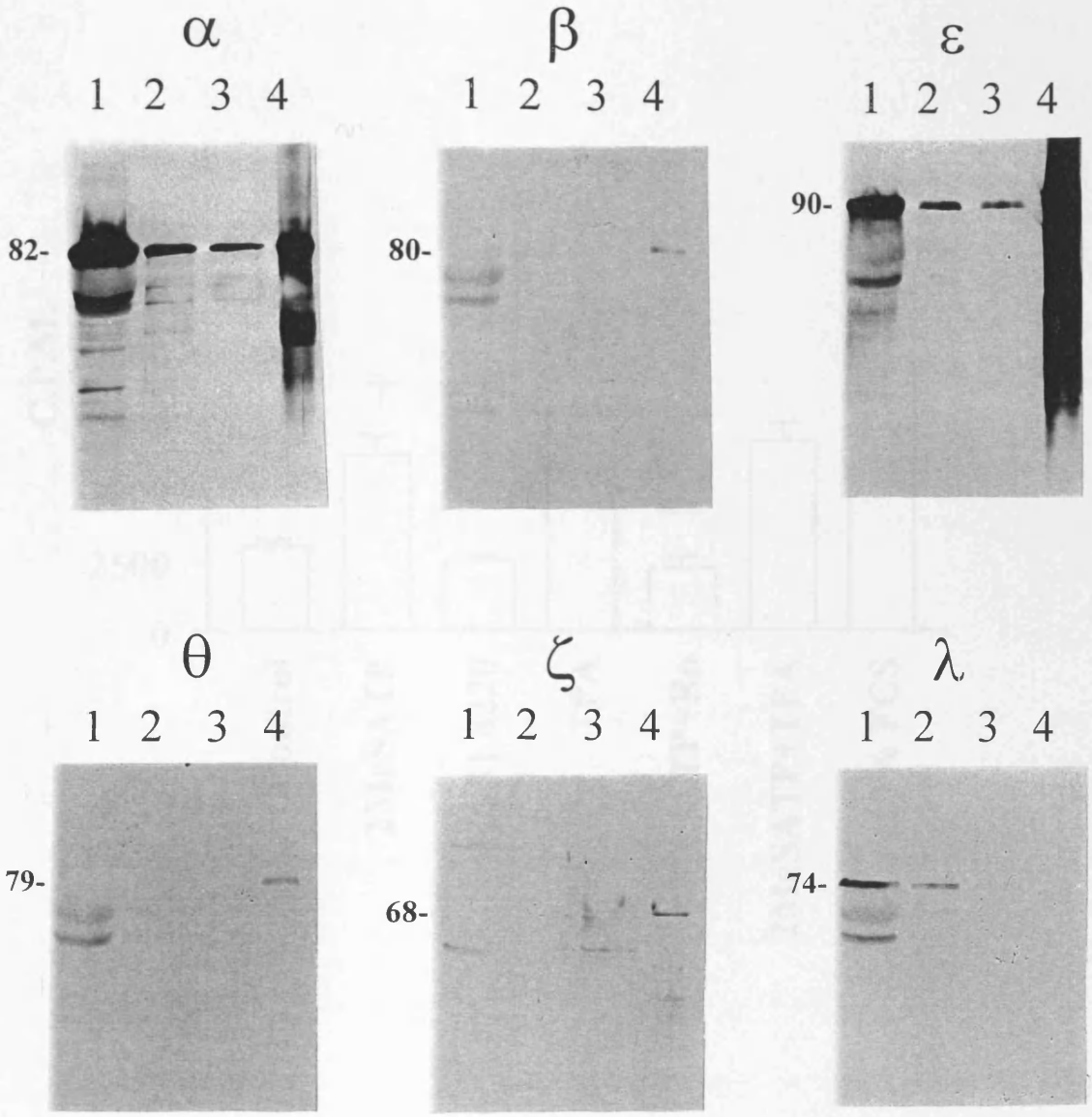


Figure 4.10

Western blotting showing presence of various PKC isoforms in 1321N1 cells.

Lanes1-4 represent wild type 1321N1 cell extract, transfected turkey $P2Y_1$ extract, transfected human $P2Y_2$ extract and rat brain extract, respectively. Blots are representative of a single experiment

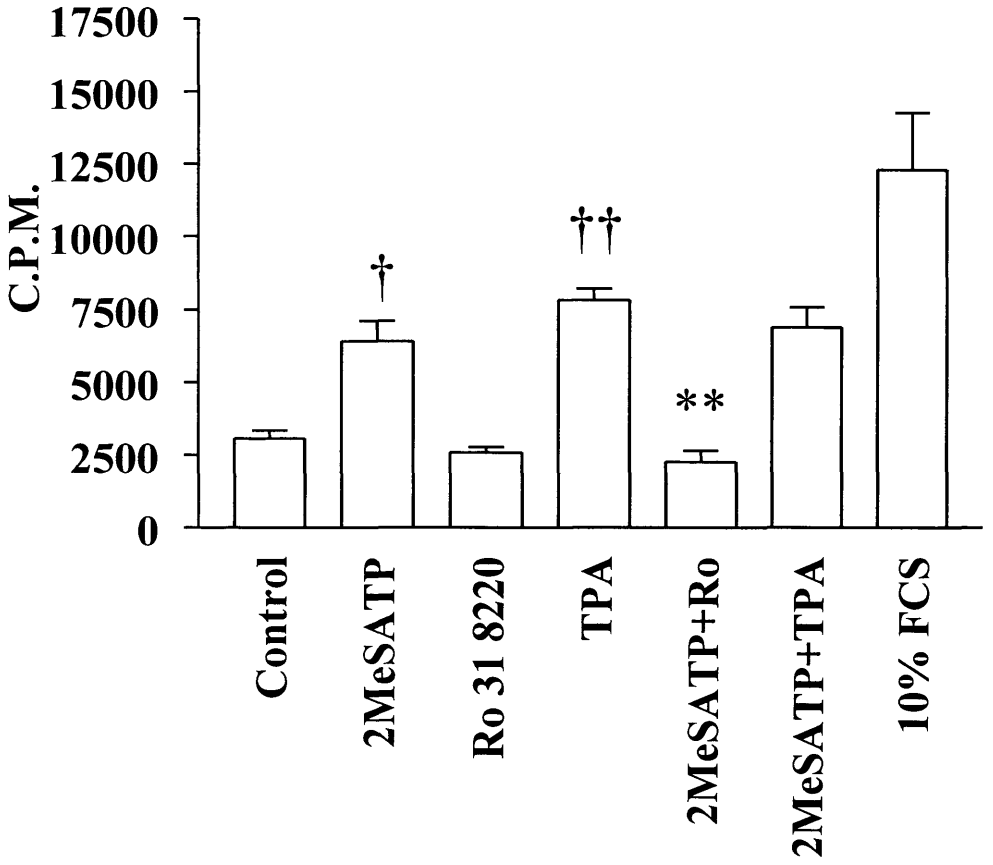


Figure 4.11

Effect of Ro 31-8220 and TPA with 30μM 2MeSATP for five minutes on MAPK phosphorylation in 1321N1 cells transfected with turkey $P2Y_1$ receptors using peptide phosphorylation assay. Data are pooled from 4 separate experiments in triplicate. †, p=<0.05 compared to control. ††, p=<0.01 compared to control. **, p=<0.01 compared to 2MeSATP

previously but on co-addition of 10μM Ro 31-8220, a general PKC inhibitor, stimulation drops back to below or near control levels (**, p=<0.01 bonferroni's post-test vs. 2MeSATP). Ro 31-8220 alone shows a slight decrease in basal levels though not significantly. TPA, an activator of PKC, shows stimulation of the MAPK pathway alone at 100nM (††, p=<0.01 bonferroni's post-test vs. control) though co-addition of 30μM 2MeSATP and 100nM TPA does not have an additive effect. 10% FCS, used as a reference stimulation, shows a further increase in MAPK activation than that seen with TPA or 2MeSATP. Data are depicted as pooled data of four separate experiments in triplicate.

4.7.2 Effect of Ro 31-8220 and TPA on MAPK activation by transfected human $P2Y_2$ receptors

Figure 4.12 depicts the effect of 10μM Ro 31-8220 and 100nM TPA with 100μM UTP on MAPK phosphorylation in 1321N1 cells transfected with human $P2Y_2$ receptors using peptide phosphorylation assay. Data are from pooled data from three separate experiments performed in triplicate. 100μM UTP gave an increase in MAPK activation as observed previously on human $P2Y_2$ cells (†, p=<0.05 bonferroni's post-test vs. control). Ro 31-8220 alone, as in the 1321N1 cells transfected with turkey $P2Y_1$, gave a slight decrease in basal levels of MAPK activity but not significantly. 100nM TPA alone gave a large increase in MAPK activation to a similar level seen with 100μM UTP alone (†††, p=<0.001 bonferroni's post-test vs. control). Addition of Ro 31-8220 together with UTP reduced levels of MAPK activity compared with UTP alone, showing an inhibitory effect of PKC inhibition on UTP activation of MAPK (**, p=<0.01, bonferroni's post-test vs. 100μM UTP).

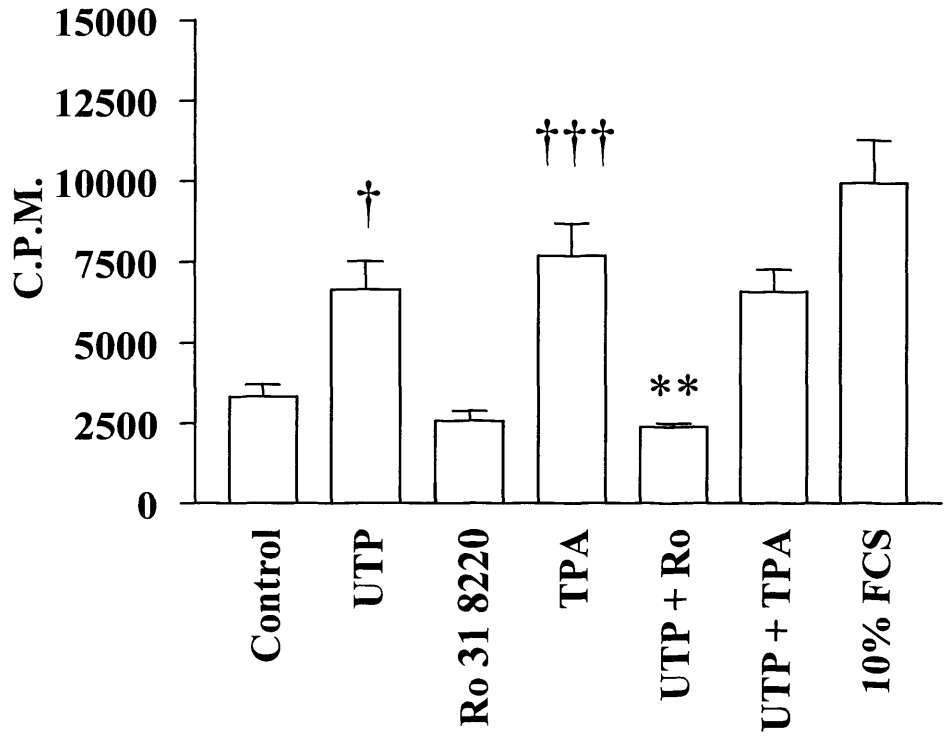


Figure 4.12

Effect of Ro 31-8220 and TPA with 100μM UTP for five minutes on MAPK phosphorylation in 1321N1 cells transfected with human $P2Y_2$ receptors using peptide phosphorylation assay. Data are pooled from 3 separate experiments in triplicate. †, p=<0.05 compared to control. †††, p=<0.001 compared to control. **, p=<0.01 compared to UTP.

UTP and TPA together gave levels equivalent to UTP levels alone. 10% FCS also gave a robust response above TPA or UTP alone.

4.7.3 Effect of Ro 31-8220 on p42/p44 MAPK activation by transfected turkey $P2Y_1$ receptors

To assess more specifically the involvement of PKC in the activation of MAPK by transfected P2Y receptors, experiments were carried out with PKC inhibitor Ro 31-8220 in a concentration dependant manner. Figure 4.13 depicts the effect of a concentration increase of Ro 31-8220 with 30μM 2MeSATP on MAPK phosphorylation in 1321N1 cells transfected with turkey $P2Y_1$ receptors using peptide phosphorylation assay. Ro 31-8220, a non-specific PKC inhibitor, effectively inhibits the activation of MAPK via agonist activation of transfected turkey $P2Y_1$ receptors with the $\log IC_{50}$ being -5.9 ± 0.24 calculated from the fitted curve. Data are pooled from three experiments each carried out in triplicate.

4.7.4 Effect of Ro 31-8220 on MAPK activation by transfected human $P2Y_2$ receptors

The effect of PKC inhibition by using Ro 31-8220 was also investigated in 1321N1 cells transfected with the human $P2Y_2$ receptor as depicted in Figure 4.14. The effect of a dose increase of Ro 31-8220 with 100μM UTP on MAPK phosphorylation in 1321N1 cells is to inhibit the UTP stimulated MAPK activation. The $\log IC_{50}$ for this inhibition was calculated as -6.5 ± 0.09. Data are pooled from three experiments each carried out in triplicate.

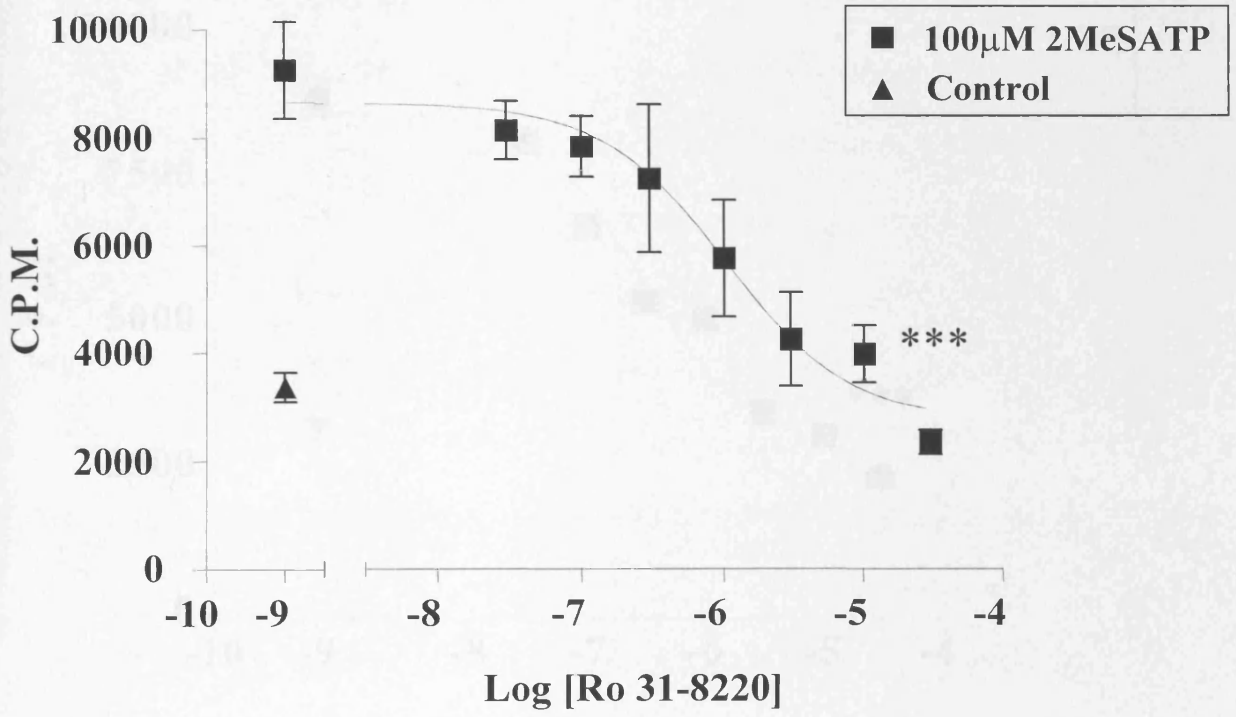


Figure 4.13

Effect of dose increase of Ro 31-8220 with 30μM 2MeSATP on MAPK phosphorylation in 1321N1 cells transfected with turkey $P2Y_1$ receptors using peptide phosphorylation assay. Pooled data *** = $p<0.001$ t-test vs. 100μM 2MeSATP (n=3).

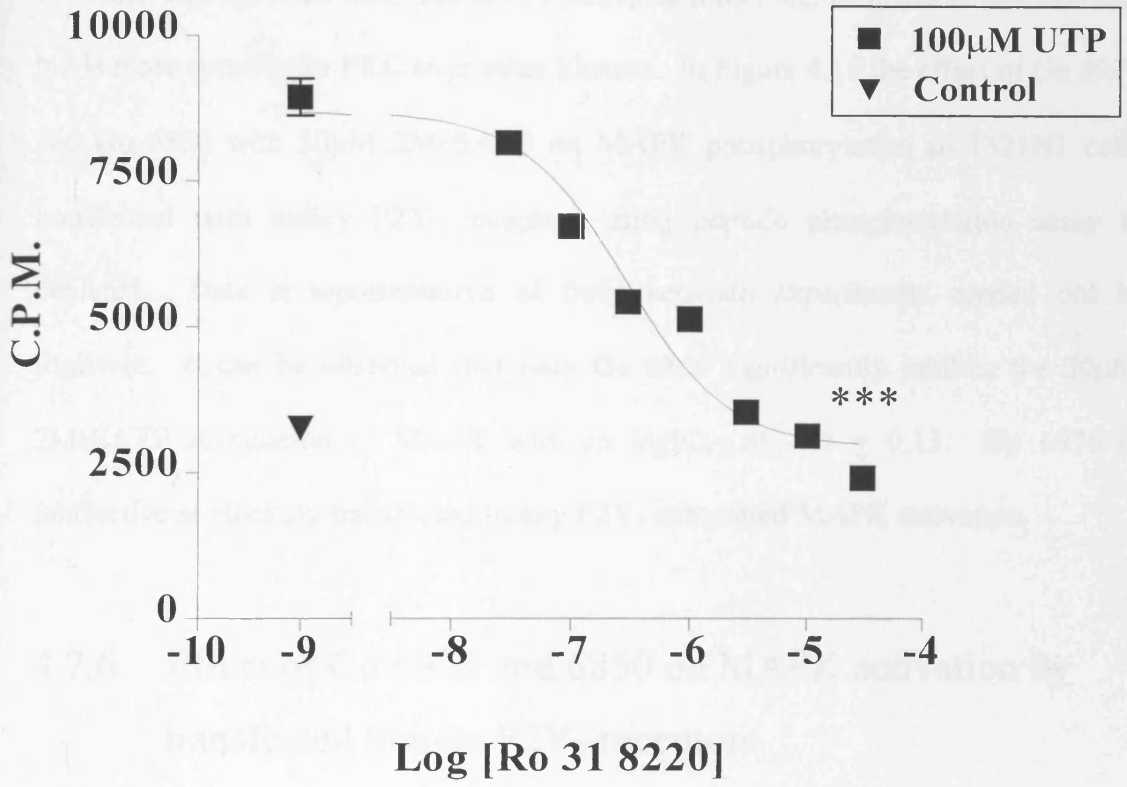


Figure 4.14
Effect of dose increase of Ro 31-8220 with 100μM UTP on MAPK phosphorylation in 1321N1 cells transfected with human $P2Y_2$ receptors using peptide phosphorylation assay. Pooled data ***, p=0.001 t-test vs. 100μM UTP (n=3).

4.7.5 Effect of Go 6976 and 6850 on MAPK activation by transfected turkey $P2Y_1$ receptors

Ro 31-8220 is a non-specific PKC inhibitor. To assess which of the PKC isoforms present in 1321N1 cells may be responsible for signalling to MAPK two other compounds were used. Go 6976 a compound that inhibits Ca^{++} sensitive PKC isoforms, and Go 6850 that, like Ro 31-8220, inhibits PKC isoforms non-selectively but is more specific for PKC over other kinases. In Figure 4.15 the effect of Go 6976 and Go 6850 with 30μM 2MeSATP on MAPK phosphorylation in 1321N1 cells transfected with turkey $P2Y_1$ receptors using peptide phosphorylation assay is depicted. Data is representative of three separate experiments carried out in triplicate. It can be observed that only Go 6850 significantly inhibits the 30μM 2MeSATP stimulation of MAPK with an $logIC_{50}$ of -7.1 $\pm$ 0.13. Go 6976 is ineffective at blocking transfected turkey $P2Y_1$ stimulated MAPK activation.

4.7.6 Effect of Go 6976 and 6850 on MAPK activation by transfected human $P2Y_2$ receptors

The transfected human $P2Y_2$ receptor was also investigated and data shown in figure 4.16 depicting the effect of Go 6976 and Go 6850 with 100μM UTP on MAPK phosphorylation in 1321N1 cells transfected with human $P2Y_2$ receptors using the peptide phosphorylation assay. Data is representative of three separate experiments carried out in triplicate. Go 6976 is ineffective on 100μM UTP stimulated MAPK activation in 1321N1 transfected with human $P2Y_2$ receptors.

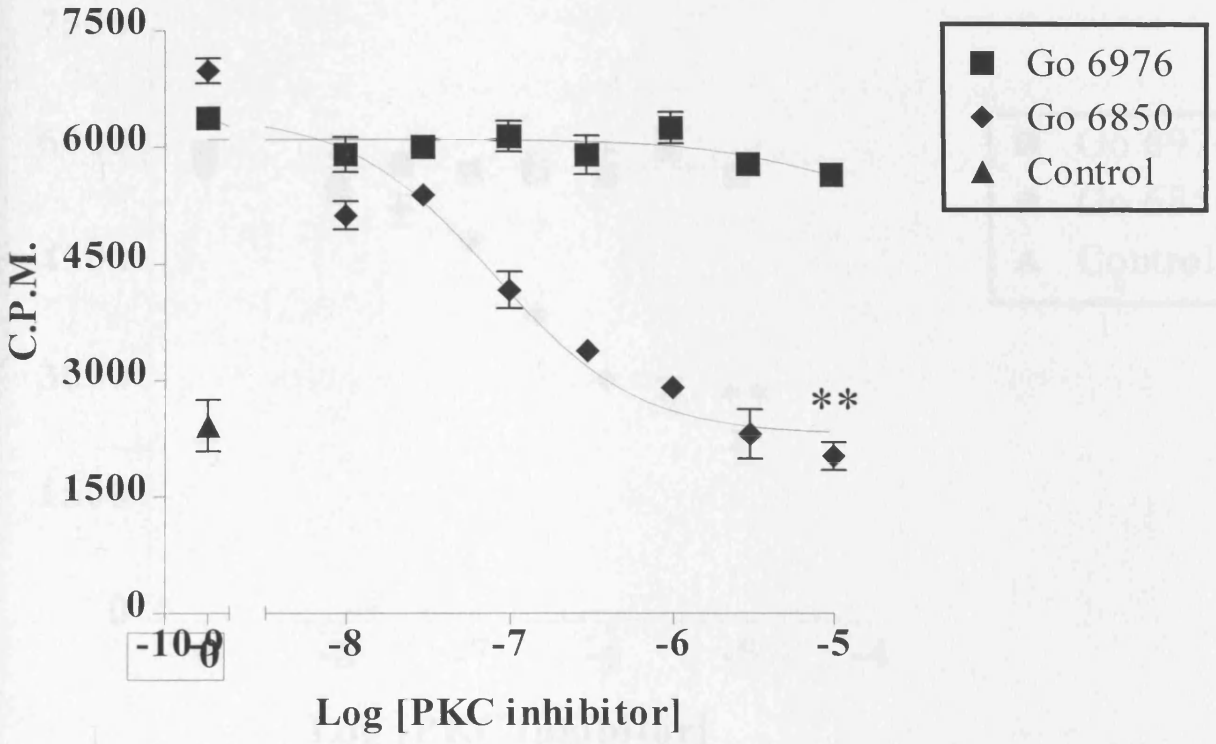


Figure 4.15

Effect of Go 6976 and Go 6850 with 30μM 2MeSATP on MAPK phosphorylation in 1321N1 cells transfected with turkey $P2Y_1$ receptors using peptide phosphorylation assay. **, p=0.01 t-test vs. 30μM 2MeSATP (n=3).

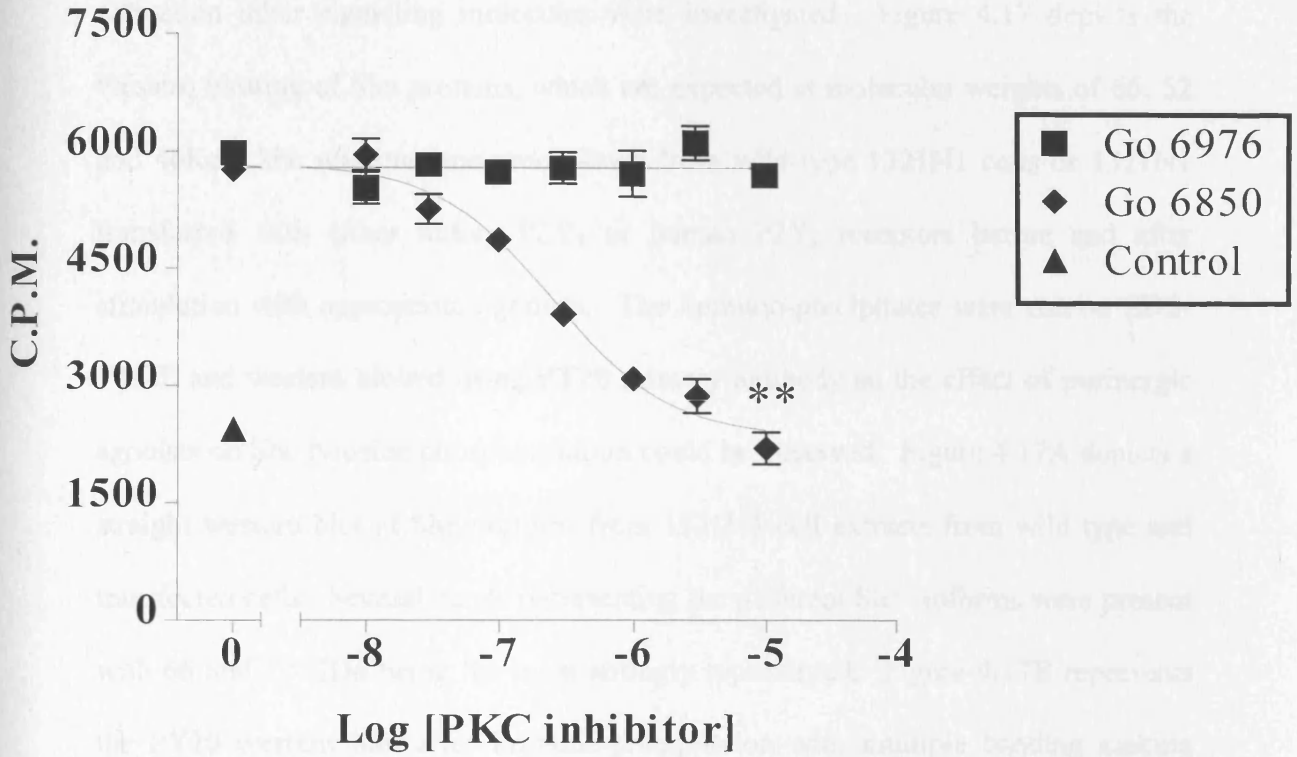


Figure 4.16
Effect of Go 6976 and Go 6850 with 100μM UTP on MAPK phosphorylation in 1321N1 cells transfected with human $P2Y_2$ receptors using peptide phosphorylation assay **, p=0.01 t-test vs. 30μM 2MeSATP (n=3).

Go 6850 inhibits UTP activated $P2Y_2$ stimulated MAPK activation with an $logIC_{50}$ of -6.6 ± 0.09.

4.8 P2Y receptor signal transduction to Shc protein

To investigate further the pathway between P2Y receptor activation and MAPK activation other signalling molecules were investigated. Figure 4.17 depicts the western blotting of Shc proteins, which are expected at molecular weights of 66, 52 and 46Kd. Shc was immuno-precipitated from wild type 1321N1 cells or 1321N1 transfected with either turkey $P2Y_1$ or human $P2Y_2$ receptors before and after stimulation with appropriate agonists. The immuno-precipitates were run on SDS-PAGE and western blotted using PY20 primary antibody so the effect of purinergic agonists on Shc tyrosine phosphorylation could be observed. Figure 4.17A depicts a straight western blot of Shc proteins from 1321N1 cell extracts from wild type and transfected cells. Several bands representing the different Shc isoforms were present with 66 and 52 KDa being the most strongly represented. Figure 4.17B represents the PY20 western blot after immuno-precipitation with multiple banding making analysis difficult. The major band observed is likely to be IgG as an artifact from the immuno-precipitation. Other banding observed at the 66Kda and approximate to 52Kda do not show an increase in tyrosine phosphorylation with stimulation. 10% FCS, used as a positive control, seems to decrease tyrosine phosphorylation of Shc, which is unexpected. This only represents two attempts to observe Shc phosphorylation and experimental procedure may need to be refined to lower IgG, e.g. by pre-clearing with an anti IgG antibody, and investigate Shc tyrosine phosphorylation more closely.

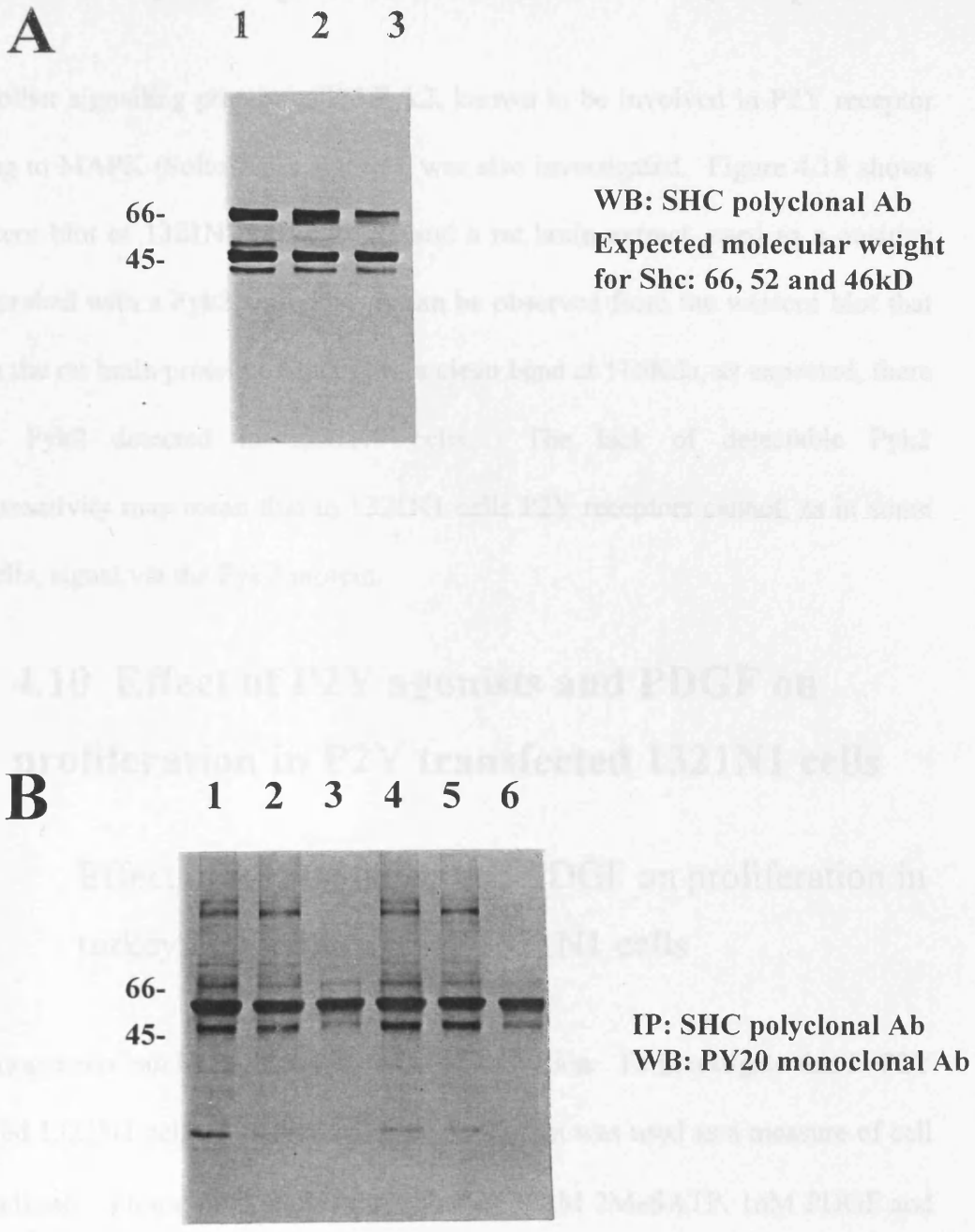


Figure 4.17

Effect of purinergic agonists on Shc tyrosine phosphorylation.

Western blot with Shc polyclonal primary antibody (A) where lanes 1-3 represents wild type, transfected t-$P2Y_1$ and transfected h-$P2Y_2$ 1321N1 cells respectively. Following immunoprecipitation with Shc polyclonal antibody a western blot with PY20 primary antibody displays tyrosine phosphorylated Shc (B) where lanes 1-3 represents t-$P2Y_1$ control, 30μM 2MeSATP and 10% FCS and lanes 4-6 represent h-$P2Y_2$ control, 100μM UTP and 10% FCS, respectively. Blots are representative of a two separate experiments.

4.9 P2Y receptor signal transduction to Pyk2 protein

Another signalling protein called Pyk2, known to be involved in P2Y receptor signalling to MAPK (Soltoff et al., 1998), was also investigated. Figure 4.18 shows the western blot of 1321N1 cell extracts and a rat brain extract, used as a positive control probed with a Pyk2 antibody. It can be observed from the western blot that although the rat brain protein extract gave a clean band at 116Kda, as expected, there was no Pyk2 detected in 1321N1 cells. The lack of detectable Pyk2 immunoreactivity may mean that in 1321N1 cells P2Y receptors cannot, as in some native cells, signal via the Pyk2 protein.

4.10 Effect of P2Y agonists and PDGF on proliferation in P2Y transfected 1321N1 cells

4.10.1 Effect of P2Y agonists and PDGF on proliferation in turkey $P2Y_1$ transfected 1321N1 cells

Mitogenesis can be regulated by MAPK activation. To investigate this in P2Y transfected 1321N1 cells, [^{3}H]thymidine incorporation was used as a measure of cell DNA synthesis. Figure 4.19 shows the effect of 30μM 2MeSATP, 1nM PDGF and 10% FCS on [^{3}H]thymidine incorporation into 1321N1 cells transfected with turkey $P2Y_1$. 30μM 2MeSATP alone had a stimulatory effect on [^{3}H]thymidine incorporation increasing 30±10.5% (mean ± s.e., $p<0.05$, t-test) above control levels. 1nM PDGF also had a stimulatory effect achieving similar stimulation levels as 2MeSATP. Co-addition of PDGF and 2MeSATP was additive (123 ± 4%) and 10% FCS, used as a positive control, was the most mitogenic increasing to 1661%

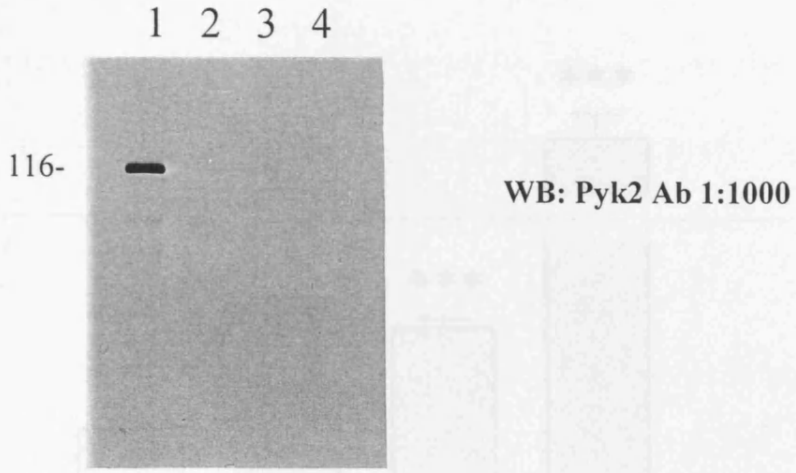


Figure 4.18

Western blot to detect presence of Pyk2 protein in 1321N1 cells.

1321N1 cells were lysed and proteins extracted from wild type, transfected turkey $P2Y_1$ and transfected human $P2Y_2$ cells and run on SDS PAGE (lanes 2-4 respectively). Rat brain extract was used in lane 1 as a positive control. Pyk2 primary antibody was used at 1:1000 for 1.5 hours. Blot shown is representative of two separate experiments.

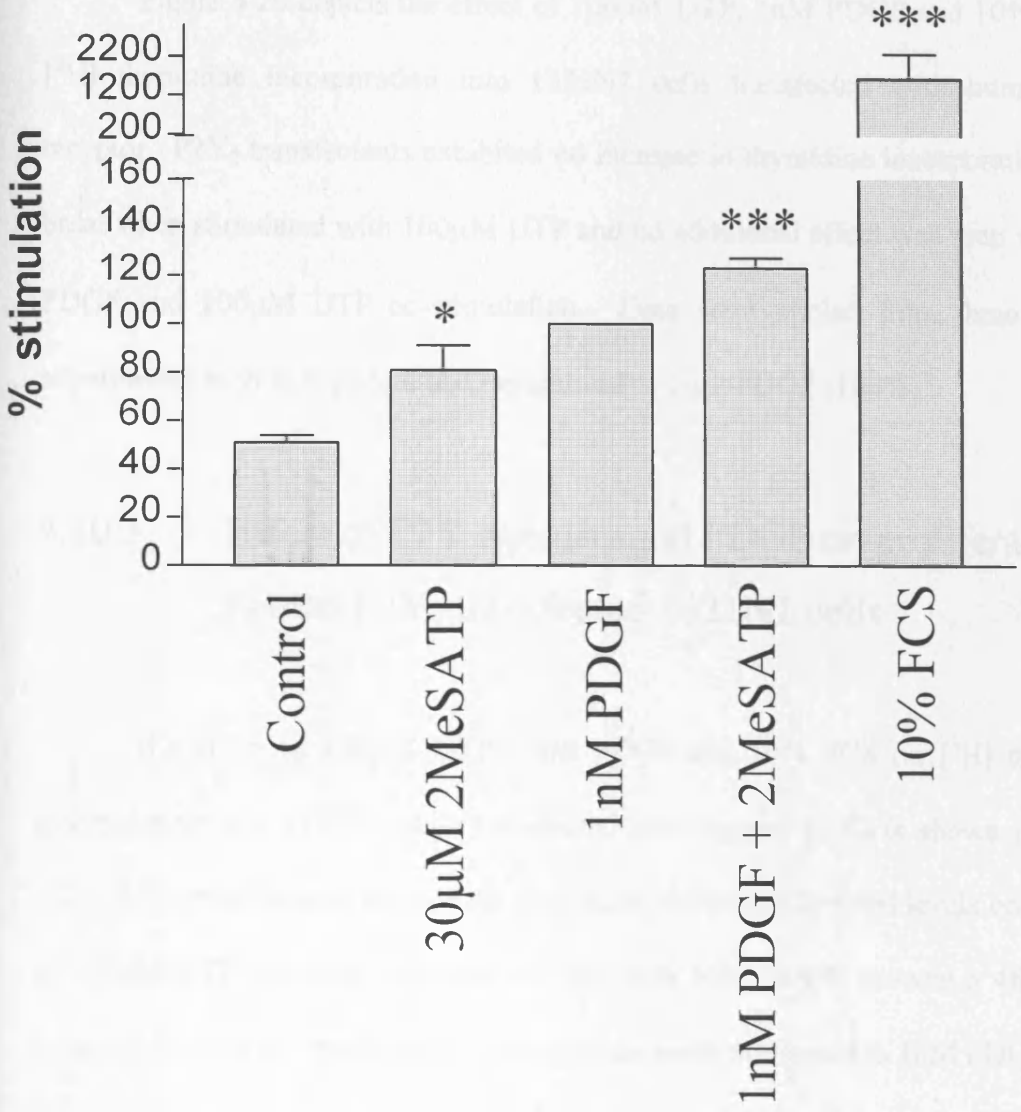


Figure 4.19

Effect of purinergic agonists, PDGF and FCS for a 1 hour stimulation on [^{3}H] thymidine incorporation into 1321N1 cells transfected with turkey $P2Y_1$ receptor (n=4). Error bars represent S.E. on data normalising to 1nM PDGF. * = $p<0.05$ ***=$p<0.001$ compared to control (t-test).

compared to 1nM PDGF (100%). Data were pooled from four separate experiments each in triplicate and normalised to 1nM PDGF (100%).

4.10.2 Effect of P2Y agonists and PDGF on proliferation in human $P2Y_2$ transfected 1321N1 cells

Figure 4.20 depicts the effect of 100μM UTP, 1nM PDGF and 10% FCS on [^{3}H] thymidine incorporation into 1321N1 cells transfected with human $P2Y_2$ receptor. $P2Y_2$ transfectants exhibited no increase in thymidine incorporation above basal when stimulated with 100μM UTP and no additional effect was seen with 1nM PDGF and 100μM UTP co-stimulation. Data were pooled from three separate experiments each in triplicate and normalised to 1nM PDGF (100%).

4.10.3 Effect of P2Y agonists and PDGF on proliferation in human $P2Y_4$ transfected 1321N1 cells

The effect of 100μM UTP, 1nM PDGF and 10% FCS on [^{3}H] thymidine incorporation into 1321N1 cells transfected with human $P2Y_4$ is shown in Figure 4.21. $P2Y_4$ transfectants showed no significant difference to basal levels on addition of 100μM UTP, however addition of UTP with 1nM PDGF showed a 46.1±5.8% (mean ± s.e., $p<0.05$, bonferroni's post-test) decrease compared to 1nM PDGF alone. Data were pooled from five separate experiments each in triplicate and normalised to 1nM PDGF (100%).

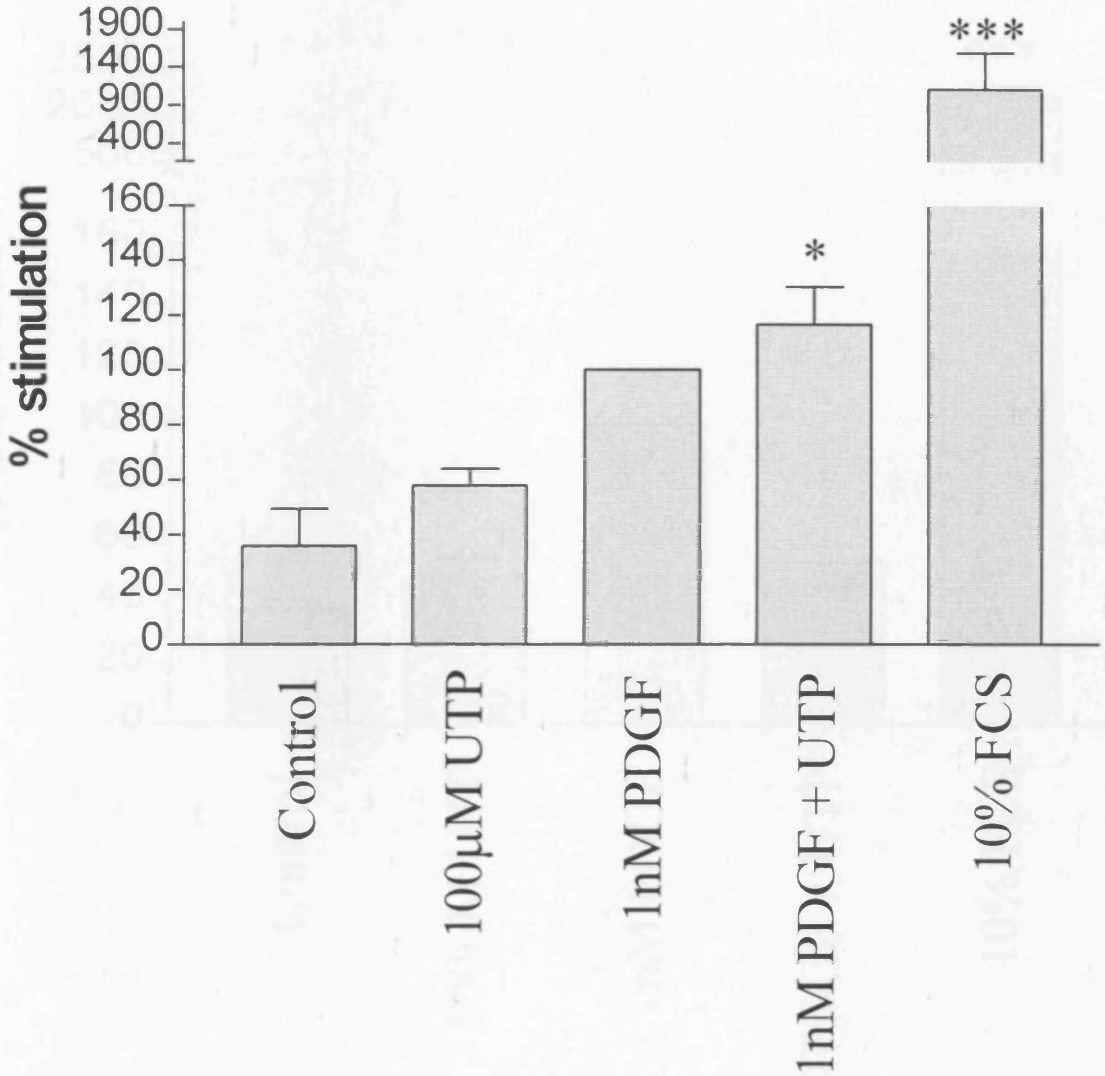


Figure 4.20

Effect of purinergic agonists, PDGF and FCS for a 1 hour stimulation on [^{3}H] thymidine incorporation into 1321N1 cells transfected with human $P2Y_2$ receptor (n=3). Error bars represent S.E. on data normalising to 1nM PDGF. * = $p<0.05$ ***=$p<0.001$ compared to control (t-test).

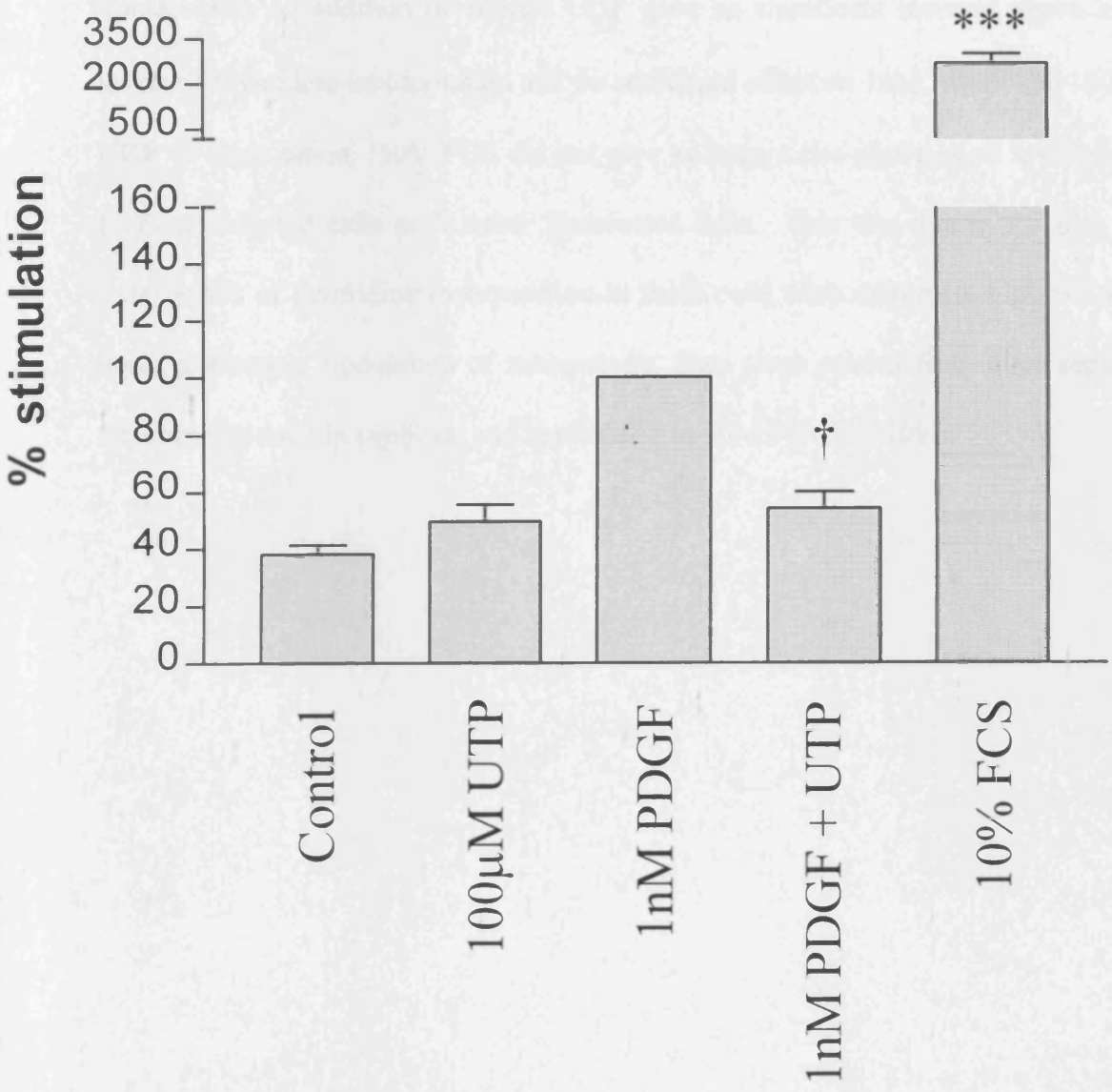


Figure 4.21

Effect of purinergic agonists, PDGF and FCS for a 1 hour stimulation on [^{3}H] thymidine incorporation into 1321N1 cells transfected with human $P2Y_4$ receptor (n=5). Error bars represent S.E. on data normalising to 1nM PDGF. ***=p<0.001 compared to control and † = p<0.05 compared to PDGF (bonferroni's post-test).

4.10.4 Effect of P2Y agonists and PDGF on proliferation in rat $P2Y_6$ transfected 1321N1 cells

Figure 4.22 represents the effect of 100μM UDP, 1nM PDGF and 10% FCS on [^{3}H] thymidine incorporation into 1321N1 cells transfected with rat $P2Y_6$. $P2Y_6$ transfectants on addition of 100μM UDP gave no significant increase above basal levels of thymidine incorporation and no additional effect on 1nM PDGF and 100μM UDP co-stimulation. 10% FCS did not give as large a rise above basal levels in rat $P2Y_6$ transfected cells as in other transfected cells. This was due to the fact that basal levels of thymidine incorporation in these cells were extremely high and may mask purinergic modulation of mitogenesis. Data were pooled from three separate experiments each in triplicate and normalised to 1nM PDGF (100%).

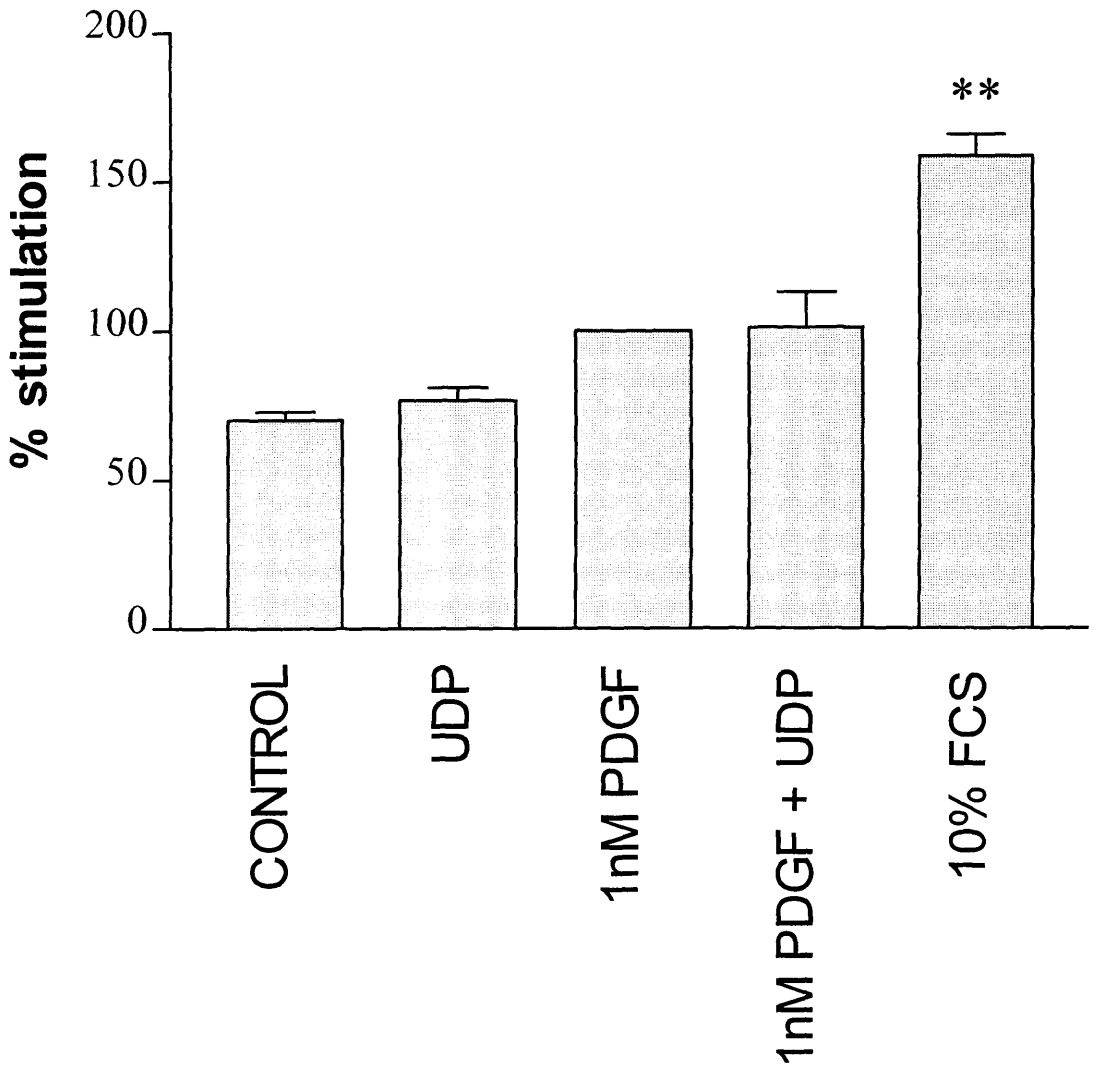


Figure 4.22
Effect of purinergic agonists, PDGF and FCS for a 1 hour stimulation on [^{3}H] thymidine incorporation into 1321N1 cells transfected with rat $P2Y_6$ receptor (n=3). Error bars represent S.E. on data normalising to 1nM PDGF. ** = $p<0.01$ compared to control (t-test).

4.11 Discussion

4.11.1 MAPK activation by P2Y receptors in 1321N1 cells

MAPK, as ERK1 and ERK2, is activated by P2Y receptors in native tissues but has not been studied in transfected cell systems when the individual receptors are isolated. The data presented in this thesis chapter clearly shows MAPK activation in 1321N1 cells by activation of turkey $P2Y_1$ and human $P2Y_2$ receptors but not human $P2Y_4$ or rat $P2Y_6$. These differences in receptor signal transduction will now be discussed.

4.11.1.1 Effect of dose and variance with time

The turkey $P2Y_1$ and human $P2Y_2$ receptor activate MAPK in a dose dependant and time dependant manner, as shown by both western blotting with a phospho-MAPK specific antibody and phosphorylation of a nonapeptide MAPK substrate. EC_{50} values for the MAPK activation of turkey $P2Y_1$ and human $P2Y_2$ receptors were similar to that obtained from total inositol (poly)phosphate assays in the same cell experimental system. The turkey $P2Y_1$ receptor has not been shown previously to link to MAPK. $P2Y_1$ receptors from other species have been linked to activation of MAPK. These include the bovine $P2Y_1$ in BAEC cells (Patel et al., 1996), rat $P2Y_1$ in aortic smooth muscle (Yu et al., 1996) and astroglial cultured $P2Y_1$ (Munsch et al., 1998). All reports indicate maximal activation of MAPK at 5 minutes with varying fold activation. Data in this thesis indicate maximal activation

also at 5 minutes and a small fold over basal increase on receptor activation. The small fold increase in MAPK activation seen in 1321N1 cells may be explained in a number of ways: 1) 1321N1 cells may have a response to MAPK in the absence of receptor activation that may explain lower fold over basal responses observed. 2) It maybe that endogenous release of agonists (Lazarowski et al., 1997a; Harden et al., 1997) have desensitised receptors preventing large responses to agonists. However, some data suggests that this is not the case at least for total inositol poly(phosphates) as robust responses are observed (Nicholas et al., 1996a;b). It maybe that the desensitisation has more impact on the signal transduction to MAPK than to PLC. 3) Another possibility is that due to high turnover of phospho-MAPK by high activity of phosphatases, high levels of phospho-MAPK are not observed. This also would not seem to be the case as activation with 10% FCS of MAPK in 1321N1 cells gives large observable responses.

$P2Y_2$ receptors have also been shown, in a wide variety of cell types, to activate MAPK. This includes smooth muscle (Erlinge et al., 1998), CHO cells (Dickenson et al., 1998), PC12 cells (Soltoff et al., 1998), astrocytes (Neary et al., 1998), rat renal inner medullary collecting duct cells (Ishikawa et al., 1997), HEK 293 cells (Gao et al., 1999) and endothelial cells. The latter includes BAEC (Patel et al., 1996), RBEC (Alberts et al., 1997) and EAhy 926 cells (Graham et al., 1996). These all show clear activation of MAPK, but as discussed in the introduction, there are many differing routes from a G protein-linked receptor to activation of the ERK-MAPK pathway. It was pertinent therefore to investigate the route to activation in 1321N1 cells of both turkey $P2Y_1$ and human $P2Y_2$ and the subject of work discussed in the next sections.

Both human $P2Y_4$ and rat $P2Y_6$ when transfected into 1321N1 cells show no link to MAPK activation. This was true for a variety of agonist concentrations and incubation times. Other reports have stated in rat aortic smooth muscle that activation of PLC and MAPK occurs via the rat $P2Y_4$ on addition of UTP and only partially by ATP indicating the presence of a $P2Y_4$-like response (Harper et al., 1998). This was not reflected in transfected human $P2Y_4$. This may be due to a species difference between the rat and human receptors, or due to the expression of the receptors in different cells.

No published evidence links native $P2Y_6$ receptors with activation of MAPK, though native responses to PLC have been shown in C6-2B cells (Nicholas et al., 1996). Large PLC responses are seen in transfected 1321N1 cells for rat $P2Y_6$ but no activation of MAPK was observed.

4.11.1.2 Effect of PD 98059

The effect of PD 98059 (Alessi et al., 1995; Dudley et al., 1995) to inhibit MEK, the MAPK kinase, is important in establishing specificity of the peptide phosphorylation assay. As the assay does not require the immunoprecipitation and purification of ERK proteins there is the possibility that other activated kinases present in the crude extract may be responsible for phosphorylation of the nonapeptide substrate, though this is a specific substrate. The inhibition observed with PD 98059 clearly shows that activation of MAPK via both transfected turkey $P2Y_1$ and human $P2Y_2$ receptors does occur and that the peptide phosphorylation is a reliable indicator of MAPK activation in 1321N1 cells. This is

also backed by evidence from western blotting that reflects observations seen with the peptide assay.

4.11.1.3 Effect of pervanadate

It has already been explained that pervanadate was used due to the lack of a detectable effect seen on general tyrosine phosphorylation possibly due to the rapid effects of phosphatases. It was thought that pervanadate may also be able to unmask larger stimulations of MAPK by transfected P2Y receptors. It was observed that pervanadate alone could stimulate MAPK, which is unsurprising as this is well documented in the literature (Morinville et al., 1998 and Huyer et al., 1997) but no additive effects were seen on co-addition of agonist and pervanadate. These data imply therefore that pervanadate is able in 1321N1 cells to inhibit tyrosine phosphatases causing an increase in MAPK phosphorylation. P2Y agonists also stimulate MAPK via turkey $P2Y_1$ and human $P2Y_2$ receptors. It also seems that pervanadate and P2Y agonists act via different pathways as no additive effects were seen. Krady et al., (1998) suggested in cultured mouse fibroblasts a different pathway for pervanadate activation of MAPK than receptor activation, bypassing MEK as it is PD 98059 insensitive. This may also be true in 1321N1 cells as agonist activation is PD 98059 sensitive but further work would establish if pervanadate was PD 98059 insensitive in 1321N1 cells.

4.11.1.4 Role of PKC in MAPK activation by transfected P2Y receptors

PKC α, ε, and λ but not β, θ, or ζ isoforms were detected in 1321N1 cells using western blotting. The activation of MAPK via both turkey $P2Y_1$ and human $P2Y_2$ receptors is sensitive to PKC inhibition using Ro 31-8220 and PKC activation using TPA. This is also reflective of data observed by Dr S.J. Charlton on PLC responses, where activation of PKC inhibited PLC and inhibition of PKC enhanced PLC responses (Charlton, 1998). Both transfected receptors therefore appear on this evidence to be linked to similar signalling pathways to MAPK activation. Dose inhibition of the MAPK responses in both transfected systems by Ro 31-8220 also shows similar results with IC_{50} results being comparable. Ro 31-8220 is however a general PKC inhibitor and therefore only informs that PKC is involved in MAPK activation from turkey $P2Y_1$ and human $P2Y_2$ receptors. To establish which PKC isoform is involved in transfected P2Y receptor MAPK signalling two other compounds were used. These were Go 6850, a non-specific PKC inhibitor and Go 6976, a specific inhibitor of Ca^{++} sensitive PKC isoforms (Martiny-Baron et al., 1993). Of the isoforms present in 1321N1 cells, α is Ca^{++} sensitive whereas ε and λ are not. The inability of Go 6976 to inhibit P2Y activated MAPK provides evidence that isoform α is not involved in P2Y receptor mediated MAPK activation. This leaves ε and λ isoforms or some other isoform not tested which include PKC γ, δ, η, ι and μ. Transfected P2Y receptors cannot be signalling through PKC γ as it is Ca^{++} sensitive. Patel et al., (1996) showed that PKC ε was the most likely candidate for P2Y signalling in BAEC cells. Soltoff et al., (1998) showed tyrosine phosphorylation of PKC δ after 15 seconds incubation with UTP or ATP indicating

$P2Y_2$ activation of PKC δ in PC12 cells. Yu et al., (1996) showed in vascular smooth muscle that P2Y receptor activation lead to MAPK activation that was dependent on PKC α and δ isoforms. For 1321N1 cells further work needs to done possibly using membrane localisation or PMA down regulation to establish definitely which isoforms are involved in transfected P2Y signalling.

4.11.2 Effect of transfected P2Y receptors on Shc and Pyk2

The pathway by which G protein-linked receptors signal to MAPK has been the subject of much work over the past few years and was discussed in detail in the introduction. Lev et al., (1995) proposed a signalling pathway linking tyrosine kinases and a number of protein factors to the activation of Ras, which couples to a kinase cascade resulting in MAPK activation. To see if these proteins were involved in the signalling pathway of transfected P2Y receptors to MAPK, western blotting analysis was undertaken. Initial experiments revealed that when the Pyk2 antibody was used on 1321N1 extracts no protein was detected implying therefore that no P2Y signalling could be occurring via Pyk2 to MAPK.

Tyrosine kinases other than Pyk2 may couple to Shc in 1321N1 cells. Therefore, Shc was investigated to see if an increase in tyrosine phosphorylation was observed on receptor activation. Shc antibody recognises three different isoforms of Shc, all of which were detected in 1321N1 cells. These are located at 66, 52 and 46 KDa in mass. 1321N1 cells showed strong banding for isoforms 66 and 52Kda. It was investigated whether using immunoprecipitation of Shc, and subsequent western blotting with PY20, receptor mediated tyrosine phosphorylation of Shc could be observed. After immunoprecipitation, western blotting probed with a Shc antibody

displayed the presence of immunoprecipitated Shc proteins. However, when using the PY20 antibody as a probe in western blotting of stimulated extracts after immunoprecipitation, no increase in tyrosine phosphorylation of Shc was observed between control and stimulated. IgG bands were also present causing complications on the western blot. This was only repeated once and would be the subject of future work.

4.11.3 Role of transfected P2Y receptors on mitogenesis

The data presented using incorporation of [^{3}H]thymidine as an indicator of mitogenesis showed that only the transfected turkey $P2Y_1$ receptor was able to achieve an increase in thymidine incorporation both in the presence and absence of PDGF. The ability of the $P2Y_1$ receptor to enhance mitogenesis is supportive of literature showing this in native cell systems (Yu et al., 1996, Wang et al., 1992). PDGF was used not only as a positive control but also to see if P2Y agonists may act as mitogenic progression factors, rather than mitogenic on their own. None of the other transfected receptors gave an increase in thymidine incorporation when activated and shows that although all the transfected receptors gave clear and substantial PLC responses this is not sufficient for mitogenesis. There is also no clear correlation between MAPK activation and mitogenesis because turkey $P2Y_1$ receptors activate both MAPK and thymidine incorporation but human $P2Y_2$ only activates MAPK and not thymidine incorporation. This is also supported by evidence using angiotensin II, which showed that MAPK is necessary but not sufficient for mitogenesis (Wilkie et al., 1996). Human $P2Y_4$ receptor activation showed no increase in MAPK activation and no effects on thymidine incorporation.

However, in the presence of PDGF-induced elevated thymidine incorporation, a decrease in incorporation was observed. Inhibition of mitogenesis by UTP has also been reported in the literature by Lemmens et al., (1996) on endothelial LLC-MK(2) cells. It may be that transfected human $P2Y_4$ receptors in 1321N1 cells represent a model of a native system that would be of great interest for future work. The mechanism behind this inhibition of mitogenesis is unclear. MAPK levels are not diminished on agonist addition compared to control levels. However, agonist addition in the presence of raised MAPK activation has yet to be carried out which would enable investigation of a role for MAPK in this inhibition. CAMP is thought to be able to control the activation of MAPK via protein kinase A (PKA) which may be able to promote MAPK activation via B-Raf (Daaka et al., 1997; Gao et al., 1999) or inhibit MAPK activation via c-Raf-1 (Vossler et al.,1997). CAMP levels are raised in 1321N1 human $P2Y_4$ transfects in the presence of forskolin (G. Roalfe and M.R. Boarder, unpublished), but further work is required to show a link between PKA and MAPK that could cause the anti-mitogenic effect observed.

Rat $P2Y_6$, although showing large PLC responses to receptor activation gave no increase in MAPK or incorporation of thymidine. It is to be noted that the basal levels for thymidine incorporation were much higher than levels seen with other transfected receptors evident by the poor increase above basal seen with 10% FCS. This may mask potential mitogenic effects of the activated $P2Y_6$ receptor in these cells. The MAPK basal level was not elevated in comparison to other transfected systems and therefore elevated MAPK cannot explain this effect which must come from a different pathway.

A summary of the observed effects of agonists on transfected P2Y receptors is shown over in Table 4.1.

Receptor Subtype	PLC/Ca^{++}	cAMP (+Forskolin)	MAPK	[^{3}H] thymidine
$tP2Y_1$	↑*	↓†	↑	↑
$hP2Y_2$	↑*	-†	↑	-
$hP2Y_4$	↑*	↑†	-	↓
$rP2Y_6$	↑	↑†	-	-

Table 4.1 Summary of signalling pathways coupled to transfected receptors.

↑ = increased activity ↓ = decreased activity - = no effect on activity

* = Data provided by Dr. S.J. Charlton † = Data provided by Grant Roalfe

Chapter 5

Regulation of signal transduction in rat brain microvascular endothelial cells by P2Y receptors

5.1 Introduction

Brain capillary endothelial cells under regulation by cell-surface receptors, including G protein-coupled receptors such as P2Y receptors, form what is known as the blood-brain barrier. This structure is critical for proper brain function and therefore characterisation of receptors and signal pathways involved in its regulation are also important. In this chapter the regulation by P2Y receptors of rat brain endothelial cells (RBEC) was investigated by looking at activation of p42/p44 MAPK. A possible role for PKC and cAMP in the control of p42/p44 MAPK by P2Y receptors was also investigated.

MAPK plays a part in tyrosine phosphorylation intracellular signalling which is known to be important in RBEC regulation of blood-brain-barrier structure and permeability (Staddon and Rubin, 1996, Staddon et al., 1995). P2Y receptor regulation of endothelial cells has previously been linked to MAPK and tyrosine phosphorylation and their role in prostacyclin release in bovine aortic endothelial cells (Bowden et al., 1995, Patel et al., 1996). It was therefore proposed to further investigate the particular P2Y receptors involved in RBEC and the nature of their linkage to the MAPK cascade.

Adenylate cyclase regulates levels of cyclic AMP, which is coupled to control of blood-brain barrier function (Rubin et al., 1991). There have been some discrepancies in published literature as to which P2Y subtypes are present in RBEC and their regulation of adenylate cyclase activity, which will be discussed in a later section. This may, in part, be explained by the different preparations of rat brain endothelial cells used. The variation between primary and passaged cells is well documented, with alterations both in receptor number and type. Using primary

unpassaged RBEC in a relatively pure preparation we used cells which we believe are more likely to show in vivo receptor expression. We have examined the responses of the primary preparations to various P2Y agonists to elucidate both the receptors responsible for, and their involvement in, regulation of cAMP.

5.2 P2Y regulation of p42/p44 MAPK in RBEC

5.2.1 Effect of P2Y agonists

Previous work has shown the presence of multiple P2Y subtypes on RBEC that are characterised as $P2Y_1$-like. These are not linked to PLC, but are able to increase $[Ca^{2+}]i$, while $P2Y_2$ receptors also present are PLC linked (Frelin et al., 1993; Vigne et al., 1994; Feolde et al., 1995). We began initial characterisation of MAPK activation by P2Y receptors on RBEC using a selection of P2Y agonists at concentrations previously shown to activate similar levels of calcium release (Albert et al., 1997).

In Figure 5.1 the result from experiments on RBEC with various agonists is depicted. The P2Y agonists were added to confluent RBEC cells and left for five minutes. After stimulation the cells were lysed as described previously and run on SDS PAGE for Western blotting with an antibody specific for the phosphorylated form of MAPK. Figure 5.1A shows a graph of pooled data from five separate experiments scanned from autoradiographs using a densitometer outlining the p42 band. This band was chosen because this form of MAPK is most heavily phosphorylated on agonist addition. Figure 5.1B shows a representative blot.

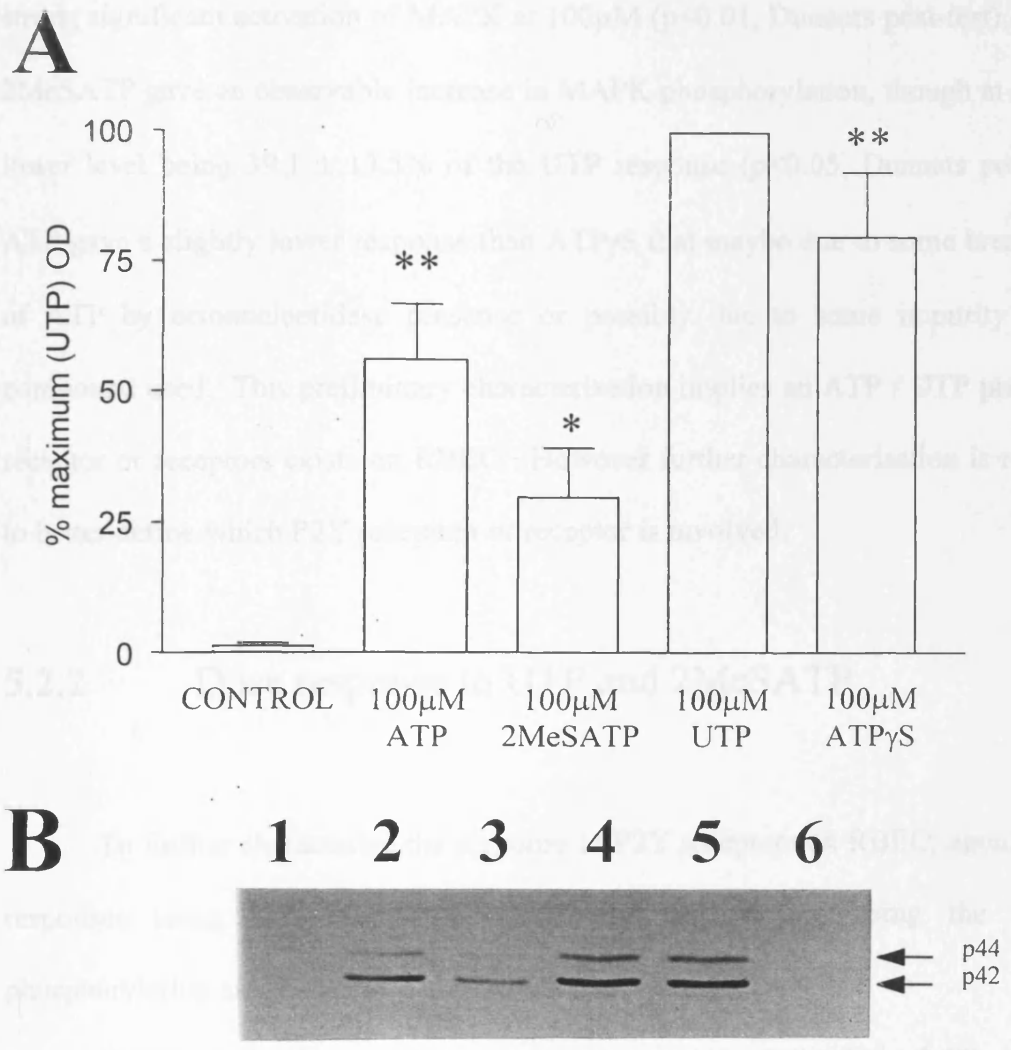


Figure 5.1

Effect of different P2Y receptor agonists on p42 MAPK phosphorylation in RBEC. Densitometric analysis of western blotting autoradiographs using an antibody specific for the phosphorylated MAPK protein (A). Data is pooled from five separate experiments and expressed as the mean ± SEM of the percentage response to 100μM UTP. Cells were stimulated for 5 minutes in each case. Representative western blot autoradiograph used for scanning (B). Lane 1 - 6 represents control, 100μM ATP, 100μM 2MeSATP, 100μM UTP, 100μM ATPγS and control, respectively. * = $p<0.05$ **=$p<0.01$ compared to control (dunnet's post-test).

The western blotting shows that under control conditions no immunoreactivity was observed. The P2Y agonists UTP, ATP and ATPγS all gave a strong significant activation of MAPK at 100μM ($p<0.01$, Dunnets post-test). 30μM 2MeSATP gave an observable increase in MAPK phosphorylation, though at a much lower level being 39.1 ± 13.5% of the UTP response ($p<0.05$, Dunnets post-test). ATP gave a slightly lower response than ATPγS that maybe due to some breakdown of ATP by ectonucleotidase presence or possibly due to some impurity of the compound used. This preliminary characterisation implies an ATP / UTP preferring receptor or receptors exists on RBEC. However further characterisation is required to better define which P2Y receptors or receptor is involved.

5.2.2 Dose response to UTP and 2MeSATP

To further characterise the response to P2Y receptors on RBEC, agonist dose responses using UTP and 2MeSATP were carried out using the peptide phosphorylation assay to detect increases in MAPK activity.

Figure 5.2 shows dose response curves generated from peptide phosphorylation assay. Data are representative of three separate experiments carried out in triplicate and expressed as a percentage of maximum UTP phosphorylation. These data reflect Figure 5.1A and 2MeSATP can be seen to be a partial agonist in this system compared with UTP (log EC_{50} are -5.70 ± 0.13 and -4.34 ± 0.31 for UTP and 2MeSATP respectively), though the accuracy of the 2MeSATP EC_{50} may be doubted as the curve did not reach a plateau.

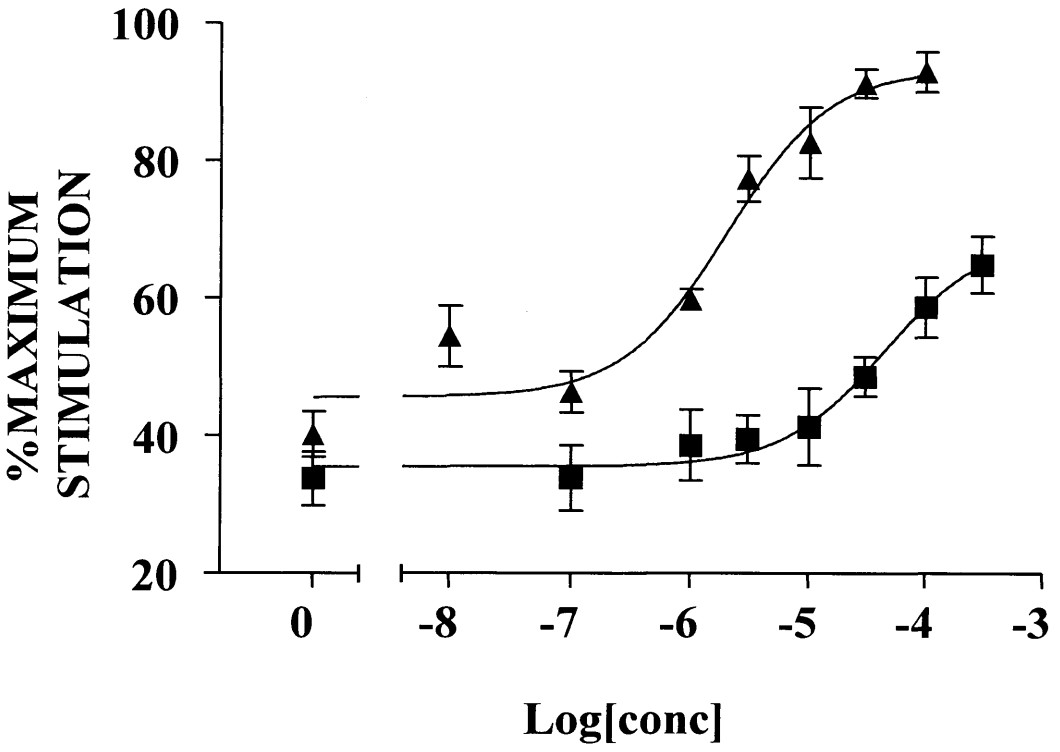


Figure 5.2
MAPK phosphorylation dose response curves on RBEC using the peptide kinase assay. Data is representative of three separate experiments pooled and expressed as the mean ± SEM of the percentage response to maximum phosphorylation by UTP. Closed triangles (▲) represent UTP and closed squares (■) represent 2MeSATP.

5.2.3 Effect of removal of extracellular calcium

Having established the most likely receptor to be a $P2Y_2$ receptor, further characterisation of the pathway to MAPK activation was required. Calcium plays a major role in cell signalling and to investigate if extracellular calcium was required for P2Y activation of MAPK 5mM EGTA, a calcium chelator, was added one minute prior and during the five minute stimulation with P2Y agonist. This short term addition of EGTA would only chelate extracellular calcium and probably not remove calcium from intracellular stores.

Figure 5.3A shows the pooled densitometer results of four separate experiments. Data has been normalised as a percentage of UTP maximal response. Figure 5.3B is a representative autoradiograph for a single experiment. Results for this experiment reflect the pattern of agonist MAPK activation seen previously. On co-addition of 5mM EGTA in the presence of the various agonists all MAPK phosphorylation was attenuated and brought down to near control levels. Statistical analysis showed 2MeSATP not to be significantly reduced (Bonferroni's multi-comparison test) though ATP, ATPγS and UTP all were significantly reduced ($p<0.01$,$p<0.001$ and $p<0.001$ respectively).

5.2.4 Modulation of MAPK activity by cAMP

Figure 5.4A represents a single experiment in triplicate investigating the effects of elevated cAMP levels using forskolin on P2Y mediated MAPK activation using the peptide phosphorylation assay. Agonists were added to the cells for five

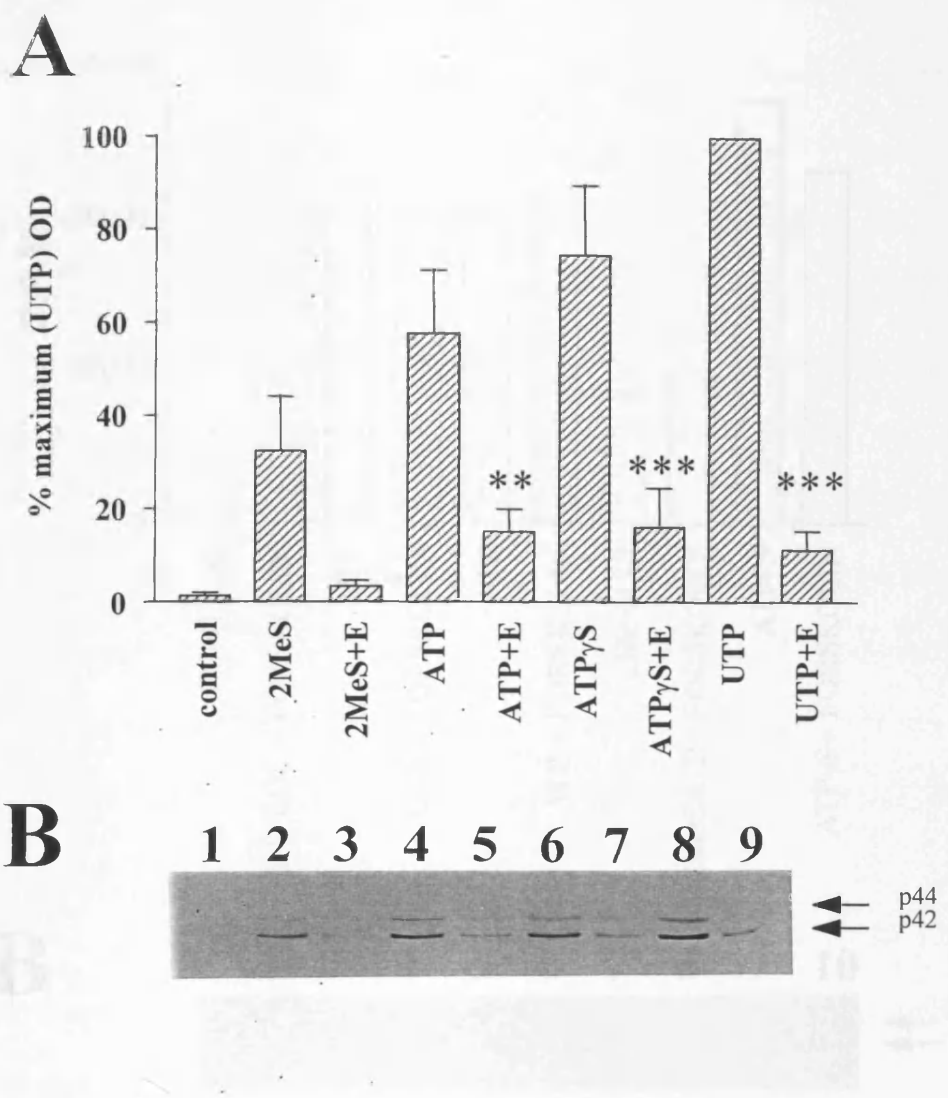


Figure 5.3

Effect of removal of extracellular calcium on P2Y agonist activation of MAPK in RBEC.

Pooled densitometry analysis from four separate experiments of phospho-MAPK antibody western blots expressed as mean ± SEM of the percentage maximum response to UTP induced phosphorylation (A). Stimulation was for 5 minutes in each case with 30μM 2MeSATP, 100μM ATP, 100μM ATPγS or 100μM UTP in the presence or absence of 5mM EGTA. Representative western blot autoradiograph used for scanning is shown (B). Lanes 1 - 9 represent control, 30μM 2MeSATP, 30μM 2MeSATP and EGTA, 100μM ATP, 100μM ATP and EGTA, 100μM ATPγS, 100μM ATPγS and EGTA, 100μM UTP and 100μM UTP and EGTA. **=p<0. 01 and ***=p<0.001 compared to agonist without EGTA (dunnet's post-test).

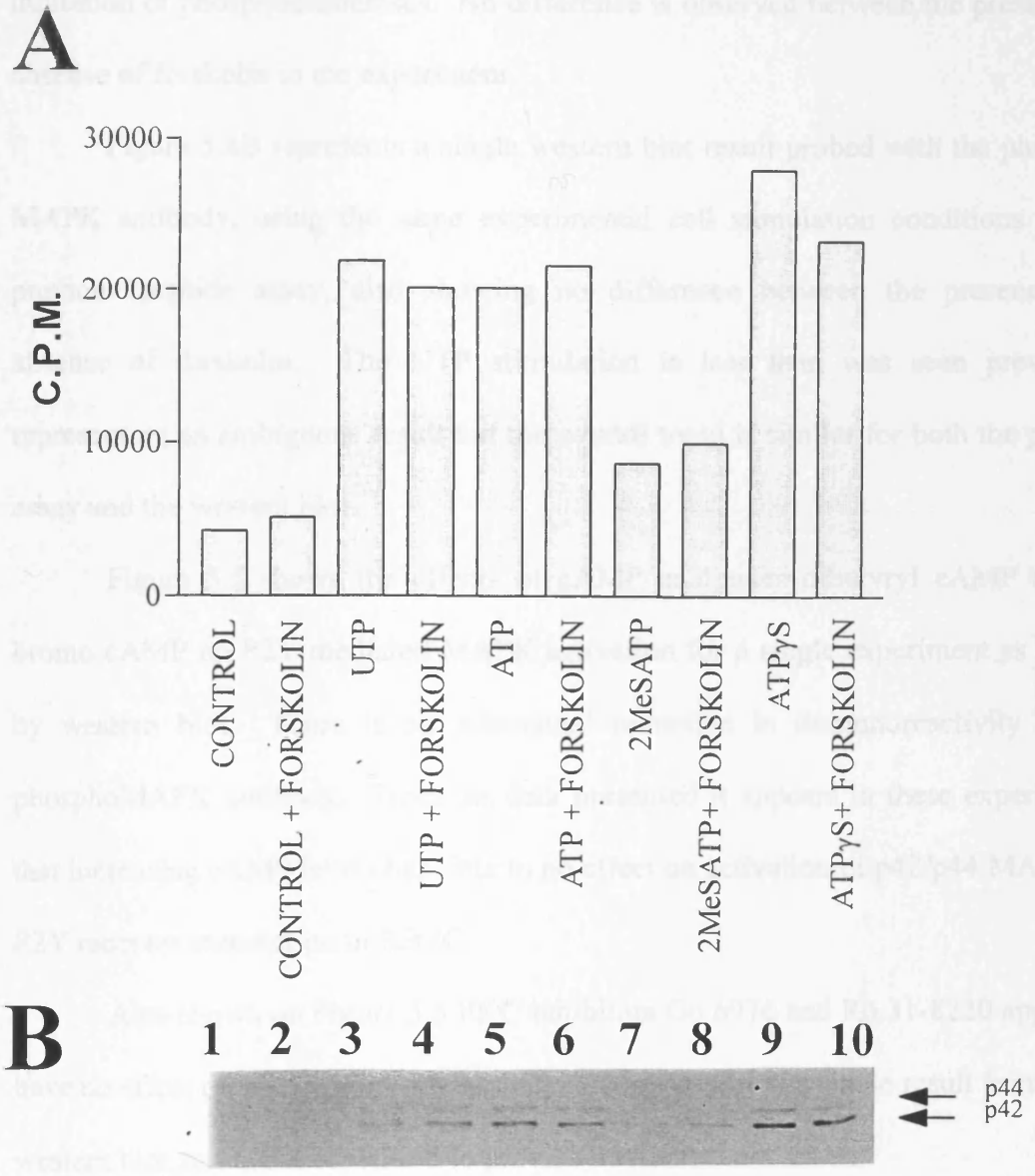


Figure 5.4

Effect of 5μM forskolin on P2Y agonist activation of MAPK in RBEC.

Peptide phosphorylation assay data is expressed as counts per minute and represents a single experiment for stimulations at 5 minutes with 100μM UTP, 100μM ATP, 100μM ATPγS or 30μM 2MeSATP in the presence or absence of 5μM forskolin (A). Phospho-MAPK antibody western blot autoradiograph of stimulations under the same conditions (B). Lanes 1 - 10 represent control, control and forskolin, 100μM UTP, 100μM UTP and forskolin, 100μM ATP, 100μM ATP and forskolin, 30μM 2MeSATP, 30μM 2MeSATP and forskolin, 100μM ATPγS and 100μM ATPγS and forskolin, respectively. Autoradiograph representative of two separate experiments.

minutes in the presence or absence of 5μM forskolin that raises cAMP levels by inhibition of phosphodiesterases. No difference is observed between the presence or absence of forskolin in the experiment.

Figure 5.4B represents a single western blot result probed with the phospho-MAPK antibody, using the same experimental cell stimulation conditions as the previous peptide assay, also showing no difference between the presence and absence of forskolin. The UTP stimulation is less than was seen previously representing an ambiguous result but the overall trend is similar for both the peptide assay and the western blot.

Figure 5.5 shows the effects of cAMP analogues dibutyryl cAMP and 8-bromo cAMP on P2Y mediated MAPK activation for a single experiment as shown by western blot. There is no substantial reduction in immunoreactivity to the phosphoMAPK antibody. From the data presented it appears in these experiments that increasing cAMP levels has little to no effect on activation of p42/p44 MAPK by P2Y receptor stimulation in RBEC.

Also shown on Figure 5.5 PKC inhibitors Go 6976 and Ro 31-8220 appear to have no effect on p42/p44 MAPK activity. However, this is a single result from a one western blot and needs repetition to provide a reliable conclusion.

5.2.5 Effect of histamine, endothelin-1 and FCS

While investigating MAPK activation in RBEC, stimulation of other receptors was used to compare with P2Y receptor activation. Figure 5.6A represents data collected for activation of MAPK shown by peptide phosphorylation assay for 100μM histamine, 100nM endothelin-1 and 10% FCS. 10% FCS increased phospho

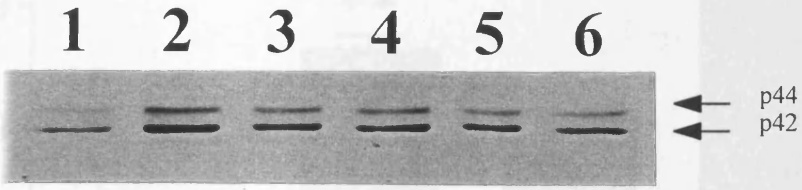


Figure 5.5

Effect of Dibutyryl cAMP, 8-Bromo cAMP, Go 6976 and Ro 31 8220 on MAPK phosphorylation in RBEC.

Autoradiograph represents a single western blot using the phospho-MAPK specific primary antibody. Lanes 1 - 6 represent control, 100μM UTP, 100μM UTP and 100μM dibutyryl cAMP, 100μM UTP and 100μM 8-Bromo cAMP, 100μM UTP and 10μM Gö 6976, and 100μM UTP and 10μM Ro 31 8220, respectively.

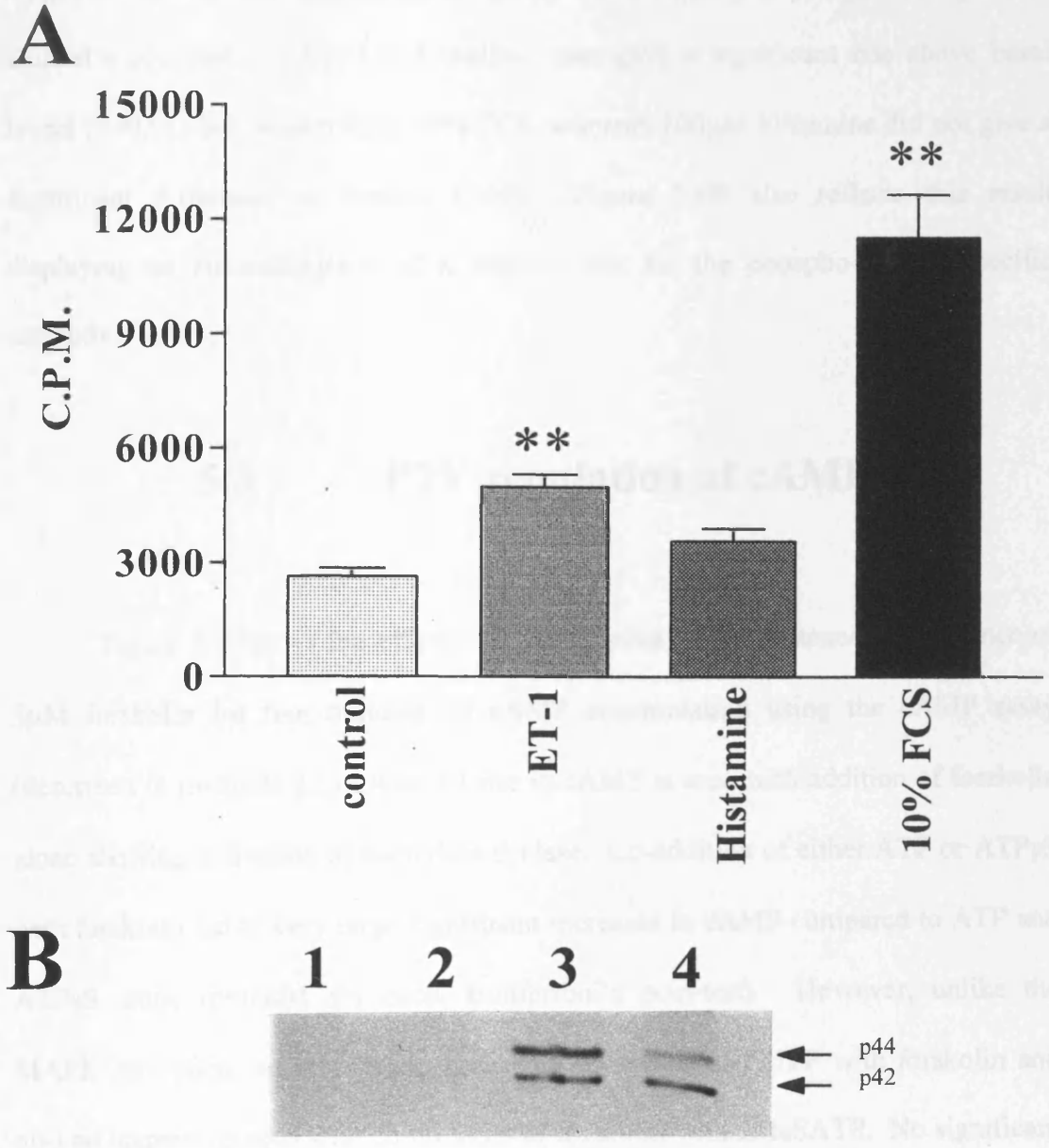


Figure 5.6

Effect of histamine and endothelin-1 activation of MAPK in RBEC.

Peptide phosphorylation assay for stimulations with BSS, 100nM ET-1, 100μM histamine and 10% FCS, expressed as mean counts per minute ± SEM and represents six separate experiments pooled (A). A Phospho-MAPK antibody western blot autoradiograph depicting the same experimental conditions (B). Lanes 1 - 4 represent control, 100μM histamine, 10% FCS, and 100nM ET-1. Stimulations were for 5 minutes in each case. Autoradiograph representative of two separate results. **=p< 0. 01 compared to control (dunnet's post-test).

-rylation significantly above basal levels of MAPK phosphorylation ($p<0.01$, Dunnet's post-test). 100nM endothelin-1 also gave a significant rise above basal levels ($p<0.01$) but weaker than 10% FCS, whereas 100μM histamine did not give a significant difference to control levels. Figure 5.6B also reflects this result displaying an autoradiograph of a western blot for the phospho-MAPK specific antibody.

5.3 P2Y regulation of cAMP

Figure 5.7 shows the effect of P2Y agonists in the presence and absence of 5μM forskolin for five minutes on cAMP accumulation using the cAMP assay (described in methods 2.7). A small rise in cAMP is seen with addition of forskolin alone showing activation of adenylate cyclase. Co-addition of either ATP or ATPγS with forskolin led to very large significant increases in cAMP compared to ATP and ATPγS alone ($p<0.001$ for each, Bonferroni's post-test). However, unlike the MAPK activation, no increase is seen with co-addition of UTP with forskolin and also no increase is seen with co-addition of forskolin with 2MeSATP. No significant difference was seen between control and agonists in the absence of forskolin.

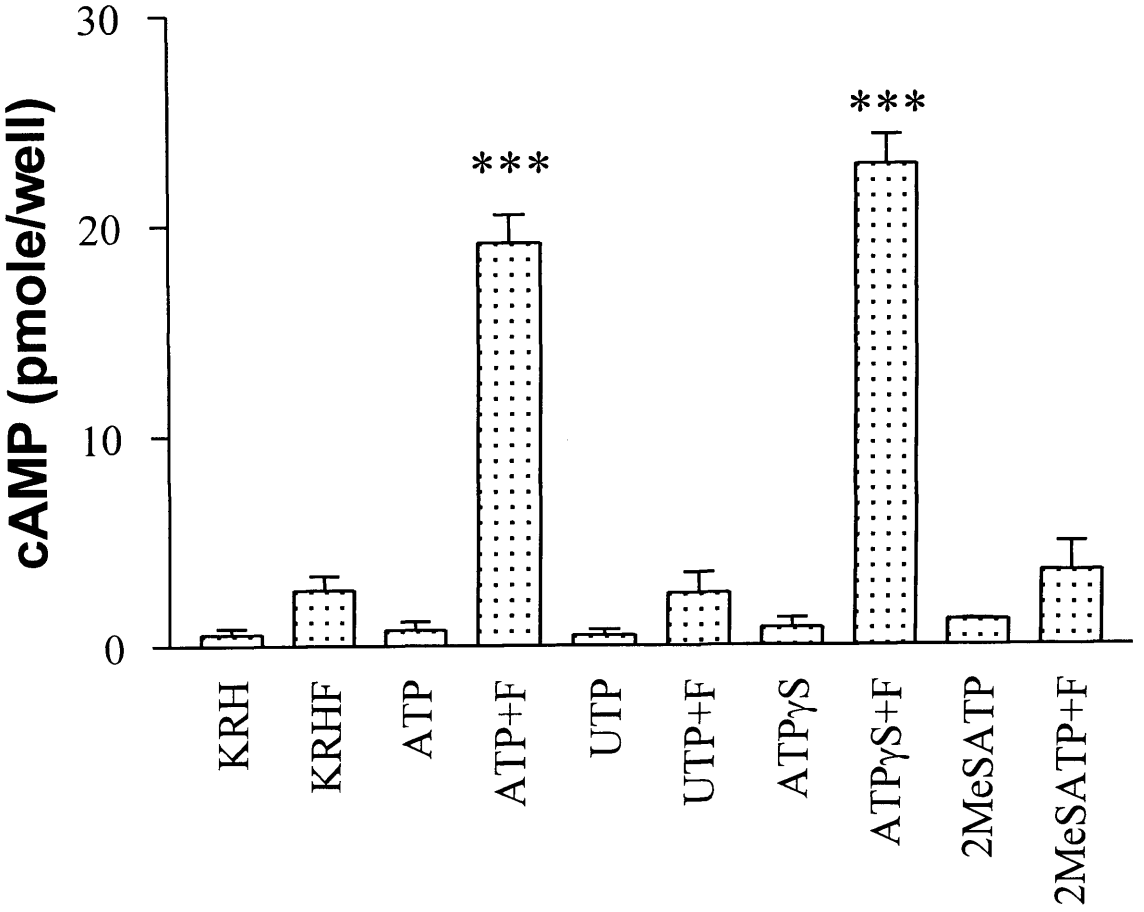


Figure 5.7

Effect of different P2Y receptor agonists in the presence or absence of 5μM forskolin on cAMP accumulation in RBEC. Data is expressed as picomoles of cAMP per well and represents mean ± SEM of three separate experiments pooled. 100μM ATP, ATPγS and UTP were used and 30μM 2MeSATP. *** = $p<0.001$ compared to agonist alone (bonferroni's post-test).

5.4 Discussion

5.4.1 p42/p44 MAPK activation in RBEC by P2Y receptors

Rat brain endothelial cells form a regulated blood-brain-barrier modulated by factors released from astrocytes. The role that P2Y receptors play in endothelial cell function has importance when it is known that astrocytes and smooth muscle release ATP locally. Therefore the presence of P2Y receptors on endothelial cells may be significant for maintenance of endothelial cell function. Activation of p42/p44 MAPK may, as well as other signalling pathways, play a key role in control of endothelial cell function and was therefore chosen for further investigation.

Initial studies showed an increase in p42/p44 MAPK activation by both UTP and ATPγS. ATP stimulation was smaller than ATPγS that may represent some form of nucleotide breakdown. UTP and ATPγS were not significantly different pointing towards involvement of the $P2Y_2$ receptor. The small stimulation observed by 2MeSATP would also be compatible with activation of a $P2Y_2$ receptor. The data presented for activation of p42/p44 MAPK in RBEC using concentration response curves for UTP and 2MeSATP is comparable with data collected for p42/p44 MAPK activation of human $P2Y_2$ receptor transfected into 1321N1 cells. Shown in a previous chapter (Figure 4.2), human $P2Y_2$ receptors gave log EC_{50} for UTP as -6.41 ± 0.21 and 2MeSATP acted as a partial agonist. Though not comparable on log EC_{50} values (-5.70 ± 0.13 for UTP on RBEC), a similar pattern of MAPK activation is observed and it can therefore be concluded that the responses seen on RBEC may most likely be attributed to the presence of a rat $P2Y_2$ receptor. It is unlikely that it is

the rat $P2Y_4$ receptor as PLC data suggests that 2MeSATP is ineffective at human transfected receptors in 1321N1 cells (Charlton, 1998) and for $[Ca^{++}]i$ at the rat $P2Y_4$ (Webb et al., 1998). 2MeSATP acts at as a partial agonist at transfected human $P2Y_2$ receptors (Charton, 1998). Recent published data by our group for RBEC using RT-PCR established the presence of $P2Y_2$, $P2Y_4$ and $P2Y_6$ but not $P2Y_1$ receptors (Anwar et al., 1999). It can be concluded from the data collected and presented here in this thesis that p42/p44 MAPK activation by UTP in RBEC is most likely to be via the $P2Y_2$ receptor present. It can not be excluded that some component of UTP stimulation of MAPK is acting via $P2Y_4$ or $P2Y_6$ receptors. $P2Y_6$ receptors are possibly responsible for part of the UTP responses and characterisation of the MAPK response to UDP would further clarify the $P2Y_6$ receptor involvement.

Removal of extracellular calcium is required for activation of p42/p44 MAPK by P2Y agonists UTP, ATP, ATPγS and 2MeSATP in RBEC. The precise function of extracellular Ca^{++} in p42/p44 MAPK activation was not determined. Further work required was limited by the availability of the RBE cells as a primary culture. It is important to note that the MAPK response is not due to a P2X receptor, since others have detected P2X receptor presence in RBEC preparations (Vigne et al., 1994; Nobles et al., 1995). In the unpassaged primary cells used here, α,β–MeATP, a P2X agonist, did not produce a rise in cytosolic Ca^{2+} or total [^{3}H]-inositol (poly)phosphates when tested (Albert et al., 1997) although this was only true for primary preparations. The small response to P2Y agonists that remain, seen with ATPγS and EGTA, maybe due to intracellular store release of Ca^{++} enabling p42/p44 activation. If the intracellular stores are depleted, attenuation of this small response may be observed, but this would be the subject of future work. Calcium is a modulator of many intracellular kinases and phosphatases (e.g. calmodulin and

calcineurin) and may therefore have indirect effects on the MAPK pathway by causing inhibition or activation of an enzyme required for the cascade.

Modulation of p42/p44 MAPK by cAMP was investigated in RBE cells. From the data presented it appears that no effect is to be observed for cAMP modulation of P2Y activated MAPK. This is however noted as preliminary data that requires further work but is interesting when considering mechanisms of MAPK modulation.

The increase in MAPK activation due to endothelin also corroborates data reported (Vigne et al., 1990, Vigne & Frelin, 1994) on endothelin activation of phosphatidylinositol hydrolysis, DNA synthesis and PLA_2 activation. It implies also that MAPK may play a role in endothelin mediated mitogenesis which may be an interesting hypothesis for further investigation in rat brain endothelial cells. Histamine has been reported to cause increases in $[Ca^{2+}]i$ without increases in total inositol phosphates or $Ins(1,4,5)P_3$ mass (Albert, unpublished). MAPK was not activated by histamine and therefore it can be hypothesised that activation of PLC is required for MAPK activation but not a rise in Ca^{2+}. Further work was limited by availability of cells but the scope for future work is evident.

5.4.2 cAMP modulation

The data reported here and published (Albert et al., 1997) show evidence of a $P2Y_2$ receptor linked to phospholipase C, Ca^{2+} and MAPK and $P2Y_1$-like receptor coupled to Ca^{2+} with no increases in mass $Ins(1,4,5)P_3$. The responses observed in Figure 5.7, show that the UTP-prefering receptor linked to MAPK, does not cause a rise in cAMP levels, as UTP is inactive. ATP and ATPγS, cause significant rises in

cAMP levels. However, 2MeSATP, a $P2Y_1$ agonist, does not. Though the responses observed for ATP may be via adenosine as a by-product of ectonucleotidase activity, the majority of the response is proposed to occur via an undefined P2Y receptor located on RBEC. ATPγS is a hydrolysis resistant ATP analogue and would be less affected by nucleotidase breakdown to adenosine. The equal levels of cAMP accumulation achieved by both ATP and ATPγS suggested that breakdown was not causing the increase in cAMP observed. Use of an adenosine antagonist would help to remove this possibility. Further work in our laboratory has made use of this and has shown that there was a component of the ATP response attributable to an adenosine receptor (Anwar et al., 1999). Adenosine deaminase completely removed the response to adenosine alone and reduced the response to ATP to about 59.2±11.0%. This meant that a receptor responsive to nucleotides was still involved in the elevation of cAMP levels. Use of the adenosine receptor antagonist 8-sulphophenyltheophylline (8-SPT) showed antagonism of the response to ATP both due to production of adenosine and of the ATP remaining. This antagonism rules out the possibility that ATP is acting via a known P2Y receptor as 8-SPT is not an antagonist at these receptors. Failure of UTP or UDP (data not shown) to produce increases in cAMP also ruled out action via $P2Y_4$ or $P2Y_6$ receptors. ATP appears to be increasing cAMP levels in RBEC via an unknown receptor that may also be activated by adenosine. This is because use of various adenosine antagonists on the adenosine response did not reflect the known pharmacology of adenosine receptors studied so far. It was proposed therefore that ATP and adenosine may be acting at the same receptor so far uncharacterised. It is also possible that the responses may be due to action at unclassified P2Y and P1 receptors. Support for a common receptor for adenosine and ATP does exist in presynaptic regulation of noradrenaline

release from peripheral nerve terminals (shinozuka et al., 1988; Kurz et al., 1993; Smith et al., 1997) and in NG108-15 cells (Matsuoka et al., 1995). The mechanism by which ATP elevates cAMP is most likely by augmentation of a G_s coupled response by forskolin as reported by Cote et al., (1993). Elevated $[Ca^{++}]i$ is unlikely to be involved as UTP increases calcium but does not increase cAMP levels. Use of the compound indomethacin showed that the increase in cAMP levels by ATP was not due to paracrine activation of adenylate cyclase due to prostaglandin release.

In conclusion the activation of p42/p44 MAPK indicates that the receptor responsible is most likely to be the $P2Y_2$ receptor. RT-PCR has showed the presence of $P2Y_2$, $P2Y_4$ and $P2Y_6$ but not $P2Y_1$ receptors though $[Ca^{++}]i$ data provides evidence of the presence of a $P2Y_1$-like receptor that does not cause an increase in PLC activity. cAMP data provided in this thesis and by colleagues has shown ATP elevation of cAMP levels. The receptor responsible for this may possibly be responsive to both ATP and adenosine produced from the partial conversion of ATP to adenosine. The results presented provide an insight into the pathways by which nucleotides may control endothelial function. It would be the subject of future work to study the precise nature of the receptors involved and further investigations into the possible control by cAMP of the MAPK cascade.

Chapter 6

Discussion

6.1 P2Y receptor research

The aim of this thesis was to examine the ability of recombinant P2Y receptors to modulate G protein activation of tyrosine kinases, MAPK and mitogenesis. It is important to discuss the background of P2Y receptor research before discussion of the results described in this thesis.

The choice of 1321N1 cells as the host cell for transfection of P2Y receptors was due to their lack of response to nucleotides and lack of known expression of endogenous P2Y receptors. Additionally, some quite early signal transduction studies have used 1321N1 cells, therefore providing some background knowledge of their signalling pathways. Initial transfection of P2Y receptors was carried out transiently in *Xenopus laevis* oocytes but mammalian cells were subsequently used to produce stable transfections, which are more desirable for their stability and ease of use. Some other mammalian cells have been discovered, after selection as a host cell, to have P2Y receptor responses. COS-7 cells, which were used to transfect the proposed $P2Y_7$ receptor (Akbar et al., 1997), were found to respond to P2Y agonists in the absence of receptor (Yokomozo et al., 1997). C6-15 glioma cells, used for initial $P2Y_6$ receptor characterisation, also had responses in the absence of the transfected receptor (Chang et al., 1995). Other cells have had limited use. For example, the non-adherent Jurkat T lymphocyte cell line had to be mechanically stirred, causing release of ATP which desensitised the bovine $P2Y_1$ receptor transfected in these cells (Henderson et al., 1995). The 1321N1 cell line remains the best choice of host cell although it is well known that disruption of these cells causes some endogenous nucleotide release (Lazarowski et al., 1997a; Nicholas et al.,

1996b). Experimental procedure was modified to minimise this effect. In these studies, prior to cell stimulation, cells were kept serum-free for 24 hours. To minimise disruption to the cells this media was not changed and cells were stimulated by addition of a small volume of highly concentrated agonist. This minimises volume changes, which lead to endogenous nucleotide release, receptor activation and possible desensitisation. This method has helped achieve high fold stimulations for the total inositol (poly) phosphate assay, which indicates PLC activity. The ability of all the transfected P2Y receptors to couple to large increases in PLC activity implies that these types of procedures enable stimulation of transfected receptors in the absence of substantial desensitisation produced by endogenous release.

Cloning of the P2Y receptors began with the chick $P2Y_1$ receptor (Webb et al., 1993) and proceeded to expand during the course of research for this thesis as discussed in Chapter One. This meant that although ideally the study of transfected P2Y receptors would occur with human receptors, or at least receptors from the same species, this was not possible due to the availability of the different 1321N1 cell transfected P2Y receptor clones. In this thesis this lead to the use of turkey $P2Y_1$, human $P2Y_2$, human $P2Y_4$ and rat $P2Y_6$ receptors in 1321N1 cells. The study of P2Y receptors had, previous to cloning, been involved in characterisation of native cell types and development of antagonists to purinergic responses. The advent of P2Y receptor cloning enabled isolation of different P2Y subtypes in a host cell. Native cells usually express more than one type of P2Y subtype making characterisation of individual receptors difficult. The study of transfected receptors is important as the study of a single defined receptor in isolation is achieved. It has to be noted that prior to beginning work with transfected receptor systems,

characterisation of signalling pathways is only useful if the pathway is coupled to the transfected receptor. Any other pattern of research would involve characterisation of the host cell rather than the events coupled to activation of the transfected receptor. However, there are problems associated with the significance and interpretation of studies on transfected receptors. For example, it is important to accept from the outset that signal transduction studies investigate both the receptor and host cell biology. As a result, the significance of this approach for understanding native receptors should be questioned. In this thesis different P2Y receptors have been transfected into a common host cell line so we can assume that the distinct responses are due to different coupling of P2Y receptors to signalling cascades. Characterisation of the transfected receptor has other problems associated with the physiological relevance of investigations. Transfected receptors may produce extremely high receptor expression that is well above any observed native cell levels. This may lead to promiscuous G-protein couplings that would, under normal conditions, have not occurred. It would be easy therefore, unless care is taken with interpretation of data, to characterise signalling pathways only relevant to the transfected cell system. This is a particular problem with P2Y receptors since the lack of acceptable radio-ligand binding procedures means that receptor number cannot be directly measured.

P2Y receptor research is still very limited by the tools available for study. No high affinity specific antagonists exist for P2Y receptors, no high affinity radio-ligands exist, and no antibodies are currently available for the P2Y receptors. These tools would be extremely useful for P2Y receptor research and will be developed in the near future.

The subtypes of P2Y receptor are divided by their relative potencies for P2Y agonists, and first studies on the cloned receptors involved their pharmacological characterisation. A profile was produced usually based on activation of PLC that provided a basis for determining the different subtypes. Problems have arisen with these profiles due to inter-conversion of nucleotides. Two key classes of enzymes control the metabolism of nucleotides at the cell surface: ectonucleotidases, that hydrolyse nucleotides (e.g. ATP→ADP), and nucleoside diphophokinase that can phosphorylate nucleotides using ATP as a phosphate donor (e.g. UDP→UTP) as has been observed for 1321N1 cells (Lazarowski et al., 1997). Prevention of misleading agonist potencies has also been contributed to by using HPLC purified agonists. It is well known that purchased agonists often contained other nucleotides as contaminants. Removal of these using HPLC purification has helped in controlling the quality of agonists used to challenge P2 receptors. However, this does not prevent the breakdown of nucleotides during the course of the experiment, achieving misleading receptor information. Regeneration systems were therefore developed to combat this problem. Hexokinase was used to convert UTP back to UDP, removing any contamination from UDP caused by the action of nucleoside diphosphokinase (Lazarowski et al., 1997b). The action of ectonucleotidases producing di- and monophosphates from triphosphates can be prevented by use of creatine phosphokinase. Using phosphocreatine as a substrate creatine phosphokinase will convert diphosphates to triphosphates. These methods, along with careful experimentation, will help to achieve the correct agonist profiles for the various P2Y subtypes, as outlined in the Introduction. In this thesis purified nucleotides were not used. Problems associated with breakdown are only important using isolated P2Y subtypes if relative agonist potency order is required. It is becomes less important

when only activation of the receptor is required to investigate the involvement of various different signalling pathways. It is noted that concentrations of particular agonists may not therefore be as precise as stated due to nucleotide conversion.

The next section considers some of the background literature on pathways to MAPK activation and P2Y receptor signalling cascades, after which the results presented in this thesis for modulation of p42/p44 MAPK will be discussed.

6.2 P2Y receptor modulation of p42/p44 MAPK

Study of P2 receptors has mainly concentrated on intracellular signal transduction activated as a result of agonist binding. The secondary messengers involved in P2Y receptor signalling have traditionally been heterotrimeric G proteins linked to the third intracellular loop of the seven transmembrane domain receptor, as was discussed in Chapter 1. On receptor activation G proteins dissociate activating PLC_{β}, catalysing PIP_2 to DAG and $Ins(1,4,5)P_3$ which respectively activate PKC and release of $[Ca^{++}]_i$. This signal transduction pathway is well studied for G protein-linked receptor families in general including the P2 receptors. Receptor tyrosine kinases (RTK) traditionally have a very different signal transduction pathway. Upon agonist binding RTK dimerise and autophosphorylate beginning a cascade of phosphorylation involving tyrosine, serine and threonine kinases and also phosphatases. Known target molecules include Shc, SOS, Grb2 and the p42/pp44 Mitogen Activated Protein Kinase (MAPK) cascade involving Ras, Raf, Mitogen Enhanced Kinase (MEK) and MAPK. As discussed in chapter one, evidence in the literature has begun to emerge that the traditional G protein-linked cascade and RTK cascade are interlinked and cross-talk can occur (Bourne et al., 1996), this will now

be discussed further. Force et al., (1991) provided initial evidence that G protein receptors could activate tyrosine kinase pathways. Working with rat renal mesangial cells provide evidence that endothelin (ET), vasopressin (VP) and angiotensin II (AII) all enhance tyrosine phosphorylation in a similar manner to EGF RTK activation. PMA, a PKC activator, increased tyrosine phosphorylation, which was inhibited by staurosporine, a PKC inhibitor that also inhibits other kinases including some tyrosine kinases. However, staurosporine did not inhibit tyrosine phosphorylation activation by ET-1, VP or AII. This indicates possible PKC dependent and independent pathways to tyrosine phosphorylation activation. A study with AII in vascular smooth muscle (Eguchi et al., 1996) reported MAPK activation that was PLC and $[Ca^{2+}]_i$ dependent, PKC independent and linked to $p21^{ras}$. Other papers on rat smooth muscle cells have also reported MAPK activation linked to AII mitogenesis, which includes the involvement of tyrosine phosphorylation and increases in tyrosine kinase activity (Wilkie et al., 1996, Morton et al., 1996). Endothelins also are reported in the literature to stimulate tyrosine phosphorylation and MAPK in astrocytes (Cazaubon et al., 1993). ET-1 and ET-3 were shown to increase tyrosine phosphorylation of p42 MAPK within minutes and ET-1 was partially PKC sensitive. Src, a protein tyrosine kinase, which may be involved in the link from G protein to MAPK, has been reported to be activated by ET-1 (Simonson et al., 1996) contributing to c-fos activation creating a role for non-receptor protein tyrosine kinases in a signal cascade from G protein-linked receptors to nuclear signalling. These reports began a discussion into the mechanisms behind G protein activation of MAPK and the involvement of protein phosphorylation cascades. Tyrosine phosphorylation and threonine phosphorylation are known to be important in MAPK activation (Anderson et al., 1990) showing a convergence of

these two phosphorylation cascades. However it appears that G_{α} and $G_{\beta\gamma}$ formed from G protein activation hold the key to important upstream events (Inglese et al., 1995). Direct G protein activation has led to increases in tyrosine phosphorylation of $p125^{FAK}$ and Paxillin (Seckl et al., 1995) indicating a controlling role in cell matrix interactions important to cell movement and division. Further work looking at protein-protein interactions has helped to refine the mechanisms of activation by comparing RTK systems. RTK when tyrosine phosphorylated form SH2 and SH3-dependent protein-protein interactions between itself, Shc, Grb2 and SOS leading to Ras dependent MAPK activation. The $G_{\beta\gamma}$-subunit has been shown to increase Shc phosphorylation (Touhara et al., 1995) and also increase the formation of the Shc, Grb2 and SOS complex (van Biesen et al., 1995). This complex, if dissociated, prevents MAPK phosphorylation, further refining the link between G protein-linked receptors and MAPK phosphorylation. G protein-linked receptors associate with different G protein isoforms (see chapter one), examples of which are G_q and G_i proteins. These different G protein isoforms are able to differentially activate the MAPK cascade (Hawes et al., 1995). Different pathways discussed so far included PKC dependent and independent G protein activation of MAPK. G_i and G_q isoforms, reported by Hawes et al., (1995), have a $p21^{ras}$ dependent and independent pathway. G_i via its $G_{\beta\gamma}$ -subunit activates MAPK in a $p21^{ras}$, $p74^{raf}$ dependent and PKC independent manner. However in G_q mediated signal transduction the $G_q\alpha$-subunit activates MAPK in a $p21^{ras}$ independent, $p74^{raf}$ and PKC dependent manner. $G_o\alpha$-subunits, like $G_q\alpha$-subunits, mediate MAPK activation by a PKC dependent mechanism (van Biesen et al., 1996). In this case MAPK activation was pertussis toxin and PKC sensitive but insensitive to a $G_{\beta\gamma}$ binding peptide βARK_{ct} and unaffected by a negative Ras mutant. Thus $G_o\alpha$-subunits are reported to mediate

MAPK activation in a PKC dependent and Ras independent pathway as shown for the $G_q\alpha$-subunit. The G_i $\beta\gamma$-subunit MAPK activation pathway becomes more complex as PI-3-K also seems to be involved (Hawes et al., 1996) acting at a point upstream of mSOS and Ras activation possibly involving Shc tyrosine phosphorylation.

The involvement of tyrosine kinases in the link between G proteins and Ras appears to be growing in importance. Evidence has been provided that one such tyrosine kinase, Pyk2, is involved with Shc, Grb2 and SOS. (Lev et al., 1995). Dikic et al., (1996) report that tyrosine phosphorylation of Pyk2 causes binding to Src via an SH2 domain leading to its activation. No exact mechanisms exists at present but from research gathered so far it is evident that Pyk2, Src, Shc, Grb2 and SOS all have important roles to play.

P2 purinergic responses involving tyrosine phosphorylation have been reported including addition of ATP to intact lymphoma cells (Allee et al., 1989). EAhy 926 human endothelial cell-line (Graham et al., 1996a, 1996b) have shown stimulation of p42 MAPK with ATP and UTP, indicating the possible presence of a $P2Y_2$ receptor. UTP activation of MAPK was PKC and extracellular calcium sensitive and $P2Y_2$ are known to link to G_i or G_o and therefore could be activating MAPK through its $G_o\alpha$-subunit as shown previously (van Biesen et al., 1996). UTP also caused activation of MKP1, a MAPK phosphatase, possibly involved in the deactivation of MAPK, though Graham et al., (1996) do not assume this to be the major MAPK dephosphorylation enzyme. Other MKP have been discovered; MKP2 (Misra-Press et al., 1995) and MKP3 (Muda et al., 1996). 2MeSATP had no effect on the EAhy 926 cell line implying either the absence of the $P2Y_1$ receptor or a $P2Y_1$ receptor that is not linked to tyrosine phosphorylation or MAPK activation.

However on bovine aortic endothelial cells (BAEC's) both UTP ($P2Y_2$ defining agonist) and 2MeSATP ($P2Y_1$ defining agonist) have been shown to activate tyrosine phosphorylation which is important for prostacyclin release (Bowden et al., 1995) and ATP responses on BAEC cells require PKC activation (Brown et al., 1996). As ATP acts at both $P2Y_1$ and $P2Y_2$ receptors it is difficult to define which receptor or if both receptors are utilising the PKC dependent pathway. These native cell responses discussed above and in Chapter One are difficult to interpret due to the presence of multiple receptor subtypes. Although some receptors have defining agonists there are no high affinity selective agonists to determine the difference between the receptor subtypes. It is therefore possible to use the transfected system available to characterise P2Y subtype receptor signalling. Some native cells have only expression of one known P2Y receptor subtype but there are further possible subtypes as yet uncharacterised that may interfere with receptor characterisation. The characterisation of $P2Y_1$ and $P2Y_2$ receptors on native cells began the formulation of the hypothesis that they maybe a differential activation of P2Y receptor activation because of their known G protein-coupling. $P2Y_1$ receptors are thought to signal through the G_q protein being pertussis toxin insensitive and $P2Y_2$ receptors being linked to $G_{i/o}$ due to sensitivity to pertussis toxin. Earlier work by Charlton (1998) has shown that human $P2Y_2$ receptors cloned into 1321N1 cells are partially sensitive to pertussis toxin. Transfected turkey $P2Y_1$ and human $P2Y_2$ receptors were treated for 16 hours with 100ng/ml of pertussis toxin and their activation of PLC was detected using total inositol (poly)phosphate assay. No effect was seen for turkey $P2Y_1$ receptors but the response was reduced in human $P2Y_2$ transfectants by 20.6 ± 3.8 % at 10 min and 27.5 ± 0.6 % at 20 min. This partial inhibition implies coupling of transfected human $P2Y_2$ receptors to more than one G

protein which is pertussis toxin insensitive. With this background information it was hypothesised that transfected $P2Y_1$ and $P2Y_2$ receptors may show activation of MAPK via different pathways as discussed previously i.e. a PKC dependent and PKC independent route or Ras dependent or independent pathway. It is also a consideration that $P2Y_1$ receptors acting via G_q and $P2Y_2$ receptors acting via G_o may both be PKC dependent. The separate pathways for G_q or G_i coupled receptor activation of p42/p44 MAPK are displayed in Figure 6.1. The data collected for the transfected P2Y receptor activation of p42/ p44 MAPK and tyrosine phosphorylation will be discussed next as well as any future work.

Initial experiments discovered that both turkey $P2Y_1$ and human $P2Y_2$ receptor activation did not lead to increased overall tyrosine phosphorylation either by concentration or with time. The lack of observed tyrosine phosphorylation, in contrast to that observed by Bowden et al., (1995), may be due to the insensitivity of the PY20 antibody used or that the tyrosine phosphorylation may have been undetected as it was only produced transiently (less than 1 minute). The use of pervanadate highlighted this problem which could occur due to the presence of highly active phosphatases. Further work would include characterisation of the mechanism behind the $P2Y_2$ receptor induced decrease in tyrosine phosphorylation due to sub-maximal pervanadate action. Detection of the phosphatase involved may help in understanding if a feed-back role is involved in maintenance of the level of tyrosine phosphorylated proteins. As discussed earlier, MKP is regulated by agonists also acting at MAPK enabling switching off an activation event to prevent over activation (Graham et al., 1996).

Work proceeded to begin characterisation of the p42/p44 MAPK pathway. As described in Chapter One and above, the route that leads to activation of MAPK

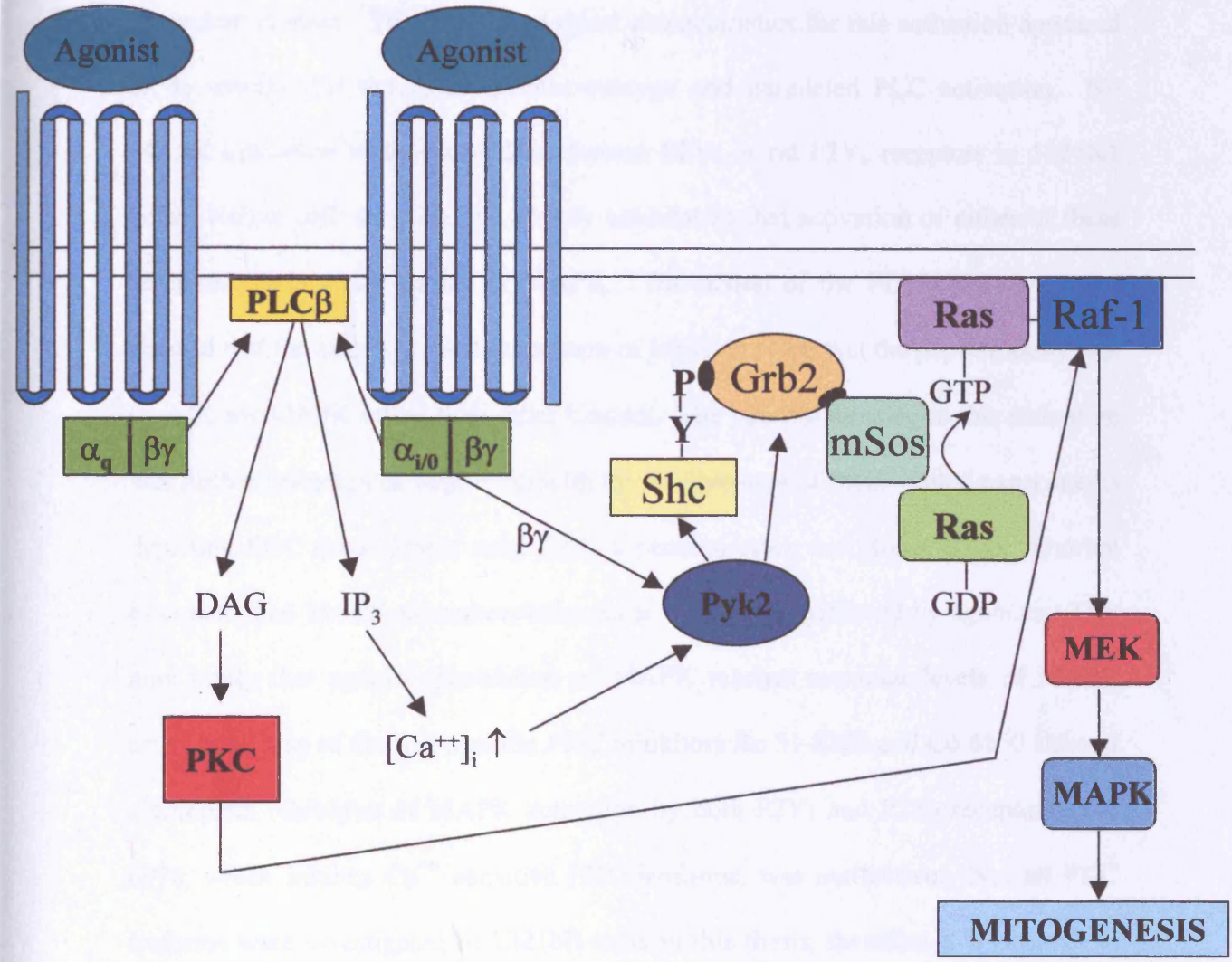


Figure 6.1
Signalling pathways for G_q and G_i linked receptors to MAPK and mitogenesis.

can be complicated involving many protein-protein interactions formed into complexes and interaction with membrane and matrix proteins. Both $P2Y_1$ and $P2Y_2$ receptors were observed to increase MAPK phosphorylation in a dose and time dependent manner. The pharmacological characteristics for this activation appeared to be specific for the P2Y receptor subtype and paralleled PLC activation. No MAPK activation was observed for human $P2Y_4$ or rat $P2Y_6$ receptors in 1321N1 cells. Native cell work has not clearly established that activation of either of these receptors leads to activation of MAPK. The action of the PD 98059 compound showed that the activity was downstream of MEK and also that the peptide assay was specific for MAPK rather than other kinases. The pathway leading to this activation was further investigated beginning with the involvement of PKC. Initial experiments depicting PKC involvement used TPA, a phorbol ester, and Ro 31-8220. Phorbol ester activated MAPK phosphorylation to similar levels achieved by agonists. This may imply that agonist stimulation of MAPK reaches maximal levels of MAPK activation. Use of the non-specific PKC inhibitors Ro 31-8220 and Go 6850 showed a complete inhibition of MAPK activation by both $P2Y_1$ and $P2Y_2$ receptors. Go 6976, which inhibits Ca^{++} sensitive PKC isoforms, was ineffective. Not all PKC isoforms were investigated in 1321N1 cells in this thesis, therefore it is difficult to say which isoforms are involved specifically. Inhibition data suggests it is a PKC isoform that is Ca^{++} insensitive, which may be the ε or λ isoforms detected or isoforms δ, η or ι not probed for. Isoform δ has been implicated in P2Y receptor signalling (Soltoff et al., 1998a) as has ε isoform (Patel et al., 1996). Therefore in 1321N1 cells P2Y receptors may be signalling through either ε, λ, δ, η or ι isoforms of PKC. Definitive coupling of PKC isoforms can be carried out using movement from cytoplasm to cell membrane, which would be the subject of further work. The

involvement of other signalling proteins was then investigated using Shc and Pyk2. Pyk2 was not detected in 1321N1 cells and therefore cannot be used for signalling. Soltoff et al., 1998a, subsequently provided evidence that $P2Y_2$ receptors in PC12 cells are coupled to PKC and Pyk2. Therefore transfected $P2Y_2$ receptors must show a different pathway to MAPK activation than that observed in PC12 cells. Evidence provided in this thesis implicates PKC as a major signalling protein in this pathway but it is unknown as to which part of the MAPK signalling cascade the PKC acts. PKC may directly act at Raf or directly at MAPK itself providing Raf-independent and dependent routes of activation. Further work would be of interest to detect the involvement of Ras or Raf in transfected P2Y receptor MAPK activation. It was unclear as to the involvement of the Shc protein. Initial experiments have provided no detectable agonist induced phosphorylation of Shc. The IgG protein present obscured interpretation of the Western blot as the IgG protein ran at a similar molecular weight to the Shc isoforms. This is an obvious area for further work, which would begin by reducing IgG levels. Though the work in this thesis has by no means fully elucidated the mechanism of MAPK activation by P2Y receptors in 1321N1 cells, it has provided a valuable body of work in initial characterisation of the signalling pathway from P2Y receptor to MAPK. Both $P2Y_1$ and $P2Y_2$ receptors activate MAPK by similar mechanisms as observed by the similarity in PKC inhibition. $P2Y_1$ receptor signalling to G_q-protein would be, as was discussed earlier, likely to involve a PKC dependent pathway. $P2Y_2$ receptor activation of G_o could account for the PKC dependent pathway observed in 1321N1 cells, which would be pertussis toxin sensitive. Preliminary experiments with MAPK activation and pertussis toxin in 1321N1 cells produced highly variable results and would be the subject of future work. It would also be an important investigation to detect P2Y

receptor coupling to the different G-protein isoforms in 1321N1 cells to help further knowledge of this pathway. It has also been observed in our laboratory that cAMP is modulated by transfected P2Y receptors (Roalfe, unpublished). This is not associated with modulation of MAPK. The scope for future work here is large, with further involvement of other kinases (e.g. PI3K) and definitive characterisation of the mechanisms of MAPK activation. Also as new P2Y subtypes are detected they too can be used in 1321N1 cells for signalling characterisation.

6.3 Transfected P2Y receptor mediated pathways to mitogenesis in 1321N1 cells

The activation of MAPK is implicated in a number of studies in mitogenesis including pathways where MAPK activation is required but not sufficient for mitogenesis (Wilkie et al., 1996). The study of the transfected turkey $P2Y_1$, human $P2Y_2$, human $P2Y_4$ and rat $P2Y_6$ receptors signalling to mitogenesis will now be discussed.

In this thesis mitogenesis was characterised by the use of the [^{3}H]thymidine incorporation assay. It should be noted that this assay more specifically characterises the incorporation of thymidine into DNA and therefore more accurately depicts DNA synthesis. An increase in DNA synthesis only indicates a possible increase in mitogenesis, as an increase in cell number is not always associated with increased DNA synthesis.

The turkey $P2Y_1$ receptor, when activated by 30μM 2MeSATP, showed increased thymidine incorporation and when activated together with PDGF showed an additive response. This suggests that the turkey $P2Y_1$ receptor is coupled to activation of mitogenesis in 1321N1 cells. Studies have been carried out in native

cells to characterise mitogenesis and have implicated the $P2Y_1$ receptor (Yu et al., 1996; Erlinge, 1998). Turkey $P2Y_1$ receptors when activated in 1321N1 cells also show increased PLC activity and increased MAPK activity.

The human $P2Y_2$ receptor when activated in 1321N1 cells does not lead to an activation of thymidine incorporation although previous experiments have shown that both MAPK and PLC are activated. This is in contrast to native cell studies which have implicated the $P2Y_2$ receptor as being the receptor involved in UTP mediated mitogenesis (Erlinge, 1998; Wilden et al., 1998). It may be that some uncoupling has occurred in 1321N1 cells disabling $P2Y_2$ activated mitogenesis.

The human $P2Y_4$ receptor provided the most interesting observation. $P2Y_4$ receptor activation did not lead to an activation of MAPK but did lead to an inhibition of [^{3}H]thymidine incorporation. Inhibition of mitogenesis by UTP has previously been observed in one native cell-line (Lemmens et al., 1996) though the mechanism of action is unclear. This phenomenon is highly interesting, and further work needs to be done to characterise the possible involvement of cAMP, as discussed at the end of Chapter 4 and later in this chapter.

Rat $P2Y_6$ receptors showed no increase in thymidine incorporation or MAPK activity, despite strong PLC activation, showing that the receptor though coupled to strong PLC activation was not coupled to MAPK or mitogenesis. It should also be noted that basal levels of thymidine incorporation were similar in all transfected cell-lines except for the transfected rat $P2Y_6$ receptor. This may be due to the nature of the transfection causing insertional inactivation of a gene necessary for cell growth control. It is also possible that the receptor may show activation due to some small endogenous release of nucleotides and because of slow desensitisation (Robaye et al., 1997) is able to increase basal thymidine incorporation. Increased basal levels may

therefore mask the actual mitogenic effect of UTP or UDP at the transfected $P2Y_6$ receptor.

Overall the results of investigations into signalling pathways from transfected P2Y receptors to mitogenesis tell us a number of things. 1) PLC and Ca^{++} may be necessary but they are not sufficient for mitogenesis since all receptors achieve strong activation of both PLC and $[Ca^{++}]$ release but do not all lead to increased mitogenesis. 2) The involvement of PKC can only be implied indirectly. Since both $P2Y_1$ and $P2Y_2$ receptors require PKC for MAPK activation it can be assumed that they both activate PKC. However, they do not both stimulate mitogenesis therefore implying that PKC may be necessary but not sufficient for mitogenesis. 3) The involvement of cAMP implied from research within our group (Roalfe, unpublished) suggests a more interesting inverse involvement. Turkey $P2Y_1$ receptors show a decrease in cAMP accumulation but an increase in mitogenesis. Human $P2Y_2$ receptors show no effect on cAMP and no increase in mitogenesis. Human $P2Y_4$ receptors show an increase in cAMP accumulation and a decrease in mitogenesis. Rat $P2Y_6$ receptors show an increased cAMP accumulation but unlike human $P2Y_4$ receptors do not show a decrease in mitogenesis. However, this may be explained either by the high basal levels, which would mask significant stimulation or inhibition, or that cAMP accumulation is not increased to sufficient levels to achieve inhibition of mitogenesis. This evidence points towards a role for cAMP in modulating P2Y mediated mitogenesis in 1321N1 cells. 4) When considering MAPK involvement it was observed that there was no case where there was increased mitogenesis in the absence of increased MAPK activity. However, there is MAPK activity in the absence of increased mitogenesis. It can be concluded that

MAPK may be necessary but not sufficient for mitogenesis by transfected P2Y receptors in 1321N1 cells.

6.4 Rat brain endothelial cell signalling

Further to work on the transfected cell systems it was also possible to widen the characterisation of P2Y receptor signalling by using rat brain endothelial cells. These cells are intrinsically difficult to prepare and grow successfully. Microvascular endothelial cells were isolated from the rat brain and cultured as primary preparations (described in Chapter Two). After plating down the microvessels, outgrowths of endothelial cells take a few days to appear and are confluent in a 35mm dish after 1-2 weeks depending on the plating conditions. Each preparation takes 6 adult rats to achieve a plating of approximately four 96-well plates or twenty-four 35mm dishes. The logistics of the preparation makes it difficult to use for experimentation due to the slow production of confluent cell layers. It must also be noted that some difficulty was had achieving pure endothelial cell monolayers, as well as times when the endothelial cell growth was not sufficient to reach confluency. This type of preparation is therefore simpler for single cell Ca^{++} experiments than for other biochemical assays. However data was collected for these cells which will be now be discussed. Coupled with research efforts within our research group it was determined that multiple P2Y receptors exist on primary preparations of rat brain endothelial cells in our hands. Characterisation with various P2Y agonists showed that the activation of MAPK was achieved with both UTP and to a much lesser effect by 2MeSATP. This, as the data for transfected human $P2Y_2$ receptors depicts, seems most likely to be via a $P2Y_2$ receptor. 2MeSATP, or its

degradation product 2MeSADP, are not active at $P2Y_4$ receptors, ruling this possibility out. Though action of the UTP and 2MeSATP could be a result of action at the $P2Y_6$ receptor, this is unlikely, based on transfected rat $P2Y_6$ data but may reflect a difference in cell types. It is to be noted that RT-PCR has detected the presence of $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptor subtypes but no $P2Y_1$ (Anwar et al., 1999). Further agonist profiling of the MAPK response to P2Y agonists would help clarify this. Other experiments on RBEC's concentrated on the pathway leading to MAPK activation and activation of the adenylate cyclase. The requirement of Ca^{++} for MAPK activation may reflect the involvement within the pathway of Ca^{++} sensitive kinase such as calmodulin or Pyk2, which is activated by Ca^{++}. Though the use of EGTA may not be the ideal way to remove extracellular calcium, as excessive EGTA will also deplete intracellular stores, it was thought that for the given time course 5mM EGTA would not significantly deplete intracellular stores. However, assuming the intracellular stores remain intact and the cell viable, MAPK activation was significantly reduced by addition of EGTA. The implication that Pyk2 may be involved would be the subject of interesting further work on RBEC. The limiting number of cells hindered completion of experiments involving cAMP and its involvement in MAPK activation. Of the data collected from three separate and different experiments, the result appears to be the same: neither forskolin nor cAMP analogues have an effect on MAPK activation. No correlation is observed for the transfected P2Y receptors or the RBE cells for a role for cAMP in modulating P2Y mediated MAPK activation. Other receptors also in RBE cells were characterised for their coupling to MAPK showing endothelin activation as reported in the literature (Vigne et al., 1990, Vigne & Frelin, 1994). The role that P2Y receptor has on modulation of cAMP levels in RBE cells was also investigated and, with RT-PCR

results produced with in this laboratory, points towards a novel P2Y receptor subtype in RBE cells. Though often discussed within the literature, the existence of a receptor responsive to both ATP and adenosine is not really known. Data points towards the presence of a dual activated receptor (sometimes referred to as P_3 but is more likely to be characterised as belonging to the P1 family due to antagonist properties) though until it is cloned and fully characterised it will remain an unknown. The signalling pathways for the receptors in RBEC's include a receptor responsive to 2MeSATP that is coupled to an increase in $[Ca^{++}]i$ mobilisation in the absence of a PLC response (Albert et al., 1997; Anwar et al., 1999). This receptor is not coupled to an increase in cAMP levels in the presence of forskolin and also not likely to be coupled to the activation of MAPK, which occurs mainly at a UTP responsive receptor. UTP causes a rise in PLC activity, increased $[Ca^{++}]i$ release, increased MAPK activity but no increase in cAMP accumulation. cAMP accumulation therefore is not sufficient for increases in MAPK activity and may play an inhibitory role to the MAPK cascade as observed in the transfected cells. An increase in $[Ca^{++}]i$ is observed with both 2MeSATP and UTP, although both do not give equal levels of MAPK activation. Extracellular calcium does appear to be required for MAPK activation in RBE cells, therefore it can be concluded that calcium release may be necessary but not sufficient for MAPK activation. Activation of PLC by UTP could lead to the increase in MAPK activity as an increase in PLC activity is observed with UTP, as is an increase in MAPK activity. Conversely, no PLC response is observed with 2MeSATP coupled with a reduced MAPK response. There may therefore be a requirement for PLC activity upstream of MAPK for increased MAPK activation. Further work would establish this as well as a possible role of PKC activated by DAG production due to PLC activity.

6.5 Conclusions

The P2Y receptors are a growing family of receptors widely implicated in various disease states and distributed around all areas of the body. Characterisation of the P2Y receptor signalling pathways is key to the understanding of these physiological and patho-physiological disease states. The work presented in this thesis contributes towards a large body of work dedicated to uncovering the complexities of P2Y receptor induced intracellular signalling. This thesis discusses the role of P2Y receptor in modulating cAMP levels and p42/p44 MAPK both in transfected cells and in the native RBE cell. This thesis provides evidence for the modulation of P2Y induced MAPK activation by PLC, PKC, Ca^{++} and by cAMP. There is also evidence that no correlation exists with observed tyrosine phosphorylation or [^{3}H] thymidine as an indicator of mitogenesis. The study of mitogenesis in transfected cells may help in the correlation with native cell systems and provide some use in characterising native cells complicated by multiple receptor expression. Transfected receptors provide an ideal system for study of P2Y receptors, which by their differential activation of the various signalling pathways, demonstrate that individual expression of P2Y subtypes leads to subtype characterisation and does not involved complex host cell characterisation.

The future of P2Y receptor research lies in a number of areas including the development of better antagonists, for which the transfected 1321N1 cells provides an easy system for testing. Mutagenesis studies aid in the characterisation of the complex formations of the agonist binding pocket enabling better agonist and antagonist design. Some research has been carried out on the mechanism of desensitisation of P2Y receptors and much work has shown that desensitisation

occurs (Robaye et al., 1997; Wilkinson et al., 1994; Hourani et al., 1993). It would be interesting to discover what role receptor phosphorylation has in this process and which kinases cause the phosphorylation. Localisation of P2Y receptors is limited by the use of low affinity radioligand binding, which may not be P2Y receptor specific. Other methods for tissue localisation include northern blotting and RT-PCR from specific tissues of interest. Development of P2Y receptor antibodies would aid tissue localisation using in situ hybridisation with fluorescent secondary antibodies. Receptor antibodies also create a number of other new investigations (e.g. receptor phosphorylation). The control of mitogenesis by P2Y receptors is perhaps the most exciting for further investigation due to problems associated with proliferation of smooth muscle in blood vessels. If further research shows similarities with the transfected cell system then a role for UTP acting at the $P2Y_4$ receptor may be useful in controlling the hyper-proliferative state induced by ATP in damaged blood vessels. Further characterisation of the signalling of P2Y receptors will remain key to the future treatment of many disease states.

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